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Influence of hydrate formation on the retention parameters of analytes in reversed-phase HPLC

Scientific specialization 1.4.2. Analytical chemistry

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Introduction

Topic Relevance

Substantiation of the topic of work: The number of organic reactions in liquid phases exceeds many times the number of reactions is gas phase. This brings up an important warning: in high performance liquid chromatography (HPLC) we should be aware of the possibility of various transformations of analytes during their chromatographic separation. First of all, this relates to the hydration of analytes.

Hydrates of organic compounds are not as well-known as hydrates of inorganic compounds, at first salts. Many of organic hydrates are unstable and their detection appears to be a complex problem. At the beginning of this work, the only information available was obtained in the PhD dissertation work of Daria A. Nikitina (June 2023): comparing the recurrent approximations of retention times of analytes in reversed phase (RP) HPLC at different separation conditions allows detecting the reversible hydration of some of them. However, in the work mentioned the verification of this approach has only been tested for acetonitrile-water eluents.

The importance and urgency of this problem forces us to consider the possibility of revealing the hydration of analytes in eluents containing another organic solvent, namely methanol. Moreover, keeping in mind the results obtained in the mentioned work of Daria Nikitina, we have the chance to compare data for both eluents.

Search the literature data on the hydration of organic compounds indicates that the formation of hydrates surprisingly is known for a large number of them. Thus, in the conditions of reversed phase HPLC separation such analytes may exist in anhydrous form, in hydrated forms, or at dynamics equilibria between both of them.

There are no direct evidences on the formation of hydrates in reversed phase HPLC. Hence, the formation of hydrates can only be established using the recurrent approximation of retention parameters of analytes in the following form:

$$t_R(C + \Delta C) = at_R(C) + b, \qquad \Delta C = \text{const}$$

where C is the content of organic solvent in an eluent. By words it means that the chemists should ensure the equal "steps" of variations of concentration ΔC of organic solvent in an eluent.

Research purpose

The main purpose of this PhD thesis is to consider the regularities and features of hydration of analytes in reversed phase HPLC with methanol-water eluents, aiming to validate a new indirect method for detecting the hydrates of organic compounds in such conditions.

Research objectives

To fulfill these purposes, it was necessary to perform the following main tasks:

1. To compare the basic relations for dependence of retention parameters of analytes on the content of organic solvent in an eluent.
2. To analyze the sources of the anomalies of recurrent approximation of retention parameters for different series of organic compounds.
3. To consider the regularities and features of retention indices of analytes of different chemical origin (polarity) on the content of organic solvent content in an eluent and physicochemical properties of analytes.
4. To detect the formation of hydrate forms of some analytes in aqueous solutions using a new indirect method, namely recurrent approximation of their retention parameters in reversed-phase HPLC.

Scientific novelty

The scientific novelty of this work is the elaboration of the concept of detecting the formation of hydrates of organic compounds in aqueous solutions for the first time, including conditions of reversed-phase high performance liquid chromatography. It is

experimentally proved that such hydration is observed not only for acetonitrile-water eluents, but for methanol-water eluents, as well.

Practical significance of the work

The practical significance of the recurrent approximation of retention parameters $t_R(C + \Delta C) = at_R(C) + b$, where C is the concentration of organic solvent in the eluent, ΔC = const, is detecting the hydration of organic compounds in aqueous solution under RP HPLC condition.

Methodology and method of research

In this work: we have prepared (synthesized) and characterized several series of organic compounds, namely N-substituted *p*-toluenesulfonamides, N-unsubstituted hydrazones of aromatic carbonyl compounds, and oximes of aromatic carbonyl compounds. We have considered the dependencies of their retention parameters on the content of organic modifier of the eluent. The key element of data processing is the recurrent approximation of retention parameters, $t_R(C)$ with the aim to reveal its anomalies.

The provisions submitted for defense

The following provisions are claimed to defend the dissertation research:

1. Comparing the basic relationships for approximation of retention parameters of analytes on the content of organic solvent in an eluent.
2. Influence of the hydrophobicity of analytes on the dependence of the retention indices on the organic solvent content in the eluent.
3. Verification of different kinds of anomalies in the recurrence approximation of retention parameters for organic compounds of different series.
4. Confirmation of the formation of hydrated forms of analytes in aqueous solutions using a new indirect method of recurrent approximation of retention parameters in reversed-phase HPLC.

Principal scientific results

The mail goal of the dissertation is considering the regularities and features of the hydration of analytes in reversed-phase HPLC with methanol-water eluents [68,123].

Different relationships for dependencies of retention parameters from content of organic solvents in an eluent were compared. It is concluded that the precision of recurrent relations is high than the precision of all previously known equations. Besides that, these relations allow approximation of net (not corrected) retention times, that excludes the necessity of preliminary determination or calculation of so-called dead time [133].

It was confirmed experimentally that hydration of analytes is possible and takes place not only in the systems “acetonitrile-water”, but in the systems “methanol-water”, as well [154]. Several series of organic compounds were synthetized and characterized, namely N-substituted *p*-toluenesulfonamides [69], hydrazones and oximes of aromatic carbonyl compounds [124]. The key element of data processing in all cases was recurrent approximation of retention parameters, $t_R(C)$, aimed for revealing the anomalies [68,71].

The influence of the hydrophobicity of analytes on the dependence of their HPLC retention indices from the content of organic solvent in an eluent was revealed first time [96,153]. The negative values of the coefficients $d\mathrm{RI}/dC$ appeared to be typical for polar analytes with highest probability of hydrate formation. Non-polar analytes are characterized by the values $d\mathrm{RI}/dC > 0$ [145,175].

It is shown that different series of organic compounds can be characterized by the absence of any anomalies of recurrent dependencies of retention times (e.g., non-substituted hydrazones of aromatic carbonyl compounds) [124], as well as manifestation of strong anomalies (oximes of aromatic carbonyl compounds).

Work Approbation and publications

Based on the materials of the thesis, 11 articles in Russian and International journals and 5 abstracts were published at international and Russian conferences.

List of Publications

The results were published in the following peer-reviewed scientific journals indexed in the Scopus and Web of Science databases:

1. Kornilova T.A., Derouiche A., Zenkevich I.G. Recurrent approximation of retention parameters as confirmation of N-substituted sulfonamides hydrate formations in reversed phase HPLC // Analytics & Control. 2020. V. 24. № 4. P. 315–322. DOI: 10.15828/analitika.2020.24.4.008. (In Russian).

2. Zenkevich I.G., Nikitina D.A., Derouiche A. Formation and chromatographic detection of organic compound hydrates // J. Analyt. Chem. 2021. V. 76. № 4. P. 493–502. DOI: 10.1134/S1061934821040146.

3. Zenkevich I.G., Derouiche A., Nikitina D.A. Detection of organic hydrates in reversed phase high performance liquid chromatography using recurrent approximation of their retention times // J. Liquid. Chromatogr. Related. Technol. 2021. V. 44. P. 588–598. DOI: 10.1080/10826076.2021.1998905.

4. Zenkevich I.G., Derouiche A., Nikitina D.A., Kornilova T.A., Khakulova A.A. Controlling the correctness of retention parameters variations in reversed phase HPLC using recurrent relations // Analytics & Control. 2021. V. 25. № 2. P. 117–125. DOI: 10.15826/analitika.2021.25.2.005. (In Russian).

5. Zenkevich I.G., Derouiche A. Analytical aspects of the dependence of the retention indices of organic compounds in reversed-phase HPLC on the content of methanol in the composition of an eluent // Analytics & Control. 2022. V. 26. № 1. P. 41–48. DOI: 10.15826/analitika.2022.26.1.004. (In Russian).

6. Zenkevich I.G., Derouiche A., Nikitina D.A. Important features of retention indices determination in reversed-phase high performance liquid chromatography // Analytics & Control. 2022. V. 26. № 1. P. 57–63. DOI: 10.15826/analitika.2022.26.1.007. (In Russian).

7. Zenkevich I.G., Nikitina D.A., Derouiche A. Revealing the hydration of sorbates based on the dependence of their retention parameters in reversed-phase HPLC on the concentration of the organic component of the eluent // Protect. Metals Phys. Chem. Surf. 2022. V. 58. № 6. P. 1156–1163. DOI: 10.1134/S2070205122060223.

8. Zenkevich I.G., Derouiche A., Nikitina D.A. Evidence for the hydration of some organic compounds during reverse-phase HPLC analysis // Molecules. 2023. V. 28(2). № 734. DOI: 10.3390/molecules280207.

9. Zenkevich I.G., Derouiche A., Nikitina D.A. Features of the dependence of the retention indices of sorbates in reversed-phase high-performance liquid chromatography on the content of organic solvents in the eluent // Rus. J. Phys. Chem. A. 2023. V. 97. № 5. P. 1007–1017. DOI: 10.1134/S0036024423050321.

10. Derouiche A., Karakashev G.V., Ukolov A.I., Zenkevich I.G. Hydrolytic stability of unsubstituted hydrazones of aromatic carbonyl compounds in reversed-phase HPLC // J. Analyt. Chem. 2023. V. 78. № 2. P. 222–230. DOI: 10.1134/S106193482302003X.

11. Derouiche A., Zenkevich I.G. Comparing the correctness of different relations for approximation of retention times in reversed phase HPLC with methanol–water eluents // Algerian. J. Chem. Eng. 2023. V. 01. P. 08–15. DOI: 10.5281/zenodo.8040658.

List of Conferences

The results of the work were presented at five all-Russian and International conferences:

1. Derouiche A., Zenkevich I.G. Features of the dependence of retention indices on the content of methanol in an eluent in reversed phase HPLC // International Student Conference “Science and Progress”. Saint-Petersburg. 2021. P. 16.
2. Derouiche A., Zenkevich I.G. Formation of hydrates of organic compounds in HPLC conditions // XXII International Conference on Chemistry for Young Scientists. Saint-Petersburg. 2021. P. 56.

3. Zenkevich I.G., Nikitina D.A., Derouiche A. Confirmation of hydrate formation organic compound under conditions of RP HPLC with using recurrent relations // All-Russian Symposium and School-Conference for Young Scientists Physical and Chemical Methods in Interdisciplinary Environmental Research. Sevastopol. 2021. P. 64–65.
4. Zenkevich I.G., Derouiche A., Nikitina D.A. Features of dependence of retention indices of analytes in RP HPLC on the content of organic component of eluent. IX All-Russian Symposium and School-Conference for Young Scientists "Kinetics and Dynamics of Sorption Processes" Sochi. 2022. P. 43–44.
5. Zenkevich I.G., Derouiche A. Recurrent approximation of retention times of reversed-phase HPLC as a method for detecting chemical transformations of analytes // All-Russian Symposium and School-Conference for Young Scientists Physical and Chemical Methods in Interdisciplinary Environmental Research. Sevastopol. 2023. P. 20–21.

Dissertation structure

The dissertation consists of introduction, literature review, experimental part, the results and their discussion, conclusion, and finally the list of references. The total volume of the thesis is 138 pages, with 22 figures and 21 tables. The list of references contains 179 items.

Correspondence to the scientific specialty

The dissertation corresponds to points 2 "Methods of chemical analysis" and 10 "Analysis of organic substances and materials" of the passport of specialty 1.4.2 Analytical chemistry (chemical sciences). The problems solved in the dissertation work also correspond to the specified specialty.

Chapter 1: Literature review

1.1. Reversed-phase HPLC as the method for separating mixtures of organic compounds

High-performance liquid chromatography commonly known as HPLC, formerly called high-pressure liquid chromatography, is an analytical technique used to separate, identify, and quantify components in complex mixtures, even in trace amounts [1]. This technique is easy to couple with mass spectrometric detection (MS). It relies with pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each sample component (analyte) interacts slightly differently with the adsorbent material, causing different rates of chromatographic zones. The result of this process is the separation of the sample's constituents.

HPLC is widely used in several fields of science and industry; especially for manufacturing (e.g., during the production process of pharmaceuticals and biologics), legal (e.g., detection of performance-enhancing drugs in urine), research (e.g., separation of components of a complex biological sample, or similar synthetic chemicals from each other) and for numerous medical purposes (e.g., detection of vitamins levels in blood serum, etc.) [2–6].

Chromatography in general can be described as a mass transfer process involving adsorption [7]. The active component of the column, the adsorbent, is usually a porous material consisting of solid particles (e.g., modified silica gel, synthetic porous polymers, etc.), with sizes from 2 to 10 μm. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is usually a mixture of solvents (e.g., water, acetonitrile or methanol, more rarely 2-propanol or tetrahydrofuran) and is referred to as the "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions between the sample components and the adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole-dipole, and ionic, most often in a combination [8,9].

HPLC differs from traditional "low pressure" liquid chromatography in that the operating pressures are significantly higher (50 to 350 bar), whereas ordinary liquid chromatography generally relies on the force of gravity to move the mobile phase to through the column. Due to the small amount of separated samples in analytical HPLC, typical column dimensions are 2.1 to 4.6 mm in diameter and 30 to 250 mm in length. HPLC columns are also made with smaller adsorbent particles (2–10 μm particle size). This gives HPLC superior resolving power (the ability to "distinguish" compounds) when separating mixtures, making it a popular chromatographic technique.

The HPLC instrument typically (Figure 1.1) includes a degasser, pumps, sampler, columns and detector. The sampler brings the mixture of analytes into the mobile phase stream which transports it to the column. The pumps deliver the desired flow rate and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component exiting the column, thereby enabling quantitative analysis of sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some designs of mechanical pumps in an HPLC instrument can mix multiple solvents together in time-changing ratios, generating a gradient in mobile phase composition. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows the temperature at which the separation is performed to be adjusted [10,11].

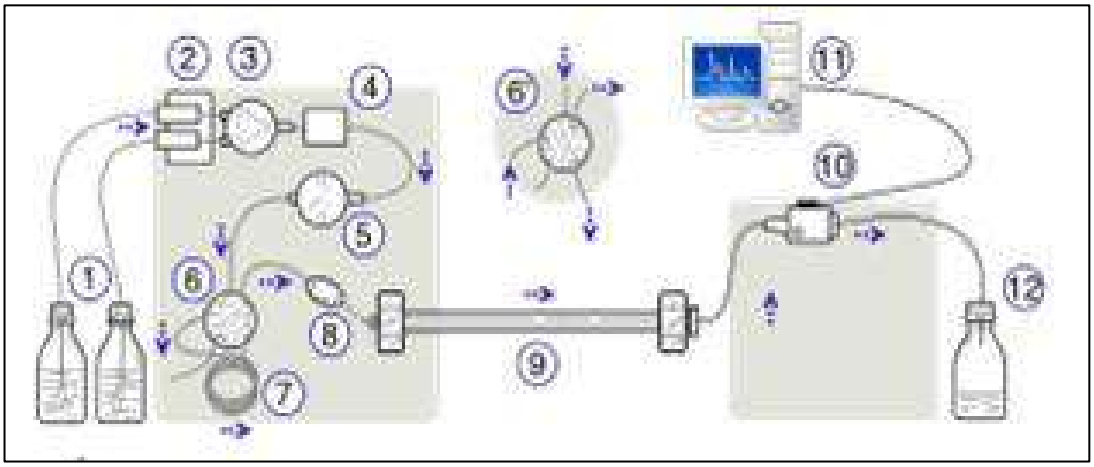


Figure 1.1. Schematic representation of an HPLC unit:

(1) Solvent reservoirs, (2) solvent degasser, (3) gradient valve, (4) mixing tank for mobile phase discharge, (5) high pressure pump, (6) switch valve in "position injection valve", (6') switching valve in "load position", (7) sample injection loop, (8) pre-column (guard column), (9) analytical column, (10) detector (i.e., UV, MS, refractometer, etc.), (11) data acquisition, (12) waste or fraction collector.

1.2. Principal features of reversed-phase HPLC (RP HPLC)

Reversed-phase high-performance liquid chromatography (RP HPLC) is the most commonly used mode of HPLC and, as the name implies, this mode is just the reverse of NP-HPLC, whereby the stationary phase is more non-polar than the eluting solvent [12]. Generally, RP HPLC has a non-polar stationary phase, a common stationary phase is silica gel that has been surface modified with dimethylalkylsilyl functional groups (the reagents are RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}). With such stationary phases, the retention time is longer for less polar molecules, while polar molecules elute more easily (at the start of the run) [13–16].

An investigator can increase retention times by adding more water to the mobile phase; thus, making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the more hydrophilic mobile phase. Likewise, a researcher can reduce the retention time by adding more organic solvent to the eluent. RP HPLC is so commonly used that it is often mistakenly called "HPLC" without further specification.

The pharmaceutical industry regularly uses RP HPLC to qualify drugs before their release [3–6].

One of theoretical concepts in RP HPLC is the principle of hydrophobic interactions, which originates from the high symmetry of the water dipole structure and plays the most important role in all life science processes. RP HPLC allows the measurement of these interactive forces. Binding of the analyte to the stationary phase is proportional to the area of contact around the nonpolar segment of the analyte molecule upon association with the C_{18} stationary phase (modified silica gel) [8,16–20].

This solvophobic effect is dominated by the force of water for "cavity reduction" around the analyte and the C_{18} chain compared to the complex of the two [21]. The energy released in this process is proportional to the surface tension of the eluent (water: 7.3×10^{-6} J /cm², methanol: 2.2×10^{-6} J/cm², acetonitrile: 2.8×10^{-6} J /cm²) [22] and the hydrophobic surface of analyte and ligand respectively. Retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution utilizes this effect by automatically reducing the polarity and surface tension of the aqueous mobile phase during analysis [23].

The structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C–H, C–C, and usually non-polar chemical bonds, such as S–S and others) is retained longer because it does not interact with the structure of water. On the other hand, analytes with a higher polar surface (conferred by the presence of polar groups, such as –OH, $–NH_2$, $–COO^-$ or $–NH_3^+$ in their structure) are less retained because they are better integrated into water. Such interactions are subject to steric effects since very large molecules may have only restricted access to the pores of the stationary phase, where interactions with surface ligands (alkyl chains) take place. Such surface clutter generally results in less retention [9].

The retention time increases with the hydrophobic (non-polar) surface [9,10]. Branched-chain compounds elute faster than their corresponding linear isomers because the overall surface area is decreased, but in RP HPLC this effect is manifested in a less

extent than in gas chromatography. Similarly, organic compounds with C–C single bonds elute later than those with a C=C or C≡C triple bond, because the double or triple bond is shorter than a C–C single bond and provide a less increments to the polarizability of molecules of organic compounds.

We can confirm this statement by reference retention indices in RP HPLC for ethylbenzene (1100 ± 10) and styrene (1058 ± 16).

Apart from the surface tension of the mobile phase (organizing force in the structure of the eluent), other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a moderate linear increase in the surface tension of aqueous solutions (about 1.5×10^{-7} J/cm² per Mole for NaCl, 2.5×10^{-7} J/cm² per Mole for $(NH_4)_2SO_4$), and because the entropy of the analyte-solvent interface is controlled by the surface, the addition of salts tends to increase the retention time. This technique is used for the gentle separation and recovery of proteins and the protection of their biological activity in protein analysis (hydrophobic interaction chromatography, HIC) [9,24].

1.2.1. Effect of pH of mobile phase

Another important factor is the pH of the mobile phase as it can alter the hydrophobic character of the analyte. For this reason, most methods use a buffering agent, such as sodium phosphate, to control the pH. Buffers serve several purposes: pH control, neutralization of charge on the silica surface of the stationary phase, and act as ion pairing agents to neutralize analyte charge. A volatile organic acid such as acetic acid, or more commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column eluent [25]. Trifluoroacetic acid or ammonium trifluoroacetate are rarely used in mass spectrometry applications due to their persistence in the detector and solvent delivery system, but can be effective in improving the retention of analytes such as carboxylic acids in applications using other detectors, as it is a fairly strong organic acid. The effects of acids and buffers vary by application but generally improve chromatographic resolution [26].

Choice of buffer is typically governed by the desired pH value. It is important that the buffer has a pK_a different from the desired pH. A rule is using the buffer with to choose a buffer with a $|pK_a - pH| > 1$ (Table 1.1) [25].

Table 1.1. HPLC buffers, pK_a values and pH range.

Buffer	Average pH values	Prohibited ranges of pK_a values
Trifluoroacetic acid (TFA)	<2 / 0.5	1.5 – 2.5
KH_2PO_4/K_2PO_4	7.2	6.2 – 8.2
KH_2PO_4/ phosphoric acid	2.1	1.1 – 3.1
Ammonium acetate*	4.8 9.2	3.8 – 5.8 8.2 – 10.2
Ammonium formate*	3.8 9.2	2.8 – 4.8 8.2 – 10.2
Ammonium hydroxide/ ammonia*	9.2	8.2 – 10.2
Potassium formate / formic acid	3.8	2.8 – 4.8
Potassium Acetate/ acetic acid	4.8	3.8 – 5.8
Borate ($H_3BO_3/Na_2B_4O_7$ $10H_2O$)	9.2	8.2 – 10.2

* Volatile buffers; can be used for LC-MS.

Reversed-phase columns are quite difficult to damage compared to normal silica columns; however, many reverse phase columns are made of silica particles derived from alkyls and should never be used with aqueous bases as they will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment [25,27]. Otherwise, the basic media can be dangerous for silica sorbents of the columns. RP HPLC columns should be flushed with clean solvent after use to remove residual acids or buffers, and stored in an appropriate solvent composition. The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be maintained.

An interesting test for the metal content within chromatographic-column is to inject a sample that is a mixture of 2,2'- and 4,4'-bipyridines. Because 2,2'-bipy can chelate metal

ions, the peak shape for 2,2'-bipy will be distorted (tailed) when metal ions are present on the silica surface [28]. The sample mixture to be separated and analyzed is introduced, in a small discrete volume (typically microliters), into the mobile phase stream percolating through the column. Sample components move through the column at different speeds, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The rate of each component depends on its chemical nature, the nature of the stationary phase (column) and the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analyte [29].

1.2.2. The composition of mobile phase

Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use mobile phases without water. The aqueous component of the mobile phase may contain acids (such as formic, trifluoroacetic or phosphoric) or salts to aid in the separation of sample components. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Phosphate buffers can be replaced with sulfate buffers when analyzing organophosphate compounds [30].

The chosen composition of the mobile phase depends on the intensity of the interactions between the various sample components "analytes" and the stationary phase (e.g., hydrophobic interactions in reversed phase HPLC). Depending on their affinity for the stationary and mobile phases, the analytes partition between them during the separation process that takes place in the column. This partitioning process is similar to that which occurs in a liquid-liquid extraction, but it is continuous rather than stepwise. In this example, using a water/acetonitrile gradient, more hydrophobic components will elute (leave the column) later, once the mobile phase is more concentrated in acetonitrile (i.e., in a mobile phase of higher elution strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column components and the sample. Often, a series of tests is performed with the sample to find the HPLC method that gives an adequate separation [31,32].

1.2.3. Modes of elution (isocratic and gradient)

The composition of the mobile phase during the chromatographic analysis can be kept constant "isocratic elution mode" or varied "gradient elution mode".

Isocratic elution is generally effective in separating sample components that are very different in their affinity for the stationary phase [33]. In gradient elution, the order of elution can change as the dimensions or flow rate changes. In gradient elution, the composition of the mobile phase typically varies from low to high elution strength. The elution strength of the mobile phase results in analyte retention times with high elution strength producing fast elution, meaning that retention times are shorter. A typical gradient profile in reversed phase chromatography may start at 5% acetonitrile (in water or aqueous buffer) and progress linearly to 95% acetonitrile in 5 (usually it is considered as “too quick”) to 25 minutes. Periods of constant mobile phase composition may be part of any gradient profile. For example, the mobile phase composition may be held constant at 5% acetonitrile for 1-3 minutes, followed by a linear change to 95% acetonitrile [34].

1.3. Dependence of retention times of analytes in RP HPLC *vs*. composition of an eluent

The early liquid chromatography procedures presuppose the use of a pure solvent as the mobile phase, and the choice of this solvent was an essential component of chromatographic enhancement. Later chromatographers discovered that by combining two solvents in the variable ratio, retention can frequently be adequately managed. Prediction of retention from the organic modifier content of the mobile phase is now widely addressed since binary mixes of a weak diluent and a strong modifier are now frequently used [35–37].

Examining the retention with various modifier concentrations, followed by fitting to an empirical or theoretical equation, is how this relationship is investigated (in both HPLC and TLC) [38]. The retention can then be extended to pure diluent or interpolated (to determine the modifier concentration producing optimum separation) (concentration equal to zero). Extrapolation in reversed-phase HPLC or TLC is a common method for determination of solute lipophilicity [39].

The first and largest group of retention models were used to predict the logarithm of the retention factor (ln$k$; where $k = (t_R - t_0)/t_0$).

One of the simplest models, the Soczewinski- Wachtmeister equation, assumes a linear dependence of the logarithms of the volume percentage of the organic modifier [38,40].

$$\ln k = aY + b \qquad (1.1)$$

where Y is the volume fraction of a modifier. In the literature this model is regularly referred to as the linear solvent strength (LSS) model [41].

Another simple proposal is the log-log dependence which assumes the linear relationship between ln$k$ and the logarithm of the volume fraction of the modifier:

$$\ln k = a\ln Y + b \qquad (1.2)$$

The mathematical properties of the logarithm prevent this dependence from being extrapolated to zero modifier content, and the constant term denotes the retention when ln Y equals zero (when $Y = 1$). This model is frequently referred to as the Snyder-Soczewinski equation [38,42] because Soczewiński developed it based on earlier findings by Snyder.

Both models are often employed in retention modeling, and there is no overarching rule dictating which model will best match the retention data in any given situation. In general, reversed-phase systems exhibit semilogarithmic dependency more frequently than normal-phase systems, which exhibit entirely logarithmic data correlation [38].

These models have a strong theoretical foundation in the chromatographic process. In reversed-phase chromatography, the intercept can be interpreted as the logarithm of the partitioning coefficient between the stationary phase and the water. The slope in equation (1.1) is strictly related to the standard free energy of solute transfer from pure water to pure organic mobile phase [43]. The nature of the adsorption is described by the slope in equation (1.2), which is near to 1 when one-point adsorption takes place.

Many variants of equation (1.1) have been presented, and this equation is a particular example of them since small curvature (nonlinearity) of the modeled dependence is relatively common in retention modeling [44]. The simplest extension is a quadratic modification proposed by Schoenmakers [45]:

$$\ln k = aY^2 + bY + c \qquad (1.3)$$

The coefficients *a* and *b* of this quadratic equation have a well-known theoretical background; they can be roughly calculated from interaction indices, solubilities, and normalized contact free energies [46]. However, this quadratic equation can only be treated as an approximation (following Taylor's theorem) of real complex dependence. Additionally, a thorough statistical comparison of linear and quadratic dependences has been provide [44]. Schoenmakers et al. [47] devised a further extension that includes a square root term:

$$\ln k = aY^2 + bY + cY^{1/2} + d \qquad (1.4)$$

and a generalization with a cubic term by Nikitas et al. [48]:

$$\ln k = aY^3 + bY^2 + cY + d \qquad (1.5)$$

The coefficients in equations (1.4) and (1.5), which were entirely provided empirically, no longer have any meaningful interpretation. A fascinating optimum three-parameter equation with a relationship to equation (1.1) was put up by Nikitas in the same study after evaluating a number of novel ideas.

$$\ln k = a - \ln(1 + bY) - cY / (1 + bY) \qquad (1.6)$$

which echoes an earlier suggestion made by Neue et al. [49]. Zapala et al. [50] provided another generalization of the Soczewinski-Wachtmeister model, which becomes eq. (1.1) when $m = 1$.

$$\ln k = aY^m + b \qquad (1.7)$$

Zenkevich [51], who recently introduced a flexible recursive method to retention modeling, suggested modeling retention linearly but in a recursive manner:

$$\ln[k(Y + \Delta Y)] = a\ln k(Y) + b, \qquad \Delta Y = \text{const} \qquad (1.8)$$

When $a = 1$, this is reduced to eq. (1.1) (linear dependence), but when $a \neq 1$ and $b = 0$, it transforms into a pure geometric progression. Other situations are intermediate; a linear recurrence can fit extremely complex nonlinear functions with acceptable accuracy. (This relationship will be characterized in more detail in the following discussion).

The second group includes the models used for modeling k itself or its reciprocal. These are:

- a linear dependence of reciprocal value of k on the volume fraction of the modifier, proposed by Row [52]:

$$1/k = aY + b \qquad (1.9)$$

a quadratic modeling of the reciprocal of k, proposed by McCann [48,53]:

$$1/k = a + bY + cY^2 \qquad (1.10)$$

- an approach proposed by Kaczmarski et al. [54]:

$$1/k = pY + q(1 - Y) \qquad (1.11)$$

- and a quite complicated, but theoretically well based equation given by Zapała [55]:

$$k = a(1 + bY + c/Y)/(1 + dY) \qquad (1.12)$$

The final group relies on the thin-layer chromatography prediction of R_F (R_F is comparable to $1/[1 + k]$). Kowalska [55–57] presented these models. The equation utilized for normal-phase retention was:

$$R_F = aY^{1/2} + b(1 - Y) + c \qquad (1.13)$$

whereas a modified form was suggested for reversed-phase retention:

$$R_F = aY^{1/2} + b(1 - Y)^{1/2} + c \qquad (1.14)$$

A new equation is proposed based on the Box-Cox transformation [58], which limits the power transformation, the natural logarithm, and the variable by one more variable, λ. It is spelled as follows [59]:

$$\ln k = a(Y^{\lambda} - 1)/\lambda + b \qquad (1.15)$$

This equation simulates a semi-logarithmic dependence similar to equation (1.1) when λ = 1. (The only difference is that the modifier fraction is shifted by 1, so a and b are not the same). When λ = 0, the parenthetical expression turns into a natural logarithm and the equation becomes the same as eq. (1.2).

1.4. Recurrent approximation of the dependences of chromatographic retention times

1.4.1. General properties of recurrence relations

It has been demonstrated that, within the bounds of certain taxonomic groups (primarily, homologous series), monotonic variations in the majority of the physicochemical properties of organic compounds (A) may be approximated by the simplest linear (first order) recurrent equations [51,60–67]:

$$A(n + 1) = aA(n) + b \qquad (1.16)$$

where the least squares approach is used to calculate the linear regression coefficients a and b. Recurrent relations (1.16) are also applicable to the approximation of the discrete properties of homologs A and equidistant values of functions with continuous attributes $B(x)$, such as temperature ($x = T$), pressure ($x = P$), concentration ($x = C$), etc. [65]:

$$B(x + \Delta x) = aB(x) + b, \quad \Delta x = \text{const} \qquad (1.17)$$

* The same relationship (1.8) where $\ln k = B$ and $Y = x$ in this case.

The retention parameters of analytes in gas chromatography and HPLC belong to these properties [51,65]. In particular, the dependence of the retention times of homologs under isothermal and isocratic separation conditions $t_R(n_C)$ on the number of carbon atoms in the molecule, can be estimated to a high degree of precision by the recurrent equation (1.19) instead of equation (1.18):

$$\log(t_R - t_0)_n = an_C + b \qquad (1.18)$$

$$t_R(n+1) = at_R(n) + b \qquad (1.19)$$

The mathematical properties and analytical applications of recurrent relations have been considered in detail in publications [51,60–67]. Recurrent relations make it possible to reveal the existence of limiting values for both discrete (A) and continuous properties $B(x)$.

If the coefficients a of recurrence relations (1.16) or (1.17) satisfy the condition $a < 0$; the values of A or $B(x)$ tend to finite limits with a hypothetically unlimited increase in arguments ($n \to \infty$ or $x \to \infty$):

$$\lim [A \text{ or } B(x)]_{n \text{ or } x \to \infty} = b/(1-a) \qquad (1.20)$$

If recurrent relations are applicable to variables A or $B(x)$, the same relationships are also applicable to the values of the monotonic functions of these variables, for example A^2, log$A$, etc.

Each point in the depicted recurrent relationships corresponds to two values of A or B, in contrast to the plots of the functions $A(n)$ or $B(x)$, where each point corresponds to one value of A or B.

1.4.1. Recurrent relation in chromatography (GC, RP HPLC)

When we apply the recurrence relation (1.17), it is not necessary to transform absolute retention times into corrected retention times with the subsequent calculation of their logarithms in accordance with the special characteristics of the recurrent relations [51,60–67].

Another illustration is the temperature dependence of the corrected gas-chromatographic retention times, which is represented by the Antoine equation:

$$\log t'_{R,n} = a/T + b \qquad (1.21)$$

but which can also be represented by the corresponding recurrent relation for uncorrected retention times: ΔT is a constant, hence:

$$t_R(T + \Delta T) = at_R(T) + b \qquad (1.22)$$

In reversed-phase HPLC, several equations for describing the functions $t_R(C)$, where C is the concentration of organic modifier in an eluent, are well known; however, all of them can also be replaced by the single recurrent relation.

$$t_R(C + \Delta C) = at_R(C) + b \qquad (1.23)$$

It is easy to check that, irrespective of the chemical nature of compounds, unique recurrent equation (1.23) can be used to approximate the concentration dependences of the retention times of sorbates in reversed phase high performance liquid chromatography with correlation coefficients no less than 0.999 for analytes that show no anomalies of their chemical composition (e.g., that form no hydrates). However, if the reversible formation of hydrates (variations of their composition, when $K_{hydr} \approx 1$) it led to the deviations of recurrent dependencies (1.23) from linearity [68–71].

1.5. Retention index systems in RP HPLC

Retention indices (RI) are among the most repeatable retention properties in chromatography [72]. This is because the effects of different separation mode parameters are balanced by accounting for the retention parameters of two reference components, often those that are closest to the target analyte:

$$RI_x = RI_n + (RI_{n+k} - RI_n)\,[f(t_{R,x}) - f(t_{R,n})] / [f(t_{R,n+k}) - f(t_{R,n})] \qquad (1.24)$$

where t_R, $t_{R,n}$ and $t_{R,n+k}$ are the retention times of the target analyte (x) and reference components with the values RI_n and RI_{n+k} assigned to them, $f(t_R) = t_R$ (linear indices), $f(t_R) = \log t_R'$ (logarithmic indices).

The RI concepts of gas chromatography [72] are also the most "popular," but there are several reasons for the interlaboratory reproducibility of such parameters, otherwise there may be some underestimation of their values from the conditions of decomposition. The main feature of gas chromatography is the temperature dependence of the indices, $d\mathrm{RI}/dT$, which is frequently considered linear, limited to the first member of the RI(T) function and the Taylor series (T_0-conventionally is selected as the standard temperature):

$$\mathrm{RI}(T) = \mathrm{RI}(T_0) + d\mathrm{RI}/dT\,(T - T_0) \qquad (1.25)$$

Many attempts have been made to approximate the dependencies of RI(T) by more complex nonlinear functions, but they have not gained widespread acceptance. One can note, among other things, the dependence of RI on the relative numbers of target and reference components [73,74], as well as (for the most polar compounds) the reality of sorption effects in chromatographic systems. The coefficients of the temperature dependence of the gas chromatographic retention indices $\beta = d\mathrm{RI}/dT$ most often satisfy the inequality $\beta > 0$ and increase with increasing in number and size of cycles for the majority of organic compounds, the carbon excitable molecules' sizes, and number of branches [75–77]. In other words, discrepancies between the topological connectivity of target sorbate molecules and reference *n*-alkane molecules can be seen in the values of β (do not contain cycles and do not have branches of the carbon chain).

In reversed-phase (RP) HPLC, the RI concept is used significantly more rarely because of a smaller range of variations in the values of these parameters, a larger number of experimental conditions that affect them [78–82]. Nevertheless, even in this method, retention indices turn out to be the most reproducible in comparison with other retention characteristics [81].

The ratio of the organic (C, vol. %) to water components of an eluent is the factor that has the largest impact on the RI readings. As a result, the coefficients representing the dependence of the results on the quantity of the organic solvent in the composition of the $d\mathrm{RI}/dC$ eluent, (conventionally standard concentration value), is the equivalent to the similar coefficients in gas chromatography:

$$RI(C) = RI(C_0) + dRI/dC\ (C - C_0) \qquad (1.26)$$

For some compounds, RI values at various C have been known to be determined, mostly to adjust their "sensitivity" to experimental conditions [80]. Contrary to gas chromatography, the dRI/dC coefficients' influencing factors were not expressly taken into account. This is most likely brought on by the HPLC's objectively less revealing retention indices. However, the values of the dRI/dC coefficients proved to be useful for elucidating the chemical nature of the analyzed compounds when detecting the formation and detection of organic compound hydrates in the HPLC using recurrent approximation of the sorbate retention parameters [69]. As a result, they merit discussion.

The selection of the list of reference compounds is the initial step in applying any RI system. With assumed values $RI_n = 100n_c$, the majority of them in GC are based on the readily available *n*-alkanes, C_nH_{2n+2}. The series on *n*-alkyl phenyl ketones, $PhCOC_nH_{2n+2}$ (also known as Smith's RI system [78,80]), is the most "popular" RI system in RP HPLC and uses the same hypothesized RI values as $RI_n = 100n_c$. Other hypotheses, however, have not yet seen widespread application. They are based on sets of *n*-alkyl benzenes, 1-nitroalkanes [83], etc.

The selection of the function $f(t_R)$ required for the calculation of retention indices with relationships presents a unique difficulty (1.24). The following general regularity is valid in isocratic regimes of HPLC elution:

$$\log(t_R - t_0)_n = an_c + b$$

It is equivalent to $$RI = a'\log(t_R - t_0) + b' \qquad (1.27)$$

As a result, the Kovats retention indices system's [84] ground function, $f(t_R)$, should equal $\log(t_R - t_0)$. The linear RI system ($f(t_R) = t_R$) [85] is preferred in a variety of gradient elution regimes (which are comparable to temperature programming in GC). The final type of this function is a generalization of the two prior types into the so-called lin-log RI system [86,87]:

$$f(t_R) = t_R + q\log(t_R - t_0) \qquad (1.28)$$

Using t_R data for at least three reference compounds, such as (in the simplest example) three successively eluted standards, the variable parameter q can be calculated:

$$q = (t_{R,\,n-1} + t_{R,\,n+1} - 2t_R) / (2\log t'_{R,\,n} - \log t'_{R,\,n-1} - \log t_{R,\,n+1}) \qquad (1.29)$$

In general, this auxiliary parameter can be calculated using any set of reference substances [88].

The RI value of the initial reference drug is related to the most significant difference between the application of RIs in GC and HPLC. Only a few (ideally inorganic) molecules are defined by RI < 100 when their calculation using formula (1.24) necessitates extrapolation, if we consider the simplest *n*-alkane (methane CH_4). For example, the list of these compounds for polymer sorbent Porapak Q is restricted by components of air (N_2, O_2 with RI ~ 50 ± 15), CO (60 ± 6), Ar (62 ± 11), NO (80 ± 16), and CF_4 (83 ± 9) [89]. By applying interpolation at the proper selection of reference *n*-alkanes, RIs computed with formula (1.24) can be used to characterize any other multitudes of potential analytes with RI > 100.

The scenario in RP HPLC is essentially different. Acetophenone, the first reference chemical in a group of *n*-alkyl phenyl ketones, has a strong hydrophobicity ($\log P$ = 1.66 ± 0.06 [90,91]). As a result, there are significally more hydrophilic organic compounds that need to be rinsed in order to avoid the common HPLC data processing issue of RI calculation by extrapolation outside the range restricted by reference compounds. Due to the existence of the general regularity (1.27), RI extrapolation in isocratic regimes is not problematic.

We can determine any RI values within the range of $t_0 < t_{R,\,X} \leq t_R$ (2) and even more by mentally calculating the retention durations for two reference components [t_R (1) and t_R (2)] and hold-up time t_0 (by extrapolation "up"). Numerous organic compounds are characterized by values RI < 800 that are published without any explanation of the factors used in their computation. The majority of them have only been determined in isocratic conditions [80].

Unfortunately, due to the lack of a suitable function RI (t_R) at the range to $t_0 < t_{R,X} \leq t_R$ (1), this most basic mode of calculations is inapplicable at all gradient elution regimes, especially in multi-step regimes mixing isocratic and gradient steps.

Various interpretations are made of the parameter t_0 significance. The true retention duration of the non-sorbable component correlates significantly more closely to gas chromatography [92] than to RP HPLC [93]. On the other hand, it can be viewed as a specific coefficient that guarantees the linearity of relations (1.27). For instance, using the maxima of the correlation coefficients as a standard, R (see the literature cited in [94]). However, the theoretical and actual values of t_0 can diverge widely, and RP HPLC is the most common example. Additionally, in the latter scenario, t_0 experimental determination typically requires more time and eluent consumption.

The formula of Peterson and Hirsch [95] is the most well-known relation for calculating t_0 from the retention times of three subsequent homologues of reference components under isothermal (GC) or isocratic (RP HPLC) conditions:

$$t_0 = (t_{R,1} \times t_{R,3} - t^2_{R,2}) / (t_{R,1} + t_{R,3} - 2t_{R,2}) \qquad (1.30)$$

This formula, which was presented for the gas chromatographic version of separation but is equally applicable in RP HPLC, results from relation (1.27), namely the requirements $\log(t_{R,2} - t_0) - \log(t_{R,1} - t_0) = \log(t_{R,3} - t_0) - \log(t_{R,2} - t_0)$ solved with respect to t_0 [96]. If so, then the values of t_0 calculated in this way naturally lead the values of the logarithms of the corrected retention times of all reference components to a linear dependence on the number of carbon atoms in the molecule, which is schematically shown in the Figure 1.2. Since the correlation coefficient in this example is $R = 1$, then the errors of the coefficients a and b can be neglected.

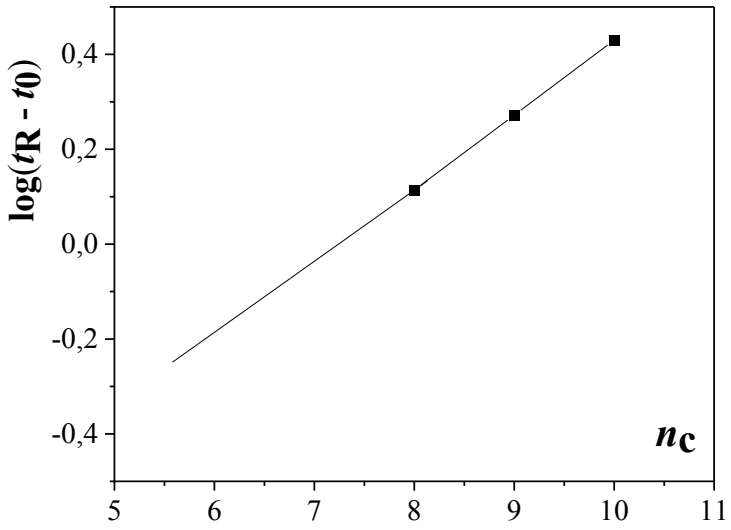


Figure 1.2. Linear dependence of the logarithms of the reference *n*-alkyl phenyl ketones adjusted retention times in RP HPLC *vs*. the number of carbon atoms in molecule. Precalculated t_0 value providing the parameters of linear regression (2.4.3) $a = 0.158$, $b = -1.1$ at the condition $R = 1$ was selected.

1.6. Literature information about hydrates of organic compounds

1.6.1. The formation of hydrates of organic compounds

The formation of hydrates (in the general case, solvates) by inorganic compounds (primarily salts) in the crystalline state and in aqueous solutions is well known [97]. Numerous inorganic hydrates can be isolated in an individual state and are described using a variety of techniques. The covalently non-bonded hydrates of different organic molecules ($X \times nH_2O$) are no less numerous, but their often-poor stability makes it difficult to characterize them:

$$X + nH_2O \rightleftharpoons X \times nH_2O \qquad (1.31)$$

Confirming the production of such hydrates can be challenging due to the limited solubility of hydrophobic chemical molecules in aqueous systems.

The potential for hydrate formation is typically not included among other features of organic compounds in the reference literature (see, for instance, papers [98–100]. Even when it appears to be absolutely important, such as in reverse phase HPLC, their formation is very occasionally taken into account. Although the analytes at the outlet of the chromatographic column are parts of aqueous-organic solutions and may exist in the form of hydrates, they are typically (by default) ascribed the structures of anhydrous forms in this analytical procedure. The reversible production of polar hydrates may be one of the principal factors causing abnormalities in the chromatographic retention of analytes in reverse phase HPLC since their characteristics differ greatly from those of their less polar non-hydrated analogs. This also explains why the retention parameters in this method can only be somewhat predicted using the computed values of the hydrophobicity factors of non-hydrated forms ($\log P$) [101]. A consistent interlaboratory scatter in the values of a substance's solubility in water is also caused by fluctuations in the amount of water that a material contains, which must be taken into account while processing data [102].

The lack of a classification for hydrates of organic compounds in the Universal Decimal Classification (UDC; accepted in Russia and some European countries) is an intriguing illustration of a rather "disdainful" attitude towards them.

Despite the comparatively seldom mention of organic compound hydrates, the creation of a vast number of compounds of various chemical natures is currently known or assumed. The hydration constants, K_{hydr} are their most objective property, however due to experimental challenges; they have only been established for a small number of substances [103]:

$$K_{\mathrm{hydr}} = \frac{[\mathrm{X} \times n\mathrm{H_2O}]}{[\mathrm{X}] \times [\mathrm{H_2O}]^n} \qquad (1.32)$$

The characteristic of hydrates is the equilibrium constant. If the equilibrium constant is much less than one $K_{hydr} << 1$, the formation of hydrates in solutions can be neglected. Otherwise, another inequality $K_{hydr} >> 1$ means forming the hydrates of high stability. If the value of equilibrium constants is close to one $K_{hydr} \approx 1$ both forms (hydrated and non-hydrated) exist in solutions together.

The direct detecting the formation of hydrates by chromatographic or mass spectrometric methods seems to be impossible due to the instability of most of them, but in reverse phase HPLC can lead to anomalies in the dependences of their retention parameters (t_R) on the content of the organic component (C) in the eluent, $t_R = f(C)$. Recurrent approximation of these dependences has recently been demonstrated to make the detection of such anomalies achievable [70,104]. One of the goals of current work is to provide a more thorough analysis of the general challenges surrounding the creation of organic compound hydrates in order to better comprehend the potential of this data processing approach. Using two anti-tumor medications as an example, characteristics of the recurrent approximation of retention parameters in HPLC are carefully addressed [70,104].

1.6.2. Typical regularities of the hydrate formation of organic compounds

Many organic compounds, both natural and synthetic, may not always have actual structures in aqueous solutions that match their nominal structural formulas for a variety of reasons, such as the production of analyte hydrates and changes in tautomeric equilibrium positions. It is best to start the problem's discussion with a variety of organic compounds for which the existence of hydrates is presumed or demonstrated in order to characterize the issue.

Table 1.2 and Table 1.3 organize the material that has been discussed. 52 substances are listed in Table 1.2 whose hydrates are unstable in aqueous solutions. For each compound, the molecular weight of the anhydrous forms, the CAS number (Chemical Abstracts Service), and some of the most distinctive characteristics (primarily, melting and boiling points, T_m and T_b) are listed. For hydrates, the CAS numbers (if any), stoichiometric compositions (the number of bound water molecules), and (rarely) some additional information are provided. Links to original publications are provided in the last column, References; however, due to a lack of information, most of the time only the names of the websites that specifically mention a given hydrate are noted.

The arrangement of the compounds corresponds to different classes of organic compounds, including aromatic hydrocarbons, carbonyl compounds, phenols, carboxylic acids, acid amides, and then compounds with varied chemical properties (without special systematization). For comparison, Table 1.2 also includes a number of chemically comparable substances (3,4-dichlorophenol, salicylic acid, theobromine, and dimethyl sulfone), for which no information on the formation of hydrates was available.

Table 1.2. Physicochemical characteristics of unstable hydrates of selected organic compounds.

Compound	M W	Anhydrous form		Hydrates		Reference
		CAS №	Properties	CAS №	Composition	
Anthracene	178	120-12-7	T_b 340 °C	188974-01-8	1 : 1	PubChem
Phenanthrene	178	85-01-8	T_b 340 °C	919080-09-4	1 : 1	PubChem, ChemSpider
Formaldehyde	30	50-00-0	T_b -25±1 °C	463-57-0 53280-35-6 53280-36-7	1 : 1, Oligomer hydrates (n:1) K_{hydr} ~10^3	[105–107] PubChem, ChemSpider
Acetaldehyde	44	75-07-0	T_b 22 °C	-	1 : 1	ChemSpider, [108,109]
Glyoxal	58	107-22-2	T_m 15 °C T_b 51 °C	4405-13-4 Trimer dihydrate	Oligomer hydrates (n:1) K_{hydr} ~10^3	PubChem, Chem Book
Benzaldehyde	106	100-52-7	T_b 179 °C	4403-72-9	2 : 1, pK_a 14.9, K_{hydr} ~ 1×10^{-2}	[103], ChemSpider, PubChem, Mol Base, etc.
Difluorochloro-acetaldehyde	114	811-96-1	T_b 17.8 °C	63034-47-9	-	ChemSpider PubChem, Mol Base, etc.
Acetone	58	67-64-1	T_b 56.1 °C	18879-06-6 Clathrate hydrate	1 : 1, 1 : n K_{hydr} ~ 1.4×10^{-3}	[103], Chemical Encyclopedia
2- Butanone	72	78-93-3	T_b 79.6 °C	-	1 : 1	PubChem
1, 1, 1-Trifuoroacetone	112	421-50-1	T_b 22 °C	-	1 : 1	Spectra Base
Cyclohexanone	98	108-94-1	T_b 155.6 °C	28553-75-5	1 : 1	ChemSpider
Acetophenone	120	98-86-2	T_b 202 °C	-	K_{hydr} ~ 6.6×10^{-6}	[103], PubChem Sigma-Aldrich
2-Hydroxyaceto phenone	136	118-93-4 582-24-1	T_m 4.5 °C T_b 213-218 °C	-	1 : 1	PubChem, ChemSpider, Sigma-Aldrich
Vanillin	152	121-33-5	T_m 81-83 °C	-	-	Hydrate Web
Benzophenone	182	119-61-9	T_m 48.5 °C T_b 305 °C	-	K_{hydr} ~ 1.7×10^{-7}	[103], Sigma-Aldrich, ChemSpider
Phenol	94	108-95-2	T_m 40.5 °C T_b 181 °C	217182-78-0 144796-97-4	1 : 1	PubChem, Sigma-Aldrich
2,3-Dichlorophenol	162	576-24-9	T_m 56 °C	848169-92-6	1 : 1	ChemSpider

Table 1.2. (Contd.)

Compound	M W	Anhydrous form		Hydrates		Reference
		CAS №	Properties	CAS №	Composition	
2,4-Dichlorophenol	162	120-83-2	T_m 41-45 °C	-	1 : 1	PubChem
3,4-Dichlorophenol	162	95-77-2	T_m 65-68 °C	-	-	-
Acetic acid	60	64-19-7	T_m 16-17 °C T_b 118-119 °C	19215-29-3 99294-94-7	1 : 1, 1 : 2	ChemSpider PubChem
Propionic acid	74	79-09-4	T_b 141.1 °C	-	1 : 1	PubChem, Sigma-Aldrich
Benzoic acid	122	65-85-0	T_m 122 °C T_b 250 °C	-	1 : 1	PubChem, Sigma - Aldrich, SynQuest
2-Hydroxybenzoic (salicylic acid)	138	69-72-7	T_m 158.6 °C T_b dec	-	-	-
3-Hydroxybenzoic acid	138	99-96-7	T_m 200-203 °C	-	1 : 1	PubChem
4-Hydroxybenzoic acid	138	99-06-9	T_m 214.5 °C	26158-92-9	1 : 1	ChemSpider, PubChem
Formamide	45	75-12-7	T_b 288 °C	56827-75-9	1 : 1	ChemSpider PubChem
Acetamide	59	60-35-5	T_m 79-81 °C T_b 221.2 °C	137547-89-3	1 : 1	PubChem
Propionamide	73	79-05-0	T_m 80 °C T_b 213 °C	-	1 : 1	PubChem
Benzamide	121	55-21-0	T_m 127-130 °C T_b 288 °C	-	1 : 1	PubChem
Urea	60	57-13-6	T_m 133 °C	163931-63-3	1 : 1, 2 : 1	PubChem ChemSpider
Ethanolamine	61	141-43-5	T_b 171 °C	922193-26-8	1 : 1	PubChem
Methylhydrazine	46	-	T_b 87-88 °C	-	1 : 1	PubChem
Triethyl phosphate	182	78-40-0	T_b 215 °C	114019-85-1	2 : 1	ChemSpider ChemSrc
Tributyl phosphate	266	126-73-8	T_b 289 °C	19517-53-4	1 : 1	ChemSpider ChemSrc
Caffeine	194	58-08-2	T_m 234 °C RI (GC) 1793±19	5743-12-4	1 : 1 RI (HPLC) 633 ± 27	PubChem, Chem-Spider Kegg Drugs, [110,111]
Theobromine	180	83-67-0	T_m 345-350 °C RI (HPLC) 586	-	-	-
Theophylline	180	58-55-9	T_m 272 °C 271-273 °C	5967-84-0	1 : 1 RI (HPLC) 580 ± 24	PubChem, [112–116]

Table 1.2. (Contd.)

Compound	M W	Anhydrous form		Hydrates		Reference
		CAS №	Properties	CAS №	Composition	
Sulfamide	172	63-74-1	T_m 165 °C	-	1 : 1 RI (HPLC) 481 ± 22	PubChem Hydrate Web
Piracetam	142	74791-74-9	RI (GC) 1649	68497-62-1	2 : 1, 1 : 1 RI (HPLC) 474	[105], ChemSpider, Hydrate Web
Dimethyl sulfoxide	78	67-68-5	T_b 189 °C	-	1 : 1, 1 : *n*	[117,118] PubChem Hydrate Web
Dimethyl sulfone	94	67-71-0	T_b 236±2 °C	-	-	-
Nitromethane	61	75-52-5	T_b 101.2 °C	-	1 : 1	PubChem
Trichloronitromethane	163	76-06-2	T_b 112 °C	-	1 : 1	PubChem
Chloroform	118	67-66-3	T_b 61.2 °C	-	1 : 1	PubChem
Acetonitrile	41	75-05-8	T_b 82 °C	128870-13-3	1 : 1	PubChem ChemSpider
1,3-Dioxolane	74	646-06-0	T_b 75.1 °C	34776-95-9	1 : 1	PubChem ChemSpider
1,4-Dioxane	88	123-91-1	T_b 101.3 °C	16468-05-6	1 : 1	ChemSpider
Benzene-1,2 dicarbo-xylic (phthalic acid)	166	88-99-3	T_m 191 °C	-	1 : 1	ChemSpider
4-Nitroaniline	138	100-01-6	T_m 48 °C	-	1 : 1	PubChem
4-Nitrophenol	139	100-02-7	T_m 113-114 °C		1 : 1	PubChem
3-Phenyl-3-ethyl-2,6-piperidinedione	217	18389-24-7 77-21-4	-	60490-74-6	1 : 1	PubChem
Phloroglucinol	126	108-73-6	T_m 215-220 °C	6099-90-7 1 : 2	1 : 1, 1 : 2	[119], PubChem

For formaldehyde, the constant of hydration $K_{hydr} = \exp(3769/T - 5.494)$, where T is the absolute temperature (K), was determined [106] in addition to K_{hydr}.

In addition to benzaldehyde, the formation of monohydrates was confirmed for numerous substituted benzaldehydes RC_6H_4CHO, where R = 4-CH_3 (CAS № 45792-02-7), 3-Cl (CAS № 85152-57-4), and 4-CF_3 (CAS № 85152-58-5); arylglyoxals of the general formula RC_6H_4COCHO, where R = 4-CH_3 (CAS № 7466-72-0), 4-OH (CAS № 197447-05-5 and 24645-80-5), 4-C_3H_7O (CAS № 99433-68-8), 3,4-$(CH_3O)_2$ (CAS № 163428- 90-8), 2-CF_3 (CAS № 745783-91-9), and 4-CF_3 (CAS № 1736-56-7); etc.

Besides that, the formation of hydrates of various carbonyl compounds was discussed by Buschmann et al. [120].

The formation of drug hydrates was considered in a special review by Heady et al. [121].

Objectively speaking, the unstable hydrates listed in Table 1.3 cannot be described by the values of any physicochemical parameters. As a result, the column "Composition" displays stoichiometric ratios between hydrate components only and, occasionally, characteristics of those compositions (such as the production of oligomer hydrates) and equilibrium constants Eq. (1.32) (known for five examples only). On the other hand, Table 1.3 illustrates the stable hydrates of organic compounds, making it easy to identify their physicochemical properties, including T_m and T_b. In many cases (8 out of 14), they are created by the nucleophilic addition of water to carbonyl groups.

After comparing the characteristics of the data in Table 1.2, we came to the conclusion that the most useful criterion for confirming the presumptions about the existence of the hydrates of organic compounds is the presence of corresponding CAS numbers (occasionally there are many such numbers). Although CAS numbers have not been allocated to 21 of the 52 compounds, it is thought that they exist. Keep in mind that the absence of these numbers does not rule out the possibility of isolating hydrates as separate substances; they can appear as the unstable parts of solutions in a state of dynamic equilibrium (1.31) including both their anhydrous forms and a solvent (water).

At room temperature, the values of K_{hydr} Eq. (1.32) are quantitative indicators of the stability of hydrates. Even with this limited knowledge, we can say that the condition $K_{hydr} \leq 10^{-2}$ is a sign of the instability of hydrates even if they are only known for five of the examples given in Table 1.2. For formaldehyde, $K_{hydr} \sim 10^3$, but the shifting of equilibrium (1.31) to the left makes sometimes impossible preparative isolation of its hydrate(s) from aqueous solutions.

The CAS numbers were predictably assigned to all of the actually existing hydrate types described in Table 1.3 in contrast to unstable hydrates. 12 of 14 hydrates in Table 1.3 have their physicochemical properties (most notably, T_b and T_m), which differed noticeably from their anhydrous equivalents' corresponding traits.

Table 1.3. Characteristics of stable hydrates of some organic compounds.

Compound	M W	Anhydrous form		Hydrates		Reference
		CAS №	Properties	CAS №	Composition	
Pyrene	202	129-00-0	T_b 145-148 °C T_m 404 °C	64201-64-5 1613-37-2	1 : 1 T_m 50-55 °C	ChemBook, ACROS Organics
1,2,3-Inadanetrione (ninhydrin)	160	938-24-9	T_m 250 °C RI 1574	485-47-2 2462-59-1	1 : 1, pK_a 8.47, RI (HPLC) 574 ± 17	Sigma-Aldrich PubChem, Merck, etc.
1,2,3,4-Tetraoxotetralin (oxoline)	188	30266-58-1	-	34333-95-4	1 : 2, log$P$= -0.55	PubChem, ChemBook, etc.
Trifluoroacetaldehyde	98	75-90-1	T_b -19.4±1.4 °C	421-53-4 33953-86-5	1 : 1 T_b 105 ± 1 °C	[122], PubChem, ChemSpider, MolBase, etc.
Pentafluoropropanal	148	422-06-0	T_b 2 °C	422-63-9	1 : 1, T_b 92 °C	Alfa Aesar, etc.
Heptafluorobutanal	198	375-02-0	T_b 28.2-29 °C	375-02-0	1 : 1, T_b 95 ± 1 °C	Alfa Aesar, etc.
Trichloroacetaldehyde	146	75-87-6	T_b 97.8 °C T_m -57.5 °C	302-17-0	1 : 1, T_b 96.3 °C T_m 57 °C, pk_a 9.66,10-11.3	PubChem, Sigma-Aldrich, etc.
Hexafluoroacetone	166	684-16-2	T_b -27.6±0.3 °C	677-71-4 10543-95-0 trihydrate 34202-69-2 Sesquihydrate 13098-39-0	1 : 1, T_b 100 ±7 °C K_{hydr} ~10^6, trihydrate T_m 18-21 °C, Sesquihydrate T_m 11-20 °C	PubChem, DrugBank, CameoChemicals ChemSpider, etc.
1,1,1-Trifluoro-3,3-dichloroacetone	180	-	-	126266-75-9 1049731-87-4	1 : 1, T_b 103 °C	PubChem, Sigma-Aldrich ChemBook, SynQuest, etc.
Glucose	180	50-99-7	T_m 150 °C	77938-63-7 14431-43-7	1 : 1, T_m 83-92 °C	ChemSpider, DuragBank, Sigma-Aldrich BioChemica
Ethylene diamine	60	107-15-3	T_b 116 °C	6780-13-8	1 : 1, T_m 118 °C n_D^{20}1.448-1.451, d_4^{20}0.96	PubChem, ChemSpider ChemBook, etc.
Piperazine	86	110-85-0	T_b 145-146 °C T_m 42-44 °C	16832-43-2 142-63-2	1 : 1, 1 : 6 T_b 125-130 °C T_m 42-46 °C	PubChem Sigma-Aldrich, DuragBank, etc.
Hydrazine	32	302-01-2	T_b 113.5-114 °C	7803-57-8	1 : 1, T_b 120-121 °C	[98–100]
Benzenesulfonic acid	158	98-11-3	T_m 51 °C	26158-00-9	T_m 42-49 °C	PubChem Sigma-Aldrich

The hydrated forms of two compounds (1,2,3,4-tetraoxotetralin and 1,1,1-trifluoro-2,2-dichloroacetone) are so stable that it is difficult to characterize their anhydrous analogues rather than the hydrates. The acidity constants (pK_a) of compounds whose anhydrous forms do not have active hydrogen atoms are another interesting confirmation of the formation of hydrates. The examples are pK_a = 8.47 for 1,2,3-indanetrione (ninhydrin), which occurs exclusively in the form of 2,2-dihydroxy-1,3-indanedione in aqueous solutions, and trichloroacetaldehyde (pK_a = 9.7–11.3) [123]. In the latter instance, attention is given to a wide range of pK_a value variations that are likely caused by changes in the ratio of the aldehyde to its hydration form depending on the concentrations of the target material and water in the solution.

K_{hydr} values for unstable hydrates are substantially lower than those for hexafluoroacetone, a fluorine derivative of carbonyl compounds, which forms a stable hydrate (which may be distilled without decomposition at atmospheric pressure). As a result, the range of K_{hydr} from 10^3 (formaldehyde) to 10^6 can be used to roughly predict the stability barrier of hydrates.

Obviously, HPLC is rather important mode of chromatographic separation. Nevertheless, some unsolved problems still exist; one of them is the detection of the formation of hydrates of organic compounds in aqueous solutions (1.31), including their formation during RP HPLC separation. However, the direct revealing the formation of hydrates by chromatographic or mass spectrometric methods seems to be impossible due to the instability of most of them.

The importance of this problem is the follows: one of the principles of analytical chemistry is one-to-one correspondence of the real forms of analytes to their chemical structures.

Chapter 2: Experimental part

2.1. Reagents and solvents

Preparations of the following compounds were used in our work: toluene, *o*-xylene, chlorobenzene and nitrobenzene ("reagent grade", for chromatography, Reakhim, Moscow), 2-methylbenzaldehyde, 4-methylbenzaldehyde, 4-methylacetophenone, 2-hydroxybenzaldehyde and 1-phenylpyrazolidine-3-one ("reagent grade ", Reakhim, Moscow), benzotriazole ("for photography", Reanal, Hungary), acetophenone, propiophenone, butyrophenone (Sigma-Aldrich Rus LLC, Russia), 2,3,5-trimethylphenol [Theodor Schuchardt, Munich, Germany (from the collection of natural compounds by PhD S. Kozhin, Leningrad State University)], phthalimide, ninhydrin, (Merck, Germany), 3-nitrophenol (indicator, British Drug Houses, LTD, UK) and *m*-toluylic acid diethylamide (DETA, insects' repellent, TU 2386-077 -00205357-2007, Luga).

N,N-substituted *p*-toluenesulfonamides (N,N-diethyl-*p*-toluenesulfonamide, N-allyl-*p*-toluenesulfonamide, N-*tert*-butyl-*p*-toluenesulfonamide, N-phenyl-*p*-toluenesulfonamide, N-hexyl-*p*-toluenesulfonamide, and N-benzyl-*p*-toluenesulfonamide) were synthesized ourselves by PhD T. Kornilova (St. Petersburg State University) from corresponding amines and *p*-toluene sulfochloride. A 2.5-fold excess of each amine was added to a 0.06 M solution of *p*-toluenesulfonyl chloride in methylene chloride, and the mixture was allowed to stand for 10 min. Then, the reaction mixtures were analyzed directly, because excess amounts of amines and their salts do not hinder UV detection of reaction products, which (except aniline) do not absorb in the near-UV region. The presence of certain amounts of *p*-toluenesulfonic acid (in the form of the anion) follows from the appearance of peaks in the region of the retention time of the non-sorbable component. Chlorobenzene was of analytical grade for GC (Reakhim, Moscow), and ninhydrin were of analytical grade (Merck, Germany).

Non-substituted hydrazones were synthesized ourselves by the interaction of the corresponding aromatic carbonyl compounds and an excess of hydrazine hydrate ("reagent

grade", 99%, Lenreaktiv) (acetophenone hydrazone, 2-methylbenzaldehyde hydrazone, 2-hydroxybenzaldehyde hydrazone, 4-methylbenzaldehyde hydrazone, 4-methylacetophenone hydrazone, propiophenone hydrazone, butyrophenone hydrazone). The preparations of 4- nitro-N-(2-hydroxypropyl) aniline, 2,4-dinitro-N-(2-hydroxypropyl) aniline, and 4-nitro-2-chloro-N-(1-pyrrolidinyl) benzene were synthesized by PhD V. Kuznetsov (Saint-Petersburg Technological Institute).

Preparations of sulfomethoxazole (a principal component of Biseptol tablets) and sulfamerazine were purchased in a pharmacy chain. Sorbate solutions were prepared in 2-propanol ("reagent grade", "Kriokhrom", St. Petersburg); before analyses the stock, solutions were additionally dissolved in an eluent. The preparations of 4-methoxybenzoylhydrazide and 1,2-*bis*(4-methoxybenzoyl)-hydrazine were synthesized by PhD E. Eliseenkov (St. Petersburg State University).

The physicochemical properties of all the compounds mentioned are summarized in Table 2.1.

Table 2.1. Principal physicochemical characteristics of selected analytes.

Compound	M W	Anhydrous form		Reference
		CAS №	Properties	
N,N-diethyl-*p*-toluenesulfonamide	227	649-15-0	log*P* = 2.2	ChemSpider
N-allyl-*p*-toluenesulfonamide	211	50487-71-3	T_m = 63 °C	PubChem, Chem BK
N-*tert*-butyl-*p*-toluenesulfonamide	227	2849-81-2	T_m = 112-113 °C T_b = 327.7 °C	ChemSpider, Spectra Base
N-phenyl-*p*-toluenesulfonamide	247	68-34-8	-	-
N-hexyl-*p*-toluenesulfonamide	255	-	-	-
N-benzyl-*p*-toluenesulfonamide	261	1576-37-0	T_m = 115 °C T_b = 419.1 °C	PubChem, Sigma-Aldrich
Toluene	92	10-88-3	T_m = -94.9 °C T_b = 110.6 °C	PubChem
o-Xylene	106	95-47-6	T_m = -25.2 °C T_b = 144.5 °C	PubChem
Chlorobenzene	112	108-90-7	T_m = -45.2 °C T_b = 131.6 °C	PubChem

Table 2.1. (Contd.)

Nitrobenzene	123	98-95-3	T_m = 5.7 °C T_b = 210.8 °C	PubChem
2-Methylbenzaldehyde	120	529-20-4	T_m = -35 °C T_b = 199-200 °C	PubChem, Sigma-Aldrich
4-Methylbenzaldehyde	120	104-87-0	T_m = -6 °C T_b = 204-205 °C	PubChem, Sigma-Aldrich
4-Methylacetophenone	134	122-00-9	T_m = -64 °C T_b = 226 °C	PubChem
2-Hydroxybenzaldehyde	122	90-02-8	T_b = 197 °C	PubChem, Alafa Aesar
1-Phenylpyrazolidine-3-one	162	92-43-3	T_b = 126 °C	PubChem, Sigma-Aldrich
Benzotriazole	119	95-14-7	T_m = 208-212 °C	PubChem
Acetophenone	120	98-86-2	T_m = 20 °C T_b = 202 °C	PubChem, Sigma-Aldrich
Propiophenone	134	93-55-0	T_m = 18.6 °C T_b = 218 °C	PubChem, ChemSpider
Butyrophenone	148	495-40-9	T_m = 12 °C T_b = 228.5 °C	PubChem
2,3,5-Trimethylphenol	136	697-82-5	T_m = 93.5 °C T_b = 230.5 °C	PubChem, Sigma-Aldrich
Phthalimide	147	85-41-6	T_m = 336 °C T_b = 238 °C	PubChem
Ninhydrin	178	485-47-2	T_m = 250 °C	PubChem, Sigma-Aldrich
3-Nitrophenol	139	554-84-7	T_m = 96.8 °C T_b = 194 °C	PubChem, Alafa Aesar
Acetophenone	120	98-86-2	T_m = 20 °C T_b = 202 °C	PubChem, Sigma-Aldrich
Propiophenone	134	93-55-0	T_m = 18.6 °C T_b = 218 °C	PubChem, ChemSpider
Butyrophenone	148	495-40-9	T_m = 12 °C T_b = 228.5 °C	PubChem
Phthalimide	147	85-41-6	T_m = 336 °C T_b = 238 °C	PubChem
Ninhydrin	178	485-47-2	T_m = 250 °C	PubChem, Sigma-Aldrich
3-Nitrophenol	139	554-84-7	T_m = 96.8 °C T_b = 194 °C	PubChem, Alafa Aesar
4 Methoxybenzoylhydrazide	166	3290-99-1	log$P$ = 0.25	PubChem, ChemSpider
1,2-*bis*(4-methoxybenzoyl) hydrazine	300	849-82-1	log$P$ = 2.3	PubChem, ECUEMI.Com

2.2. Synthesis of analytes

2.2.1. N-substituted *p*-toluenesulfonamides

N-substituted *p*-toluenesulfonamides were synthesized from *p*-toluenesulfonyl chloride (99%, Acros Organics, Belgium), which is the most readily available reagent among sulfonyl chlorides, and the following amines: allylamine (I, analytical grade, Merck, Germany), diethylamine (II, reagent grade, Angarsk Chemical Plant, Russia), *tert*-butylamine (III, 99%, Acros Organics, Belgium), aniline (IV, "reagent grade," Berezniki Chemical Plant, Russia), benzylamine (V, Merck, Germany), and *n*-hexylamine (VI, available as hydrochloride, Reakhim, Russia). *n*-Hexylamine hydrochloride was preliminarily converted to the free base by adding the excess of sodium hydroxide to its aqueous solution. Free hexylamine base was extracted (twice) with methylene chloride (reagent grade, Vekton, Russia) from the aqueous solution and dried over calcium hydroxide [69].

$$CH_3–C_6H_4SO_2Cl + 2HNRR' \rightarrow CH_3–C_6H_4–SO_2–NRR' + RR'NH\times HCl$$

where R = H, $-CH_2-CH=CH_2$ (I), $-C_2H_5$ (II), *tert*-C_4H_9 (III), $-C_6H_5$ (IV), $CH_2C_6H_5$ (V), *n*–C_6H_{13} (VI).

2.2.2. Non substituted hydrazones

Non-substituted hydrazones of aromatic aldehydes and ketones ($RR'C=N–NH_2$) were synthesized by the interaction of corresponding carbonyl compounds with an excess of hydrazine hydrate "reagent grade", 99%, Lenreaktiv. With an excess of carbonyl compounds, the parallel formation of azines becomes significant [124].

2.2.3. Oximes of aromatic carbonyl compounds

The oximes of aromatic carbonyl compounds were synthesized by interaction of hydroxylamine sulfate (Reakhim, Moscow) in alkaline medium with the following substituted benzaldehydes: (*a*) 2-methyl- (Lancaster), (*b*) 4-methyl-, (*c*) 2-hydroxy- (Aldrich, USA), (*d*) 4-hydroxy-, (*e*) 2-methoxy- (Fluka, UK), (*f*) 4-methoxy- (Reakhim, Kiev plant), (*g*) 3-hydroxy-4-methoxy (vanillin, Ferak Berlin, Germany), (*h*) 4-hydroxy-3-methoxy (isovanillin, Janssens Chimica, Belgium), (*i*) 3,4-dimethoxy (veratroic aldehyde, Acros, Belgium), as well as ketones $C_6H_5COC_nH_{2n+1}$: (*k*) acetophenone, (*l*) propiophenone and (*m*) butyrophenone (Sigma-Aldrich Rus LLC, Russia). The same ketones were used as reference components in the determination of retention indices.

$$Ar–RCO + NH_2OH \times H_2SO_4 + 2NaOH \rightarrow Ar–CR=NOH\ (a–m) + Na_2SO_4 + 2H_2O$$

To approximately 100 μL (or 100 mg for solids) of aldehyde (0.65–0.80 mM) was added 2–4 mL of isopropyl alcohol (h.p., KryoKhrom, St. Petersburg), approximately 100 mg of hydroxylamine sulfate (0.80 mM), and 40 mg of anhydrous sodium carbonate soda (1 mM). The reaction mixtures were incubated at room temperature for 24 hours with periodic stirring and further diluted 10^3-fold with eluent to be injected into the chromatograph.

2.3. Reversed phase high performance liquid chromatography analytical conditions

Regime (A): Chromatographic analysis of the reaction mixtures was carried out with a "Stayer-M" chromatograph with UV detector. Column: Phenomenex C18, 250 × 4.6 mm, sorbent particle size 5 μm. Mobile phase components: bidistilled deionized water [GFL distiller (Germany) and deionizer D-301(Aquilon)] with a resistivity of 18.2 MΩ.cm and methanol (99.8%, HPLC Grade, J.T. Baker, USA, or grade "0", "Kriokhrom", St. Petersburg). Eluent flow rate was 1.0 mL min^{-1}, column temperature 30 °C. Sample volume was 20 μl.

Regime (B): Shimadzu LC-20 Prominence liquid chromatography with a diode-array detector and Phenomenex C18 column 250 mm long and 4.6 mm i.d. with a sorbent particle

size of 5 μm in water–methanol mobile phases in several isocratic modes with (5% *v/v* or 10% in our cases) concentration steps of the organic component at an eluent flow rate of 1.0 mL min^{-1} and column temperature of 30 °C. The samples were injected using SIL-20A/AC autosampler; the sample volume was 20 μl.

All the samples for analyses were prepared by dissolving individual compounds or reaction mixtures in the corresponding mobile phase.

We used isocratic elution modes with different methanol content in an eluent. Variation step of methanol concentration (ΔC) was chosen equal to (5% *v/v* or 10% in our cases). Increasing the C values decreases the total number of points for establishing the dependence $t_R(C)$, since decreasing the C values appeared to be time and solvent consuming modes.

The number of replicate injections of each sample in all the regimes (A)–(B) was 2–3. The inter-injection variations of the retention times of the target analytes in all the cases did not exceed 0.01–0.02 min.

UV detection in our work was carried out at the wavelengths of 220 and 254 nm. Calculation of the parameters of recurrent dependences in the mode isocratic elution with a rate of change in the concentration of CH_3OH ΔC = (5% or 10% in our cases), to determine the retention times of the different organic compounds and plotting were carried out using Origin software (versions 4.1 and 8.1).

2.4. Measuring retention times and retention indices

The determination of the dependence of retention times of analytes *vs.* composition of an eluent seems to be the principal approach in the characterization of chromatographic properties of analytes.

In all modes, at least three parallel determinations of retention times were carried out for each sample. The retention parameters were statistically processed using Excel software (Microsoft Office, 2010).

The retention times of non-sorbable components (t_0) required for calculating the logarithmic retention indices were estimated from the retention times of three reference components using the Peterson and Hirsch formula [95]:

$$t_0 = (t_{R,1} \times t_{R,3} - t^2_{R,2}) / (t_{R,1} + t_{R,3} - 2t_{R,2}) \qquad (1.30)$$

To determine the retention indices, three reference *n*-alkyl phenyl ketones $C_6H_5COC_nH_{2n+1}$ with $n = 1 \div 3$ were added to all samples. Retention indices were calculated either using a programmable calculator (logarithmic indices) or the QBasic program (linear-logarithmic indices).

All our results for methanol-water eluents were compared with the results independently obtained by Darya A. Nikitina for acetonitrile-water eluents [70].

2.5. Revealing possible experimental errors in determining the retention parameters

When the column pressure is extremely high, as it is in the case of some methanol/water eluents, it is difficult to fix or estimate the flow values of an eluent through the column. One of our instruments (namely Stayer-M) was excluded from using just for this reason because the flow of an eluent did not correspond to the selected values.

The low reproducibility of retention times can be illustrated with data for N-*tert*-butyl-*p*-toluenesulfonamide.

Table 2.2. Retention times of N-*tert*-butyl-*p*-toluenesulfonamide measured with a "Stayer-M" HPLC instrument (the example of low reproducibility).

C (CH_3OH) Volume %	N-*tert*-butyl-*p*-toluenesulfonamide		
	t_{R1}(min)	t_{R2}(min)	t_{R3}(min)
85	3.400	3.381	3.374
80	3.766	-	3.735
75	4.306	4.294	4.292
70	5.150	5.129	5.115
65	6.546	6.528	6.555
60	8.754	8.730	8.720
55	12.728	12.648	12.661
50	-	19.587	19.535

This reproducibility is insufficient for characterizing analytes using recurrence relationships and was the reason for using different chromatographic equipment.

Retention times measured using the Shimadzu instrument are summarized in the section Results and Discussion.

Chapter 3: Results and discussion

At the starting moment of our work the following information on the problem under consideration was available:

– The applications of recurrent dependencies in gas and high-performance liquid chromatography were proposed, namely their using in approximation of chromatographic retention times at different conditions of separations.

– On the examples of several synthetic anti-tumor drugs of complex structures and few specially synthesized N-substituted *p*-toluenesulfonamides it was shown the following [51,60–67]:

i. If the analyte demonstrates no chemical transformations at the different composition of an eluent (only acetonitrile-water systems were tested), the recurrent approximation of its retention times manifests no anomalies and it is linear with correlation coefficients exceeding 0.999.

ii. If the chemical nature of analyte at the different compositions of eluent is changed, the plots of recurrent approximations of its retention times indicate anomalies. Namely, the points corresponded to the highest content of water in an eluent are located downward the regression line.

It was interpreted as the sign of the formation of hydrates of analytes during their HPLC separation [68–71].

We can note an important feature of this work, which consists in the fact that some of the considered compounds were simultaneously characterized in the work of Darya A. Nikitina using water-acetonitrile eluents. This makes it possible to compare the obtained data for eluents of different compositions [68,125].

3.1. Basic relations for characterizing the dependence of analyte retention parameters on eluent composition.

Recurrence approximation of retention parameters

In reversed-phase high-performance liquid chromatography (RP HPLC) there is no single general equation for the dependence of the retention parameters on the concentration of an organic component in an eluent, $t_R(C)$. Several approximation functions were proposed in the literature [126–132]. These approximations are used for compounds of different chemical origin under different elution conditions.

We have selected five relations for approximating the dependences $t_R(C)$ most often used in analytical practice. To compare them, we have selected five analytes listed in Table 3.1.

Table 3.1. Molecular weights and CAS numbers of selected analytes.

Compound	Molecular weight (Da)	CAS №
Toluene	92	10-88-3
Nitrobenzene	123	98-95-3
3-Nitrophenol	139	554-84-7
N-allyl-*p*-toluenesulfonamide	211	50487-71-3
N-*tert*-butyl-*p*-toluenesulfonamide	227	2849-81-2

In all equations considered below, the variable C means the volume fraction of the organic modifier in an eluent; coefficients a, b (and c, if necessary) are calculated by LSM. It is important that most of the models considered below imply using not the net retention parameters (t_R) but adjusted retention times (t'_R).

Let us start our discussion with the simplest model: hyperbolic correlation (3.1) based on the Scott–Kuchera approach and proposed by Row [52]:

$$1/t'_R = aC + b \qquad (3.1)$$

The second and the most important group of retention models is based on Soczewinski–Wachtmeister equation (3.2), which assumes linear dependence of the logarithms of the adjusted retention times on the volume fraction of the modifier [38,40]:

$$\log t'_R = aC + b \qquad (3.2)$$

In the literature, this model is frequently referred to as the linear solvent strength (LSS) model [41].

The next approach is the log–log dependence, which assumes the linear relationship between $\log t'_R$ and the logarithm of the volume fraction of the modifier. This model is often named as the Snyder–Soczewinski equation (3.3) because it was developed by Soczewinski from earlier findings by Snyder [38,42]:

$$\log t'_R = a\log C + b \qquad (3.3)$$

The reasonable extension of the last two models is the second–degree polynomial form (3.4) proposed by Schoenmakers [45]:

$$\log t'_R = aC^2 + bC + c \qquad (3.4)$$

In addition, the $t_R(C)$ dependences in RP HPLC can be approximated by so-called recurrent relations recently proposed by Zenkevich [51,60–67]:

$$t_R(C + \Delta C) = at_R(C) + b, \qquad (3.5)$$

where ΔC = const is a constant increment of the organic component concentration in an eluent (in our work, we chose ΔC = 5%).

The mathematical properties of recurrent relations (3.5) were considered in [63–67]. The important advantage of recurrences is their applicability directly to net retention times, without their conversion to the adjusted parameters.

The retention times of the theoretically nonsorbable component were precalculated using the well-known Peterson–Hirsch relation (3.6) [95] based on the retention times of three reference *n*-alkyl phenyl ketones $C_6H_5COC_nH_{2n+1}$ (n = 1 ÷ 3) added to all samples:

$$t_0 = (t_{R,1} \times t_{R,3} - t^2_{R,2}) / (t_{R,1} + t_{R,3} - 2t_{R,2}) \qquad (3.6)$$

Table 3.2 includes the net retention times of the selected analytes and the retention times of the unsorbed component (dead time), precalculated at different methanol concentrations in an eluent ($50 \le C \le 75$ %, *v/v*).

Table 3.2. Average values of net retention time (min) of the selected analytes and the retention time of the unsorbable component (dead time, t_0), measured at different methanol concentrations in the eluent.

C (CH_3OH) % vol.	t_R			t_0
	toluene	nitrobenzene	3-nitrophenol	
75	7.262	4.121	3.604	1.548
70	9.178	4.622	3.932	1.604
65	12.223	5.329	4.440	1.620
60	16.278	6.267	5.076	1.606
55	22.618	7.626	6.050	1.543
50	30.621	9.525	7.429	1.515
C(CH_3OH) % vol.	t_R			t_0
	N-allyl-*p*-toluenesulfonamide		N-*tert*-butyl-*p*-toluenesulfonamide	
75	3.611		4.297	1.551
70	4.035		5.131	1.603
65	4.712		6.543	1.622
60	5.702		8.735	1.604
55	7.387		12.679	1.518

Table 3.3 contains the results of processing the retention data for various analytes using different approximation equations (3.1) – (3.5), namely, coefficients of linear regression (*a*, *b*, and *c*, if necessary), correlation coefficients (*R*), and the estimated accuracy of the minimal and maximal t_R values ($\Delta t_{R,\,min}$ and $\Delta t_{R,\,max}$).

Table 3.3. Parameters of different approximations of dependence $t_R(C)$ (regression and correlation coefficients, R) and the differences between the precalculated and experimental minimal and maximal retention times for each analyte, $\Delta t_R = t_{R,\,cal} - t_{R,\,exp}$, min.

Approximation equation	Coefficients	R	$\Delta t_{R,\,min}$	$\Delta t_{R,\,max}$
Toluene				
$1/t'_R = aC + b$	a = 0.006 b = -0.259	0.98	0.54	-129.1*
$\log t'_R = aC + b$	a = -0.027 b = 2.893	-0.9994	0.04	1.02
$\log t'_R = a\log C + b$	a = -4.066 b = 8.388	-0.9991	0.16	2.4
$\log t'_R = aC^2 + bC + c$	a = 9.571 ×10^{-5} b = -0.041 c = 3.260	0.9995	0.08	1.6
$t'_R(C + \Delta C) = at'_R(C) + b$	a = 0.711 b = 0.129	0.999	-0.39	1.5
$t_R(C + \Delta C) = at_R(C) + b$	a = 0.713 b = 0.545	0.9991	-0.33	1.3
Nitrobenzene				
$1/t'_R = aC + b$	a = 0.011 b = -0.421	0.997	0.29	3.4
$\log t'_R = aC + b$	a = -0.020 b = 1.879	-0.995	-0.17	-0.91
$\log t'_R = a\log C + b$	a = -2.836 b = 5.716	-0.9993	-0.11	-0.20
$\log t'_R = aC^2 + bC + c$	a = 2.5×10^{-4} b = -0.051 c = 2.838	0.9998	0.08	-0.26
$t'_R(C + \Delta C) = at'_R(C) + b$	a = 0.703 b = 0.426	0.9997	0.05	0.15
$t_R(C + \Delta C) = at_R(C) + b$	a = 0.714 b = 0.826	0.9999	0.01	0.02

Table 3.3. (Contd.)

3-Nitrophenol				
$1/t'_R = aC + b$	a = 0.113 b = -0.488	0.998	0.02	1.5
$\log t'_R = aC + b$	a = -0.019 b = 1.675	-0.992	-0.18	-0.59
$\log t'_R = a\log C + b$	a = -2.649 b = 5.262	-0.997	-0.16	-0.14
$\log t'_R = aC^2 + bC + c$	$a = 3.171\times10^{-4}$ b = -0.058 c = 2.891	0.9996	-0.22	0.37
$t'_R(C + \Delta C) = at'_R(C) + b$	a = 0.686 b = 0.426	0.9993	-0.06	0.16
$t_R(C + \Delta C) = at_R(C) + b$	a = 0.701 b = 0.848	0.9997	-0.00	-0.02
N-allyl-*p*-toluenesulfonamide				
$1/t'_R = aC + b$	a = 0.015 b = -0.656	0.998	0.01	2.3
$\log t'_R = aC + b$	a = -0.025 b = 2.157	-0.98	-0.16	-1.7
$\log t'_R = a\log C + b$	a = -3.596 b = 7.028	-0.996	-0.25	-0.91
$\log t'_R = aC^2 + bC + c$	$a = 4.743\times10^{-4}$ b = -0.085 c = 3.987	0.9999	-0.16	-0.35
$t'_R(C + \Delta C) = at'_R(C) + b$	a = 0.574 b = 0.607	0.9998	0.00	0.64
$t_R(C + \Delta C) = at_R(C) + b$	a = 0.616 b = 1.152	0.9998	0.04	-0.11

Table 3.3.(Contd.)

N-*tert*-butyl-*p*-toluenesulfonamide				
$1/t'_R = aC + b$	a = 0.013 b = -0.593	0.98	0.66	40.7*
$\log t'_R = aC + b$	a = -0.033 b = 2.862	-0.993	-0.70	-4.1
$\log t'_R = a\log C + b$	a = -4.694 b = 9.217	-0.998	-0.28	-1.8
$\log t'_R = aC^2 + bC + c$	$a = 5.064\times10^{-4}$ b = -0.096 c = 4.803	0.9999	0.16	1.8
$t'_R(C + \Delta C) = at'_R(C) + b$	a = 0.576 b = 0.733	0.9999	-0.01	-0.04
$t_R(C + \Delta C) = at_R(C) + b$	a = 0.579 b = 1.377	0.9998	0.09	-0.28

* Anomalous outlier which was not used in averaging the results.

The quantities Δt_R presented in Table 3.3 were calculated from the results obtained using all the relations (3.4) – (3.5). This approach to the estimation of the approximation accuracy has been proposed for the first time. It implies hypothetical exclusion of the maximal and the minimal t_R values from each total data set, followed by precalculation of these values using the remaining retention data and calculation of the differences ($t_{R,\,cal} - t_{R,\,exp}$) for both of them. In other words, for evaluating the $\Delta t_{R,\,min}$ values we deleted the t_R-point corresponding to the highest methanol concentration in the eluent, after which we estimated the coefficients a, b and then calculated $t_{R,\,cal}$ or $t'_{R,\,cal}$.

On the contrary, to evaluate $\Delta t_{R,\,max}$, we deleted the point corresponding to the low concentration of methanol in the eluent, after which we estimated the coefficients a, b (and c, if necessary) and then calculated $t_{R,\,cal}$ or $t'_{R,\,cal}$.

Figure 3.1 (*a–f*) presents the plots of all the relations approximating the dependences of the retention times on the methanol concentration in the eluent for N-allyl-*p*-toluenesulfonamide selected as an example.

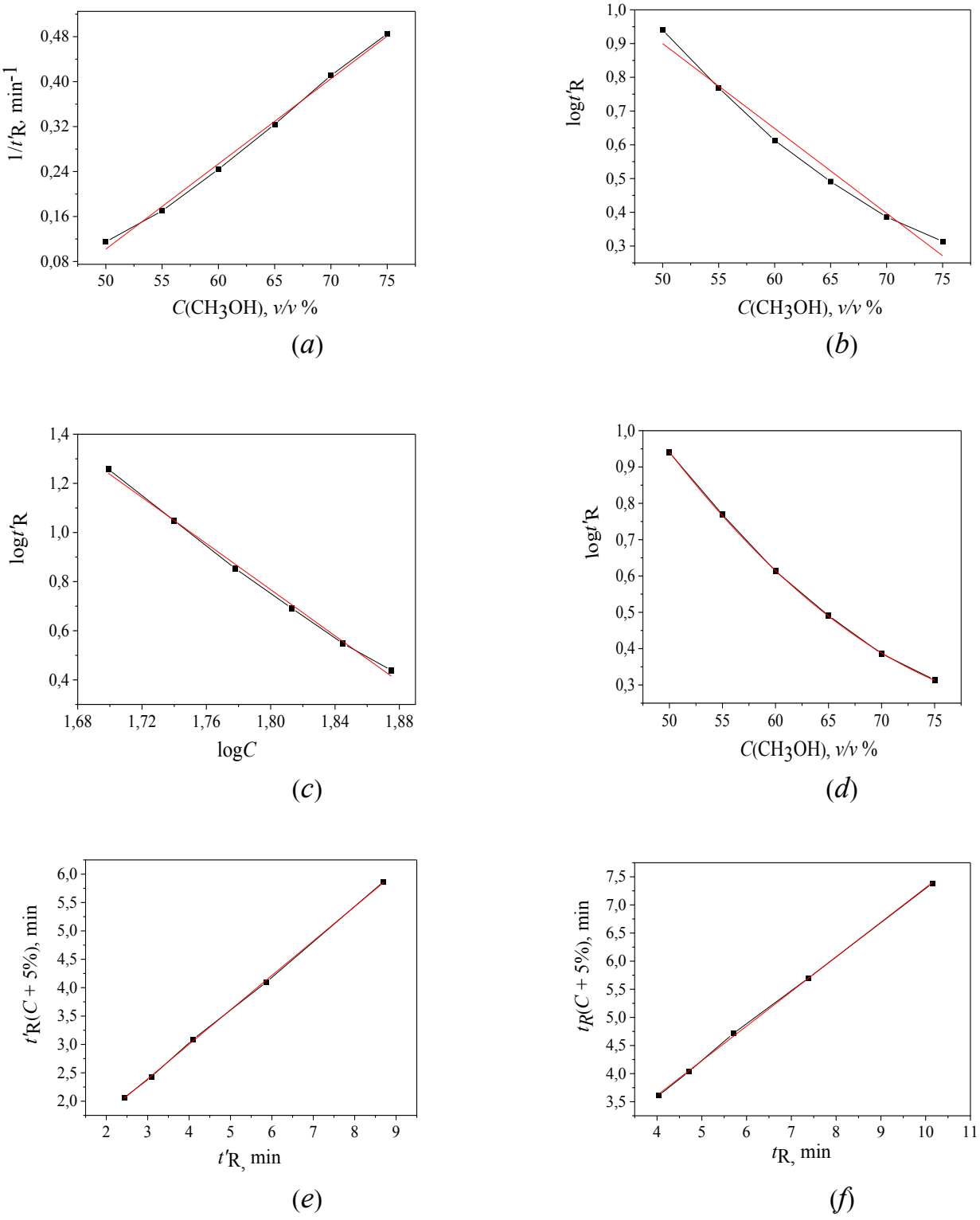


Figure 3.1. (*a–f*). The plots for different approximations for the dependences $t_R(C)$ of N-allyl-*p*-toluenesulfonamide on the methanol concentration in the eluent. (*a*) relation 3.1, (*b*) relation 3.2, (*c*) relation 3.3, (*d*) relation 3.4, (*e*) relation 3.5, (*f*) relation 3.5.

Comparison of the results allows the following conclusions:

The values of the correlation coefficients in Table 3.3 (0.99–0.9999) do not allow reliable conclusions on the preference of any approximation model. All *R*-values seem to be acceptable.

Relatively low absolute values of the correlation coefficients (Table 3.3) were obtained only with approximation function (3.1) for toluene and N-*tert*-butyl-*p*-toluenesulfonamide ($R = 0.98$) and with function (3.2) for N-allyl-*p*-toluenesulfonamide ($R = -0.98$).

Because comparison of the *R*-values appeared to be non-informative, we have decided to complete our characterization with comparing the values of Δt_R for all the approximation relations and all the analytes chosen. The "raw" results are presented in Table 3.3.

It also seems reasonable to average all the $\Delta t_{R,\,min}$ and $\Delta t_{R,\,max}$ values estimated for different analytes. Such averages $\Sigma\Delta t_R/N$ ($N = 5$) characterize specifically the approximation abilities of different equations and are compared in Table 3.4.

Table 3.4. Averaged accuracy of the minimal and maximal t_R values ($\Delta t_{R,\,min}$ and $\Delta t_{R,\,max}$, min) for different approximation models.

Approximation equation	Average value $\Delta t_{R,\,min}$	Average value $\Delta t_{R,\,max}$
$1/t'_R = aC + b$	0.30	2.4*
$\log t'_R = aC + b$	0.25	1.7
$\log t'_R = a\log C + b$	0.19	1.1
$\log t'_R = aC^2 + bC + c$	0.14	0.88
$t'_R(C + \Delta C) = at'_R(C) + b$	0.10	0.50
$t_R(C + \Delta C) = at_R(C) + b$	0.09	0.35

* Anomalous outliers which were not used in averaging the results.

Table 3.4 presents the averaged accuracy $\Sigma\Delta t_R/N$ for the minimal and maximal t_R values for all the approximation functions selected in our work.

The largest $\Sigma\Delta t_R/N$ values demonstrate the minimal precision of the hyperbolic equation (Row model), because $\Delta t_{R,\,min} = 0.30$ min and $\Delta t_{R,\,max} = 2.4$ min. The Soczewinski–Wachtmeister and Snyder–Soczewinski models are characterized by medium precision, as well as the polynomial approach, and recurrent relations ensure the highest precision. It is important that two kinds of recurrences should be compared [133].

The first of them implies using the adjusted retention times (t'_R) by analogy with other approximation relations. However, this is not necessary, because the unique properties of recurrences allow approximation of net retention times (t_R). The results are shown in the last line of Table 3.4: $\Delta t_{R,\,min} = 0.09$ min (5 s), and $\Delta t_{R,\,max} = 0.35$ min (it is equivalent to 21 s). Such values are comparable with the widths of chromatographic peaks for standard RP HPLC columns.

The fact of better precision of $\Delta t_{R,\,min}$ and $\Delta t_{R,\,max}$ estimates in the last two lines of Table 3.4 appeared to be rather interesting. It can be attributed to the features of the dead time (t_0) calculation with Eq. (3.6). The retention times of three reference *n*-alkyl phenyl ketones contain some additional uncertainties influencing t_0 evaluation. If we use net retention times, such influence is excluded.

i. To simplify the search for the preferable approximation models, we have applied the newly proposed criterion: comparison of the minimal and maximal experimental t_R values in data sets with the values precalculated after their hypothetical exclusion from these sets (Δt_R). The next step is calculating the average values, $\Sigma\Delta t_R/N$, which characterize not the individual analytes but the overall accuracy of different approximation equations [133].

ii. The recurrent approximation of retention times, namely $t_R(C + \Delta C) = at_R(C) + b$, $\Delta C = \text{const}$, provides the highest precision of the approximated t_R-values compared to other models. It is applicable to both adjusted ($t_R' = t_R - t_0$) and net retention times. The accuracy of such approximations appeared to be better in the case of net retention times, which seems to be a unique feature of recurrent functions. This anomaly is attributable to the use of

retention times of three consecutive *n*-alkyl phenyl ketones for evaluating t_0-values, because these data contain additional uncertainties [133].

3.2. Hydration of analytes in RP HPLC is the main reason of deviations of the recurrence approximation of retention parameters from the linearity. Comparing the various factors influencing on these deviations

3.2.1. Recurrent approximation of retention parameters for a series of N-substituted *p*-toluenesulfonamides at different concentrations of methanol in an eluent under the conditions of RP HPLC analysis

Many organic compounds exist in a single form in aqueous media, the confirmation of the correspondence of the detected forms of analytes to their molecular structures seems to be one of the principal problems of analytical chemistry. The information on the formation and existence of organic hydrates is important for chemical analysis. Unfortunately, the direct detection of such hydrates by chromatographic and mass-spectrometric methods seems to be rather difficult or even impossible due to the following reasons:

i. Most of hydrates are non-volatile;

ii. If we use electrospray as the detector in HPLC, we cannot distinguish the hydrated and non-hydrated forms of analytes (both of them give the similar fragments after ES-ionization);

iii. So far as most of organic compounds are volatile, the equilibria between hydrated and non-hydrated forms may be shifted to the left;

$$X + nH_2O \rightleftharpoons X \times nH_2O \qquad (1.31)$$

iv. So far as the solubility of organic compounds in water usually is small, hence the concentration of their hydrated forms maybe several times smaller that makes their detection more complex.

However, there is an indirect way of the detection of hydrates based on revealing the anomalies in the dependences of the retention parameters of the analytes on the concentration of the organic modifier in the eluent.

The recurrent approximation of retention times of analytes in RP HPLC, corresponds to the equation (3.5):

$$t_R(C + \Delta C) = at_R(C) + b,\ \Delta C = \text{const} \qquad (3.5)$$

where ΔC = const – constant increment of concentration of methanol (5% *v/v* or 10% in our cases), *a* and *b* – coefficients calculated by Least Squares Method, allows us to reveal the organic compounds forming the hydrates in an eluent. The criterion of their formation is the deviation of recurrent approximation above linearity (correlation coefficient $R < 0.999$), especially for points corresponding to the maximal water content of the eluent. If the analyte exists in the same hydrated or non-hydrated forms within the whole range of the eluent compositions, the recurrent dependences show no anomalies ($R > 0.999$).

Recurrent approximation of the temperature dependence of the solubility of inorganic salts in water as the applicability test of the approach considered

Generally, considering the solubility of inorganic salts in water is appropriate because of high solution concentrations, which simplifies revealing the specific features of the temperature and concentration dependences of the solubility. The reference data on the solubility of inorganic salts in water (*r*, g/100 mL) usually cover the temperature range $0 \le T \le 100^{\circ}$C [100]. Within this interval, there are both salts existing in the non-hydrate state (e.g., NH_4Cl, KBr, $NaNO_2$, etc.) and salts forming stable hydrates ($AlCl_3 \bullet 6H_2O$, $CuSO_4 \bullet 5H_2O$, $Mg(NO_3)_2 \bullet 6H_2O$, etc.). For all the salts that exist in the same form at different temperatures of aqueous solutions, recurrence relation (Equation (1.31)) provides linear data approximations.

$$r(T + \Delta T) = ar(T) + b,\ \Delta T = \text{const} \qquad (3.8)$$

The data for ammonium sulfate $(NH_4)_2SO_4$ as an example are plotted in Figure 3.2. Plot (*a*) shows the nonlinear temperature dependence of its solubility within the

temperature range $0 \le T \le 100$°C, $\Delta T = 20$°C; the initial data on the solubility are listed in the figure caption. Another figure (*b*) presents the plot of recurrent approximation of the same data; the correlation coefficient $R = 0.9998$ corresponds to the practically "ideal" linear dependence. Other regression parameters are listed in the figure caption. Similar linear dependences are observed for salts forming stable hydrates with fixed water content.

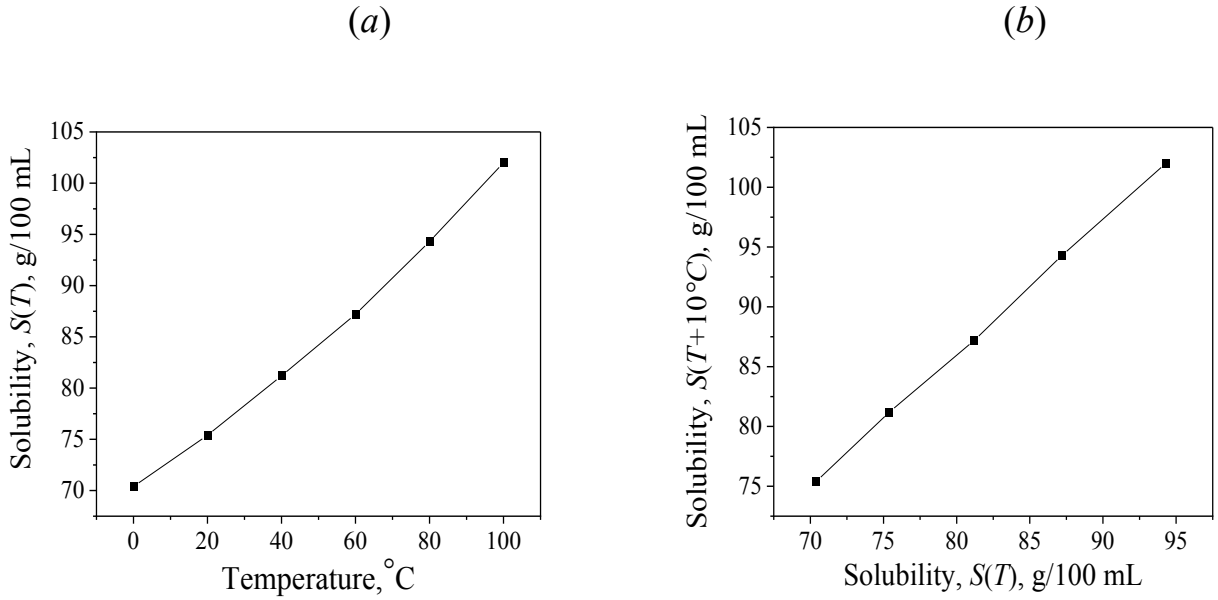


Figure 3.2. (*a*) Nonlinear temperature dependence of the solubility of ammonium sulfate $(NH_4)_2SO_4$ in water (forms no hydrates). Initial data, [100] *r*, g/100 mL (*T*, °C): 70.4 (0), 75.4 (20), 81.2 (40), 87.4 (60), 94.3 (80), 102 (100). (*b*) Recurrent approximation of solubility, $r(T + 20°C) = ar(T) + b$. Parameters of recurrent regression: $a = 1.112 \pm 0.01$, $b = -2.85 \pm 0.9$, $R = 0.9998$, $S_0 = 0.1$.

The salt speciation in solutions may be different because of the hydrate formation depending on the temperature, variations of the hydrate composition, or reactions of salts with water (hydrolysis). An example of such salts is lithium bromide. It exists as anhydrous salt (CAS № 7550-35-8), monohydrate (CAS № 85017-82-9), dihydrate (no CAS №), and hydrate with an uncertain number of water molecules (CAS № 23303-71-1). In such cases, the plot of the recurrent dependence shows strong anomalies (Figure 3.3 *a* and *b*). However, the results obtained do not allow an unambiguous understanding of the chemical sense of the transformation of LiBr in an aqueous solution at approximately 40°C: Most probably, it is the interconversion of the hydrate (below 40°C) and anhydrous form (above

40°C), but changes in hydrate composition (di–, mono–, etc.) cannot be ruled out as well. The recurrent plot for LiBr contains two linear sections: the right part for high *r*-values at high temperatures (six points) and the left one for low *r*-values at low temperatures. The parameters of the linear regression for the right part are given in the caption to this figure. Numerous examples of using the recurrent approximation of the data on solubility of inorganic salts in water are considered in [134].

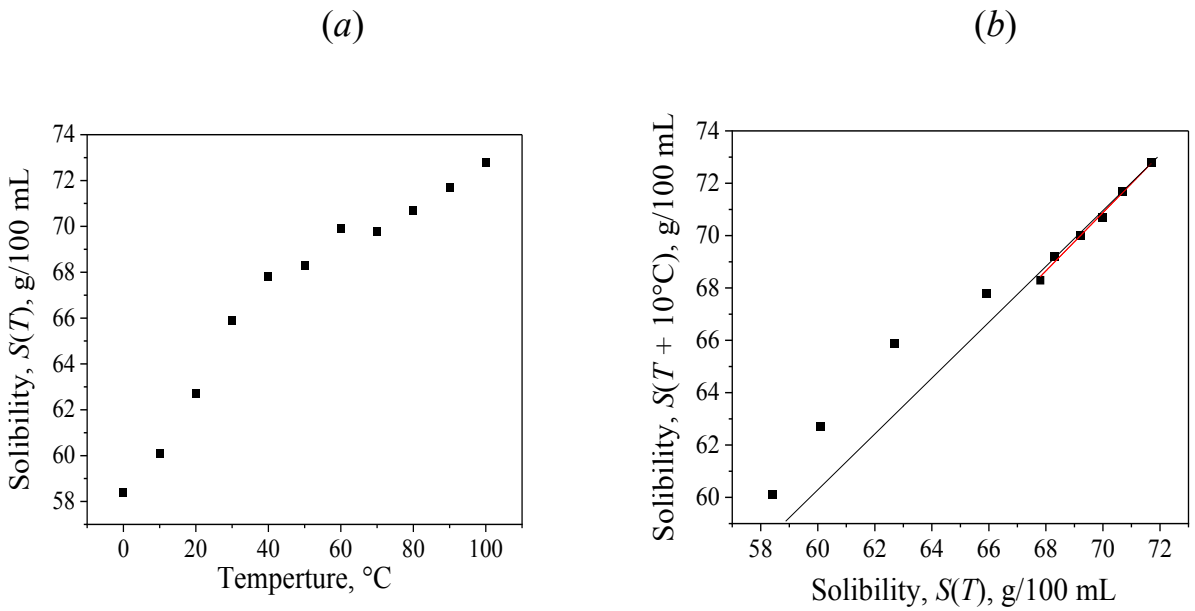
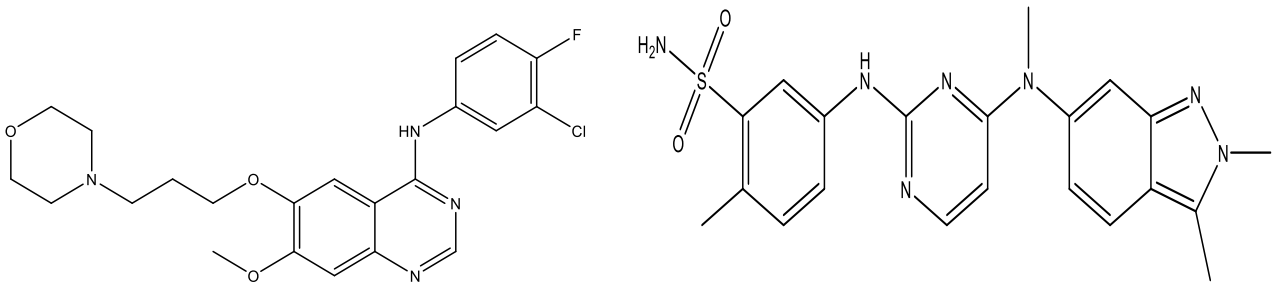


Figure 3.3. (*a*) Nonlinear temperature dependence of the solubility of lithium bromide in water (exists in different forms at $T < 40$°C and at $T > 40$°C). Initial data, [100] *r*, g/100 mL (*T*, °C), $\Delta T = 10$°C: 58.4 (0), 60.1 (10), 62.7 (20), 65.9 (30), 67.8 (40), 68.3 (50), 69.9 (60), 69.8 (70), 70.7 (80), 71.7 (90), 72.8 (100). (*b*) Recurrent approximation of solubility, $r(T + 10°C) = ar(T) + b$, with two linear portions: the left portion belongs to the more hydrated form existing at $T < 40$°C, and the right portion, to the less hydrated form existing at $T > 40$°C. Regression parameters for less hydrated form: $a = 1.11 \pm 0.05$, $b = -6.95 \pm 3.29$, $R = 0.9996$, $S_0 = 0.09$.



Gefitinib (***a***) Pazopanib (***b***)

Figure 3.4. Structural formulas of two antitumor drugs Gefitinib (***a***), and Pazopanib (***b***)

The examples of the previous considering the series of drugs (complex polyfunctional organic compounds) with acetonitrile as the organic solvent in the eluent are plotted in Figure 3.5 *a* and *b* [70]. For compound with the trivial name Gefitinib (*a*), the linear dependence without anomalies is observed; i.e., this compound forms no hydrates at the acetonitrile concentration in the eluent in the range within 35 to 65% *v/v*. On the contrary, the similar recurrent presentation of retention data for Pazopanib (*b*) reveals two points that correspond to the maximal water content of the eluent and deviate “downward” from the regression line. Hence, the chemical form of this compound in the eluent with high water content differs from that at low water content. The presence of other constituents in the eluent has no effect on this anomaly [70].

Gefitinib (*a*) Pazopanib (*b*)

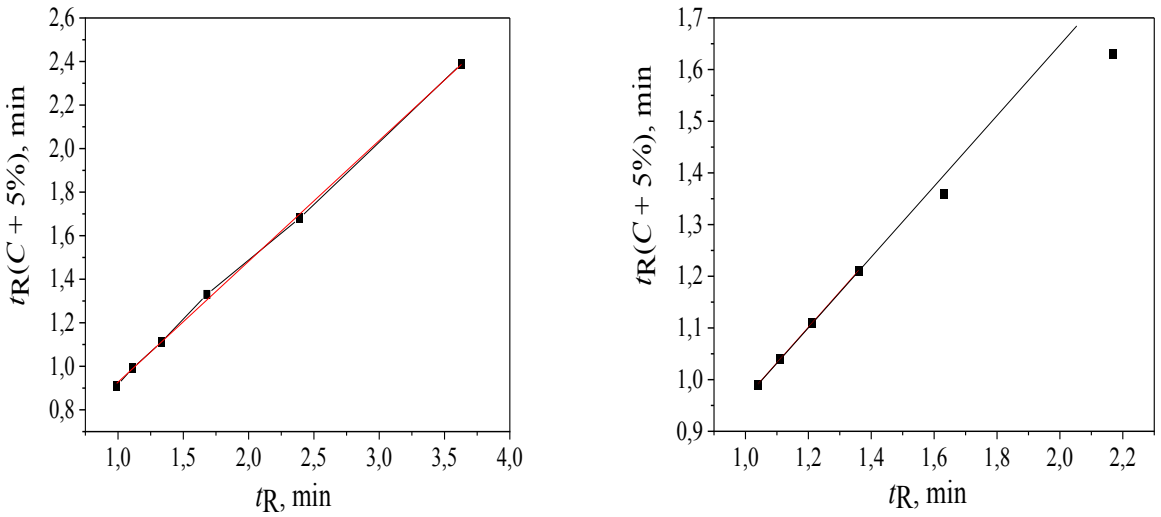


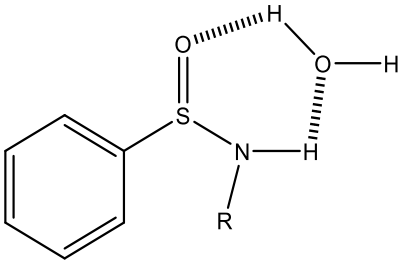
Figure 3.5. (*a*) Linear recurrent approximation of the retention times for Gefitinib with acetonitrile as an organic modifier of the eluent. t_R, min (*C*, % *v/v*): 3.63 (35), 2.39 (40), 1.68 (45), 1.33 (50), 1.11 (55), 0.99 (60), 0.91 (65). Linear regression parameters: $a = 0.555 \pm 0.008$, $b = 0.37 \pm 0.02$, $R = 0.9996$, $S_0 = 0.02$. (*b*) Recurrent approximation of the retention times for Pazopanib. t_R, min (*C*, % *v/v*): 2.17 (20), 1.63 (25), 1.36 (30), 1.21 (35), 1.11 (40), 1.04 (45), 0.99 (50). Linear regression parameters: (without two points for maximal water content) $a = 0.687 \pm 0.008$, $b = 0.277 \pm 0.009$, $R = 0.99987$, $S_0 = 0.002$.

The most reasonable explanation of this anomaly is the formation of hydrate of compound (*b*). Apparently, the structural factor responsible for this feature is the presence of polar sulfonamide functional group, $–SO_2–NH_2$.

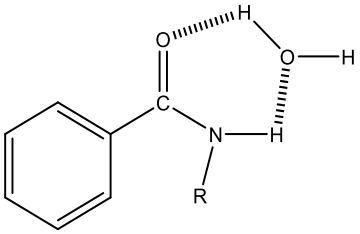
Published literature data show that the formation of hydrates in the solid-state and, hence, in an aqueous solution is typical of some carboxamides with polar structural fragments –CO–NH– [135] and of the majority of sulfonamides containing more polar groups $–SO_2–NH–$ [136–139].

The reverse formation of hydrates is typical for an unexpectedly high number of organic compounds [68], which is often neglected. The reason for the stability of such hydrates may be the coordination of water molecules with the oxygen and nitrogen atoms

of the –NH–S=O structural fragment that contains two π (the S=O double bond) and four *p* (the electron pairs localized on the oxygen and nitrogen atoms) electrons which leads to the formation of a six-membered cycle like it is shown below. In accordance with the Hückel rule, six π or *p* electrons in such a cycle form a relatively stable aromatic system (structure I):



Structure I



Structure II

The presence of a similar structural fragment explains the existence of hydrates of carboxylic acid amides (structure II). Thus, e.g., caffeine (CAS № 58-08-2) forms a stable monohydrate (CAS № 5743-12-4), which should be taken into account when determining this compound in various objects.

The advantage of these objects is the possibility of chromatographic analysis of reaction mixtures directly without isolation of the target sulfonamides because they are the sole reaction products. All the samples contain variable amounts of hydrophilic *p*-toluenesulfonic acid, but its peak has a retention time close to the "dead time" and does not interfere with the peaks of the target sulfonamides.

Table 3.5 includes the net retention times of some characterized N-substituted *p*-toluenesulfonamides, determined at different methanol content in an eluent ($50 \leq C \leq 85$ %, *v/v*). All compounds are listed in the ascending order of their molecular weights (MW). Besides that, the number of so called "active hydrogen atoms" ($N\{H\}$) is indicated for every compound.

Table 3.5. Retention times (min) of N-substituted *p*-toluenesulfonamides at various content of methanol in the eluent, ($50 \leq C \leq 85$ %, *v/v*). Standard deviations of all the values are ± 0.01 – 0.02 min.

Analyte	MW	*N*{H}	Content of methanol in the eluent, vol %							
			50	55	60	65	70	75	80	85
N-allyl-*p*-toluenesulfonamide	211	1	10.15	7.39	5.70	4.71	4.04	3.61	3.33	3.11
N,N-diethyl-*p*-toluenesulfonamide	227	0	20.76	13.47	9.28	6.30	5.41	4.51	3.91	3.51
N-*tert*-butyl-*p*-toluenesulfonamide	227	1	19.56	12.68	8.74	6.54	5.13	4.30	3.75	3.38
N-phenyl-*p*-toluenesulfonamide	247	1	19.05	12.03	8.17	6.10	4.80	4.06	3.57	3.26
N-hexyl-*p*-toluenesulfonamide	255	1	25.75	15.56	10.11	7.22	5.43	4.43	3.79	3.40
N-benzyl-*p*-toluenesulfonamide	261	1	—	47.54	25.43	15.56	9.76	6.82	5.10	4.12

* The point t_R = have been corrected as $t_R = 6.30 \rightarrow 6.60$, $t_R = 25.43 \rightarrow 25.70$, respectively (N,N-diethyl-*p*-toluenesulfonamide).

The general shape of $t_R(C)$ dependences does not differ from their form for other organic compounds (decreasing approximated by exponential or hyperbolic functions). The nonlinear dependences $t_R(C)$ for all characterized N-substituted *p*-toluenesulfonamides are plotted in Figure 3.6 (*a–f*).

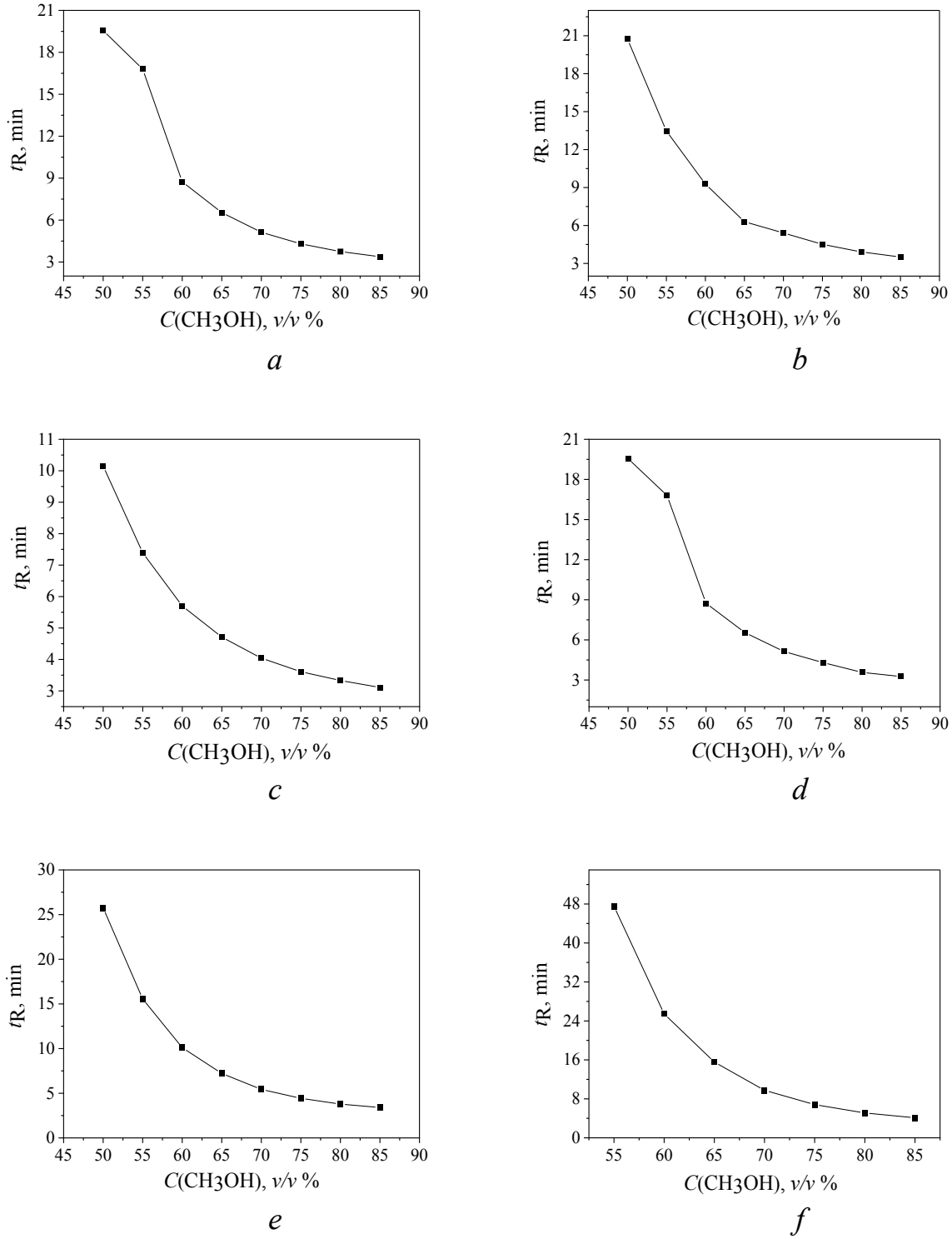


Figure 3.6. Dependencies of net retention times of (*a*) N-allyl-, (*b*) N,N-diethyl, (*c*) N-*tert*-butyl, (*d*) N-phenyl, (*e*) N-hexyl, and (*f*) N-benzyl-*p*-toluenesulfonamides on the content of methanol in an eluent ($50 \leq C \leq 85$ %, *v/v*).

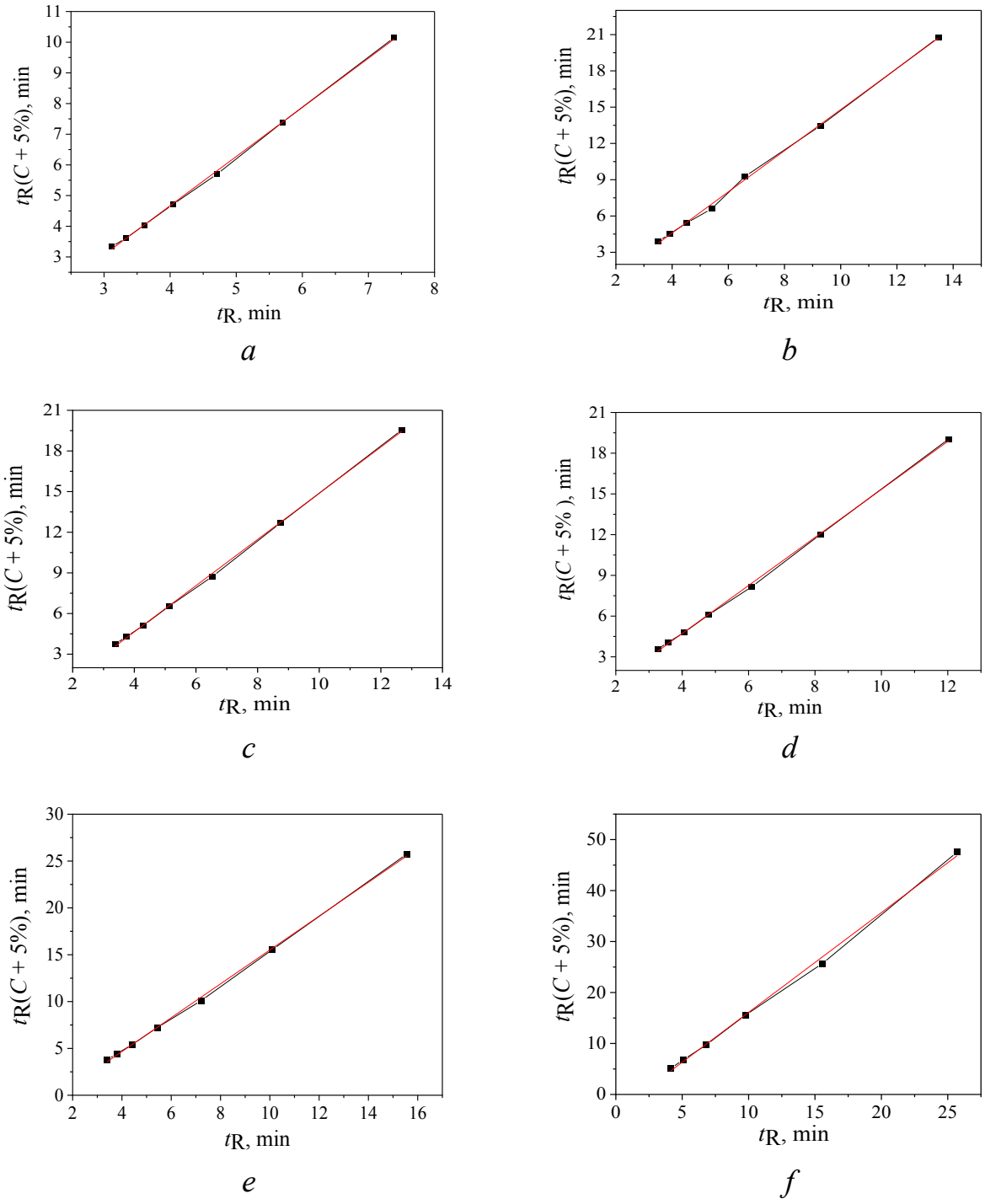


Figure 3.7. Typical plots of recurrent approximation of net retention times for (*a*) N-allyl, (*b*) N,N-diethyl, (*c*) N-*tert*-butyl, (*d*) N-phenyl, (*e*) N-hexyl, and (*f*) N-benzyl-*p*-toluenesulfonamides with methanol as an organic modifier of the eluent.

All points characterizing the approximation dependence (3.5) for different N-substituted *p*-toluenesulfonamide [Figure 3.7 (*a–f*)] correspond to a linear dependence with correlation coefficient *R* usually not less than 0.999.

Table 3.6. Parameters of the recurrent approximation of retention parameters $t_R(C)$.

Analyte	*a*	*b*	S_0	*R*
N-allyl-*p*-toluenesulfonamide	1.602	-1.736	0.018	0.9997
N,N-diethyl-*p*-toluenesulfonamide	1.702	-2.215	0.026	0.9994
N-*tert*-butyl-*p*-toluenesulfonamide	1.704	-2.170	0.018	0.9997
N-phenyl-*p*-toluenesulfonamide	1.769	-2.357	0.020	0.9996
N-hexyl-*p*-toluenesulfonamide	1.807	-2.565	0.020	0.9996
N-benzyl-*p*-toluenesulfonamide	1.956	-3.455	0.043	0.9990

The parameters of the linear recurrence relations (3.5) are presented in Table 3.6 for all characterized N-substituted *p*-toluenesulfonamides, namely the values of the coefficients *a* and *b*, correlation coefficients *R* and general dispersion S_0 (in other words – sum of residuals).

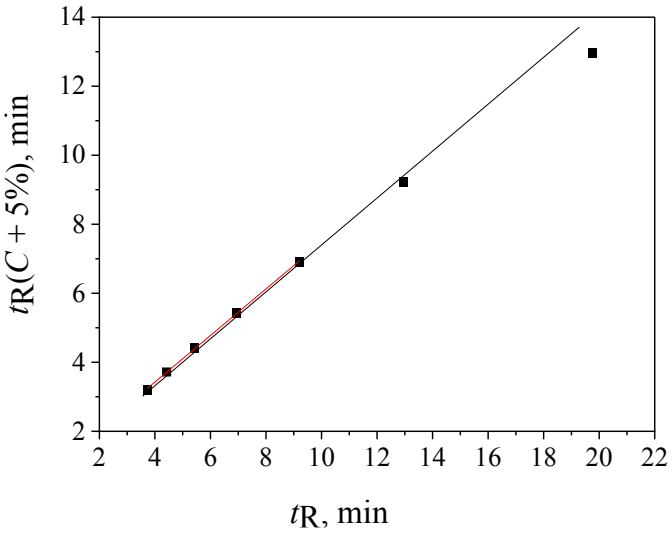


Figure 3.8. Typical plot of the linear approximation of retention parameters of N-hexyl-*p*-toluenesulfonamides at different content of acetonitrile in the eluent, t_R, min (C, % *v/v*): 3.20 (85), 3.72 (80), 4.42 (75), 5.42 (70), 6.92 (65), 9.21 (60), 12.96 (55), 19.75 (50). Regression parameters (two points for maximal water content are excluded): $a = 0.675 \pm 0.008$, $b = 0.73 \pm 0.05$, $R = 0.9998$, $S_0 = 0.03$.

The recurrent approximation of net retention times of N-hexyl-*p*-toluenesulfonamides at different content of acetonitrile in the eluent presented in graphical form in Figure 3.8 in the range from 50 to 85% *v/v* (seven points) [125]. Two points of this plot, corresponding to the maximal water content of the eluent, deviate from the regression line similarly to Figure 3.7 Comparing with the plots for N-hexyl-*p*-toluenesulfonamide when we use methanol as an organic modifier of the eluent [Figure 3.7 (*f*)], it becomes a linear with correlation coefficient R more than 0.999.

With acetonitrile as an organic modifier of the eluent, all the sulfonamides in more or less extent demonstrate the deviations from the linearity of recurrent approximations of their HPLC retention times [125]. That is, these anomalies do not depend on the hydrophilic/hydrophobic properties of the analytes, which are determined by substituents at the nitrogen atom. However, if we take methanol instead of acetonitrile as an organic modifier of the eluent, the recurrent approximations for all the sulfonamides (Figure 3.7) become practically linear.

3.2.2. Features of methanol as an organic component of the eluent in reversed-phase HPLC

Considering the differences in the properties of acetonitrile and methanol, it should be noted that organic solvents containing hydroxyl groups, namely, methanol, ethanol, simplest polyethylene glycols, etc., inhibit the formation of so-called gas hydrates of light hydrocarbons [140–144]. If methanol destroys such gas hydrates, it can destroy the hydrates of more complex organic compounds as well.

In other words, methanol, whose concentration in an eluent is many times higher than the content of target analytes, forms a more stable monohydrate (CAS № 118249-86-1 and 151900-28-5) and hence efficiently prevents the formation of hydrates of other compounds. The free energy of methanol hydration was evaluated experimentally as –5.1 kcal mol^{-1} [142]. This means that this hydrate is not a stable chemical compound under ambient conditions; this value is close to the energies of typical hydrogen bonds. In other words, the influence of methanol on the formation of hydrates can be illustrated as follows. For the hydration constants of analyte (X) and methanol (MeOH), we have two Equations (3.9) and (3.10):

$$K_X = [X{\bullet}H_2O]/\{[X] \times [H_2O]\} \quad (3.9)$$

$$K_{MeOH} = [MeOH{\bullet}H_2O]/\{[MeOH] \times [H_2O]\} \quad (3.10)$$

Combining them, we can express the ratio of hydrated and non-hydrated forms of an analyte (X) by the following inequality:

$$[X{\bullet}H_2O]/[X] = K_X[H_2O] = (K_X/K_{MeOH})\,([MeOH{\bullet}H_2O]/[MeOH]) \quad (3.11)$$

Thus, if $K_X << K_{MeOH}$, and $[MeOH] \approx [H_2O]$; hence, $[MeOH{\bullet}H_2O] \approx [H_2O]$, and the fraction of the hydrated form of analyte in the presence of methanol should be $[X{\bullet}H_2O]/[X] << 1$. Such influence of methanol in the eluent confirms indirectly that the nonlinearity of the recurrent approximation of HPLC retention times for some analytes with acetonitrile as an organic modifier of the eluent is caused specifically by the formation

of hydrates, whereas the linearity of such dependencies in the case of methanol is caused by the decomposition of such hydrates.

To additionally confirm the formation of analyte hydrates specifically in eluents, it seems reasonable to exclude the influence of the HPLC column polarity on the anomalies of chromatographic retention. For this purpose, all measurements for N-substituted *p*-toluenesulfonamides with acetonitrile as an organic modifier of the eluent were duplicated using two columns of different polarity: a column packed with nonpolar silica gel, 120 EC-C18 (A), and a column packed with a more polar sorbent, 120 EC-CN (B) [125]. The features of the recurrent approximations of the retention parameters remained the same; the features observed do not depend on the HPLC column polarity and are determined by the eluent composition [68].

3.3. Retention indices in reversed-phase HPLC. Dependence of retention indices of various compounds on the content of organic solvents in the eluent and coefficients *d*RI/*dC*

If we analyze the selected compounds at isocratic conditions, all of them may be characterized by logarithmic (Kovats) retention indices [72]:

$$RI_x = RI_n + (RI_{n+1} - RI_n) \times [\log(t_{R,x}') - \log(t_{R,n}')]/[\log(t_{R,n+1}') - \log(t_{R,n}')] \quad (3.12)$$

where $t_{R,x}$, $t_{R,n}$, and $t_{R,n+1}$ are the net retention times of the target analyte and the two reference compounds (*n*-alkyl phenyl ketones) eluted immediately before and immediately after, and RI_x, RI_n, and RI_{n+1} are their retention Indices; the asterisks mean conversion of net retention times to the adjusted retention times, $t_R' = t_R - t_0$, where t_0 is the retention time of theoretically unsorbed component (dead time or hold-up time).

The retention indices (RI) of some N-substituted *p*-toluenesulfonamides determined using methanol as the organic component of an eluent ($50 \le C \le 85\%$, *v/v*) are listed in Table 3.7 Other symbols are the same as those in Table 3.5.

Table 3.7. Retention indices of some N-substituted *p*-toluenesulfonamides as a function of the content of methanol in the eluent ($50 \leq C \leq 85$ %, *v/v*). Standard deviations of all the values are ± 1 – 2 i.u.

Analyte	MW	*N*{H}	Content of methanol in the eluent, vol %							
			50	55	60	65	70	75	80	85
N-allyl-*p*-toluenesulfonamide	211	1	852	838	823	808	792	772	756	732
N,N-diethyl-*p*-toluenesulfonamide	227	0	978	964	950	936	920	903	885	862
N-*tert*-butyl-*p*-toluenesulfonamide	227	1	968	952	935	918	898	876	852	824
N-phenyl-*p*-toluenesulfonamide	247	1	963	942	918	895	869	842	813	782
N-hexyl-*p*-toluenesulfonamide	255	1	1225	1205	1185	1165	1140	1110	1075	1029
N-benzyl-*p*-toluenesulfonamide	261	1	1014	993	972	948	921	894	860	828

Comparing with the recurrent approximation of retention times, these data provide no information on the reversible formation of hydrates in an eluent. Hence, RI values should be transformed into more informative parameters. For that we have used another criterion for revealing the hydrates formation, it is the dependence of HPLC retention indices RI on the concentration of the organic modifier in an eluent RI = *f*(*C*), namely:

$$\mathrm{RI} \approx aC + b, \qquad (3.13)$$

C = is the concentration of methanol ($50 \leq C \leq 85$ %, *v/v*), *a* and *b* are coefficients calculated by LSM.

The dependencies of the retention indices in RP HPLC on the concentration of methanol in an eluent RI = $f(C)$ for N-allyl-*p*-toluenesulfonamide and N-phenyl-*p*-toluenesulfonamide are plotted in Figure 3.9. Good linearity is observed (R = –0.997, and –0.998, respectively); with $d\mathrm{RI}/dC < 0$. The deviations from linearity for some analytes are caused by their tautomeric transformations or prototropic equilibria.

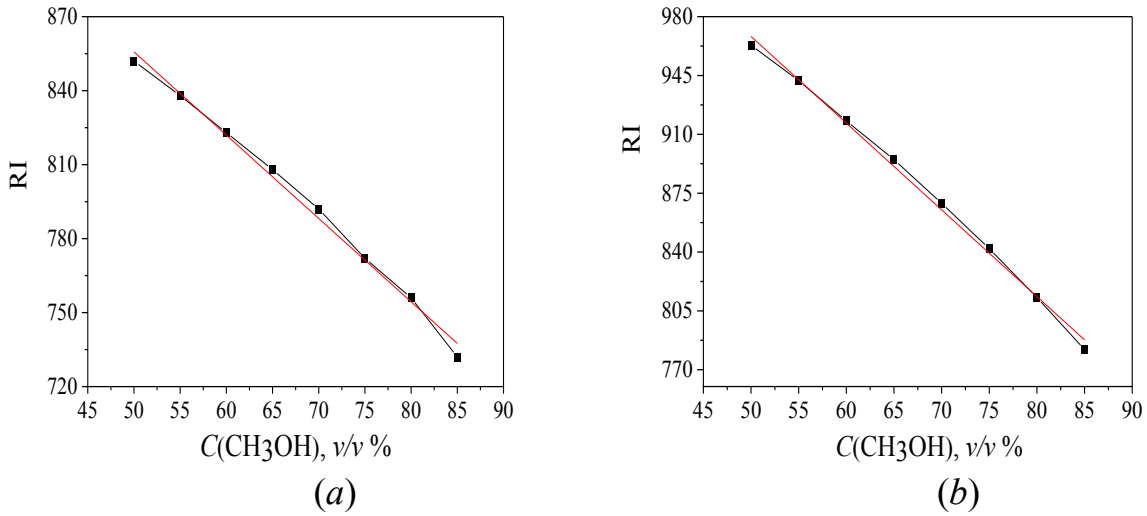


Figure 3.9. The dependencies of the retention indices in RP HPLC on the concentration of methanol in an eluent for N-allyl-*p*-toluenesulfonamide (*a*) and N-phenyl-*p*-toluenesulfonamide (*b*).

The coefficients $d\mathrm{RI}/dC$ of all other N-substituted *p*-toluenesulfonamides as a function of the content of methanol in the eluent ($50 \le C \le 85$ %, *v/v*) are presented in Table 3.8.

Table 3.8. The data illustrating the correlation of concentration coefficients of retention indices (dRI/dC) with different physicochemical characteristics of analytes: hydrophobicity factor (log$P$), homologous increments of log$P$ ($i_{\log P}$), and homologous increments (i_{RI}).

Analyte	MW	$N\{H\}$	dRI/dC	R	log$P$*	$i_{\log P}$	i_{RI} for RI (70)
N-allyl-*p*-toluenesulfonamide	211	1	–3.4 ± 0.1	–0.997	2.26 ± 0.32	–5.84	–708
N,N-diethyl-*p*-toluenesulfonamide	227	0	–3.3 ± 0.1	–0.996	2.87 ± 0.28	–5.77	–680
N-*tert*-butyl-*p*-toluenesulfonamide	227	1	–4.2 ± 0.2	–0.995	2.66 ± 0.32	–5.98	–702
N-phenyl-*p*-toluenesulfonamide	247	1	–5.2 ± 0.1	–0.998	3.04 ± 0.29	–6.14	–831
N-hexyl-*p*-toluenesulfonamide	255	1	–5.4 ± 0.3	–0.987	4.09 ± 0.30	–5.64	–660
N-benzyl-*p*-toluenesulfonamide	261	1	–5.3 ± 0.2	–0.996	3.21 ± 0.32	–6.51	–879

* Calculated log$P$ values (using ACD software) are shown with confidence intervals.

The detailed discussion of the variables $i_{\log P}$ and i_{RI} is the subject of the sections 3.4.2 and 3.4.3 respectively.

The coefficients dRI/dC are determined in RP HPLC first time, all other N-substituted *p*-toluenesulfonamides have a good linearity and the coefficients dRI/dC have negative, large absolute values.

Another example of organic compounds with different hydrophobicity are presented in Table 3.9 with the aim to evaluate the effect of their hydrophobicity/hydrophilicity on the retention indices in RP HPLC as a function of the concentration of the organic modifier in the eluent.

Table 3.9 presents retention times (min) of 17 organic compounds at different content of methanol in the eluent ($50 \leq C \leq 85$ %, *v/v*). All compounds are ranked by increasing their molecular weights.

Table 3.9. Retention times (min) of some organic compounds at various content of methanol in the eluent ($50 \leq C \leq 85$ %, *v/v*).

Analyte	MW	*N*{H}	Content of methanol in the eluent, vol %							
			50	55	60	65	70	75	80	85
Toluene	92	0	30.62	22.62	16.28	12.22	9.18	7.26	5.91	—
o-Xylene	106	0	55.52	37.30	25.16	17.34	12.40	9.25	7.15	—
Chlorobenzene	112	0	31.03	21.60	15.23	11.29	8.47	6.75	5.51	4.62
Benzotriazole	119	1	4.45	3.98	3.65	3.42	3.25	3.13	3.04	—
Nitrobenzene	123	0	9.53	7.63	6.27	5.33	4.62	4.12	3.74	—
2,3,5-Trimethylphenol	136	1	9.79	7.80	6.37	5.42	4.66	4.16	3.78	—
3-Nitrophenol	139	1	7.43	6.05	5.08	4.44	3.93	3.60	3.37	—
Phthalimide	147	1	4.55	4.06	3.71	3.46	3.29	3.16	3.06	—
1-Phenylpyrazolidin-3-one	162	1	6.67	5.40	4.55	4.00	3.62	3.35	3.16	—
4-Methoxybenzoylhydrazide	166	3	—	—	—	29.33	17.23	11.11	7.51	—
Ninhydrin (hydrate)	178	2	3.99	3.70	3.46	3.29	3.16	3.06	2.97	—
Diethyl-*m*-toluamide	191	0	16.59	11.36	8.27	6.47	5.19	4.42	3.90	—
4-Nitro-N-(2-hydroxypropyl) aniline	196	2	8.02	6.45	5.35	4.73	3.57	3.29	3.17	—
4-Nitro-2-chloro-N-(1-pyrrolidinyl) benzene	226	0	82.31	49.69	30.46	19.81	13.14	9.30	6.88	—
2,4-Dinitro-N-(2-hydroxypropyl) aniline	228	2	10.05	7.61	5.98	4.97	4.24	3.77	3.44	—
Sulfamethoxazole (hydrate)	253	3	3.35	3.14	2.98	—	—	—	—	—
1,2-*bis*(4-methoxybenzoyl) hydrazine	300	2	—	—	—	36.83	20.56	12.61	8.13	—

Table 3.10 contains the RI values of 17 organic compounds of different hydrophobicity. The RI values determined for methanol–water systems the content of methanol was varied in the range ($50 \leq C \leq 85$ %, *v/v*).

Table 3.10. Retention indices of some organic compounds as a function of the content of methanol in the eluent ($50 \leq C \leq 85$ %, *v/v*).

Analyte	MW	*N*{H}	Content of methanol in the eluent, vol %							
			50	55	60	65	70	75	80	85
Toluene	92	0	1052	1071	1088	1107	1127	1146	1176	—
o-Xylene	106	0	1150	1166	1186	1202	1230	1254	1292	—
Chlorobenzene	112	0	1046	1057	1067	1078	1090	1105	1127	1144
Benzotriazole	119	1	688	686	684	684	684	680	681	—
Nitrobenzene	123	0	847	849	854	856	860	857	852	—
2,3,5-Trimethylphenol	136	1	852	854	858	862	862	864	860	—
3-Nitrophenol	139	1	799	796	793	791	784	776	768	—
Phthalimide	147	1	693	692	691	689	690	686	686	—
1-Phenylpyrazolidin-3-one*	162	1	730	714	692	670	*732*	*729*	*715*	—
*4-Methoxybenzoylhydrazide****	166	3	—	—	—	1306	1309	1312	1312	—
*Ninhydrin (hydrate)****	178	2	662	664	664	666	668	664	663	—
Diethyl-*m*-toluamide	191	0	948	935	927	920	910	896	884	—
4-Nitro-N-(2-hydroxypropyl) aniline**	196	2	768	760	746	740	726	716	700	—
4-Nitro-2-chloro-N-(1-pyrrolidinyl) benzene*	226	0	1166	1169	1169	1174	*1240*	*1256*	*1271*	—
2,4-Dinitro-N-(2-hydroxypropyl) aniline**	228	2	810	800	780	760	*811*	*804*	*786*	—
*Sulfamethoxazole (hydrate)****	253	3	611	608	601	—	—	—	—	—
*1,2-bis(4-methoxybenzoyl) hydrazine*****	300	2	—	—	—	1364	1364	1364	1361	—

* Compounds with anomalies of RI(*C*) dependences [ranges of anomalous RI(*C*) values are italicized];

** Polar nitroanilines are characterized by the formation of hydrates in aqueous solutions;

*** The RIs of sulfamethoxazole at the content of methanol in the eluent of 40 and 45% are 614 and 613, respectively;

**** The most probable structure of the main impurity in the 4-methoxybenzoylhydrazide sample.

The coefficients *d*RI/*dC* of 17 organic compounds of various hydrophobicity as a function of the content of methanol in the eluent ($50 \leq C \leq 85$ %, *v/v*) are presented in Table 3.11.

Table 3.11. The data illustrating the correlation of concentration coefficients of retention indices (dRI/dC) of some organic compounds with their different physicochemical characteristics: hydrophobicity factor (log$P$), homologous increments of log$P$ ($i_{\log P}$), and homologous increments of retention indices (i_{RI}).

Analyte	MW	$N\{\mathrm{H}\}$	dRI/dC	R	log$P$*	$i_{\log P}$	i_{RI} for RI (70)
Toluene	92	0	4.0 ± 0.1	0.997	2.71	–0.53	527
o-Xylene	106	0	4.6 ± 0.3**	0.990	3.12	–0.66	530
Chlorobenzene	112	0	2.8 ± 0.2	0.990	2.90	–1.42	290
Benzotriazole	119	1	–0.24 ± 0.04	–0.93	1.44	–2.34	–116
Nitrobenzene	123	0	0.3 ± 0.2	0.62**	1.83	–2.49	60
2,3,5-Trimethylphenol	136	1	0.3 ± 0.1	0.83	2.73	–2.13	–34
3-Nitrophenol	139	1	–1.0 ± 0.1	–0.96	2.00	–2.86	–116
Phthalimide	147	1	–0.24 ± 0.04	–0.95	1.15	–4.25	–310
1-Phenylpyrazolidin-3-one	162	1	–4.0 ± 0.2	–0.997	0.89	–5.05	–368
4-Methoxybenzoylhydrazide***	166	3	0.3 ± 0.3	0.944	0.25 ± 0.24	–5.69	204
Ninhydrin (hydrate)***	178	2	0.05 ± 0.08	0.27**	0.67	–5.81	–532
Diethyl-*m*-toluamide	191	0	–2.0 ± 0.1	–0.995	2.18	–4.84	–390
4-Nitro-N-(2-hydroxypropyl) aniline	196	2	–2.2 ± 0.1	–0.995	1.61 ± 0.50	–5.95	–732
4-Nitro-2-chloro-N-(1-pyrrolidinyl) benzene	226	0	0.5 ± 0.1	0.93	3.92 ± 0.35	–4.72	–360
2,4-Dinitro-N-(2-hydroxypropyl) aniline	228	2	–3.4 ± 0.3	–0.990	2.13 ± 0.53	–6.51	–789
Sulfamethoxazole (hydrate)	253	3	–0.4 ± 0.1	–0.98	0.89	–8.83	–1189
1,2-*bis*(4-methoxybenzoyl) hydrazine	300	2	–0.4 ± 0.3	–0.78	2.54 ± 0.59	–11.70	–736

* Calculated log$P$ values (using ACD software) are shown with confidence intervals;

** Low correlation coefficient values indicate the nonlinearity of the dRI/dC dependences;

*** Here and below, the names of compounds containing two or three active hydrogen atoms in the molecule that explains the anomalies in the $d\mathrm{RI}/dC$–$i_{\log P}$ dependences are italicized.

It should be noted that not the RI values and, specifically, the coefficients $d\mathrm{RI}/dC$ can be considered for additional confirming the formation of hydrates of analytes in an eluent. Comparing these coefficients (data are presented in Table 3.11) shows that the minimal values of $d\mathrm{RI}/dC$ belong to the most polar analytes, such as the 1-phenylpyrazolidin-3-one, and diethyl-*m*-toluamide. On the contrary, maximal values belong to less polar analytes, such as hydrocarbons (toluene, *o*-xylene) and their chloroderivatives (chlorobenzene) [145].

Collecting the data of $d\mathrm{RI}/dC$ for selected compounds in Table 3.8 with data from Table 3.11, three distinct sub-groups can be observed: low ($d\mathrm{RI}/dC \leq -1.0$), close to zero ($-0.4 \leq d\mathrm{RI}/dC \leq 0.3$), and high ($\geq 1.6$). The first subgroup (nine most polar compounds) includes six sulfonamides with polar fragments –SO_2–N< (Table 3.8), one amide (–CO–N<), one cyclic hydrazide (–CO–NH–N<), and nitrophenol (Table 3.11). The third subgroup includes only nonpolar compounds. Thus, we can conclude that the main factor that determines the sign and absolute values of the coefficients $d\mathrm{RI}/dC$ is the polarity of the analytes. The most negative values belong to the most polar sulfonamides, for which the probability of hydrate formation is maximal [145].

The set of compounds in the middle subgroup seems to be rather unusual. It contains four medium-polarity analytes (nitrobenzene, and trimethylphenol) and four polar compounds: sulfamethoxazole (stable hydrate exists), ninhydrin (the same), 1H-benzotriazole, and phthalimide (formation of hydrates is rather probable).

At the same time, the absolute values of the coefficients $d\mathrm{RI}/dC$ are not as large as those for the analytes of the first subgroup. If the main reason for large negative $d\mathrm{RI}/dC$ values is the strong dependence of the equilibrium (of hydration equation (1.31)) on the content of the organic solvent in the eluent, then a lack of such dependence may be caused by the fact that the position of this equilibrium is independent of the solvent composition [145].

3.4. Correlations of *d*RI/*dC* coefficients with values of various physicochemical properties of analytes and their molecular parameters

3.4.1. Factors of hydrophobicity

First of all, it is of interest to check the dependence of *d*RI/*dC* parameters on hydrophobicity factors (logarithms of distribution coefficients in the 1–octanol-water heterophase system, log*P*), since the correlation of analyte retention parameters with their log*P* values is one of the main ways to estimate retention parameters in HPLC [146–150]. The log*P* values for all compounds are given in Table 3.8 and Table 3.11.

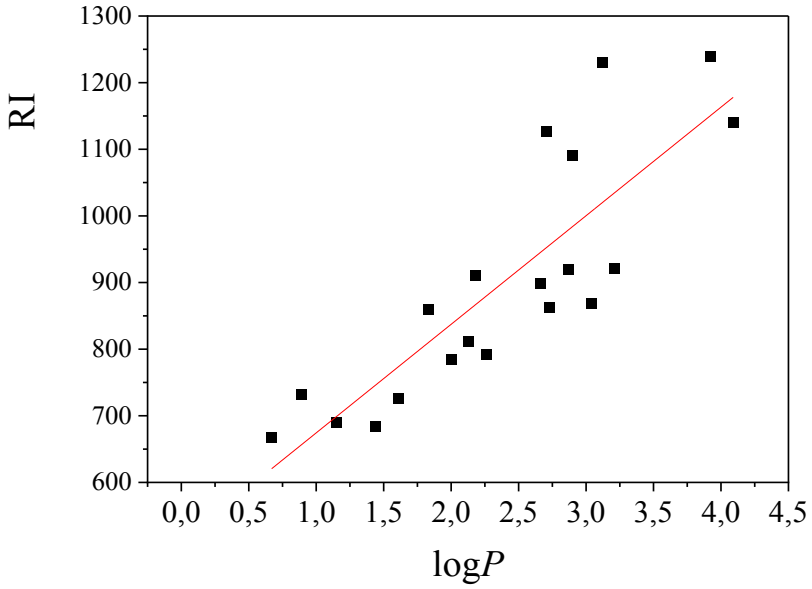


Figure 3.10. Dependence of the retention indices of various compounds in RP HPLC (eluent containing 70% CH_3OH) on the values of their hydrophobicity factors (log*P*) as a typical example of correlation in RP HPLC; linear regression parameters: $a = 163 \pm 24$, $b = 511 \pm 61$, $R = 0.847$, and $S_0 = 69$.

In the case under consideration, this is most clearly illustrated by Figure 3.10, which presents the plot of linear regression RI(log*P*) for all analytes (regression parameters are indicated in the figure caption). Experimental log*P* values were used for all compounds; in case of inconsistency of data from several sources, their arithmetic averages were calculated. The expected symbatic variation of both values is clearly observed (correlation coefficient $R = 0.847$), although for analytical purposes this dependence is of little use

because of the low accuracy of the obtained estimates. The value of S_0, representing the average accuracy of RI estimates in the selected range of log*P* variations, reaches 69 index units (i.e.). If we check the similar correlation of *d*RI/*dC* parameters with log*P* values (Figure 3.11), we should recognize its absence.

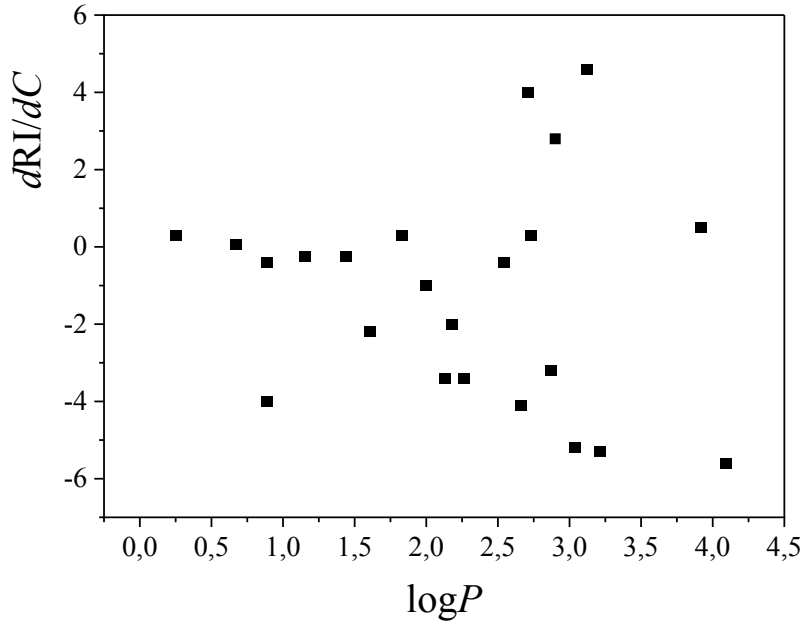


Figure 3.11. Graphical verification of the possible dependence of coefficients *d*RI/*dC* of selected analytes vs. their hydrophobicity factors, log*P*. There is no correlation.

To verify the correlation under consideration for a small number of objects, that allows us to exclude it from a more detailed consideration in the future. Similar results (no correlation) are obtained when *d*RI/*dC* is examined for its influence on the retention indices values, RI.

One of the traits of the polarity of organic compounds is thought to be the log*P* values. Nevertheless, these values grow by the homologous difference CH_2 increment when shifting from one homologue within any sub-group to the next. Because of this, there is a paradox wherein the log*P* values of polar compounds might be higher than those of less polar compounds.

The exclusion of these paradoxes can be achieved through the utilization of more informative values, such as dipole moments, whose additive dependency on the amount of methylene moieties in the molecules (or on the location of a specific homolog in the corresponding series) is not obvious. In the case of hydrophobicity, it seems sense to purposefully remove this reliance by converting values to their so-called homological increments, $i_{\log P}$.

3.4.2. Homologous increments of hydrophobicity factors

Homologous increments of additive properties (A) were originally proposed to represent chromatographic data for their joint interpretation with mass spectra in gas chromatography–mass spectrometry [151]:

$$i_A = A - x\Delta A(CH_2) \qquad (3.14)$$

where x is the integer quotient of the division of molecular mass number M by 14 (it is the mass number of the homological difference), $x = \text{int}(M/14)$, and $\Delta A(CH_2)$ is the increment of property A for the homological difference of CH_2.

Transforming the values of various properties, A into their homological increments allows us to characterize complete series, rather than individual homologs. To achieve the goal, the additive components of these qualities for set x of the homological differences of CH_2 have to be deducted from the values of A. In the context of hydrophobicity factors, expression (3.14) can be written:

$$i_{\log P} = \log P - x\Delta\log P(CH_2) \qquad (3.15)$$

The only difficulty in using this relation is the need to preliminarily determine the increment of hydrophobicity for homologous difference $\Delta(CH_2)$. Additionally, rather than utilizing computed values (such as those generated using the ACD software module), it is preferable to ascertain this increment using experimental data on the differences between the values of adjacent homologs of the same series [$\Delta\log P = \log P(n + 1) - \log P(n)$]. In this instance, $\Delta\log P = 0.54 \pm 0.07$ was obtained by averaging the available experimental (not

precalculated) values for alkylarenes (12 consecutive homologs), 2-alkanones (7), and alkyl phenyl ketones (5) [146].

Table 3.8 and Table 3.11 provides the $i_{\log P}$ values that were determined using the relation (3.15). The plots of dependence of the $d\mathrm{RI}/dC$ coefficients of the analytes under consideration on the $i_{\log P}$ values are presented in Figure 3.12. The parameters of the linear regression equation are indicated in the caption to this figure. The correlation coefficient (R = 0.835) is still lower than the value R = 0.847 for the RI(log$P$) dependence (Figure 3.10), despite the figure confirming symbatic variation of both components.

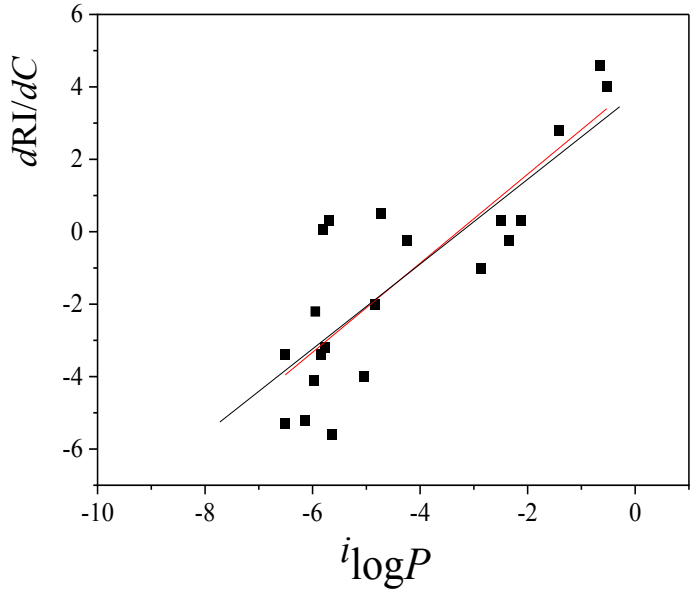


Figure 3.12. Dependence of the coefficients $d\mathrm{RI}/dC$ of the discussed sorbates (water–methanol eluents) on the values of the homologous increments of hydrophobicity factors $i_{\log P}$; linear regression equation parameters: a = 1.23 ± 0.19, b = 4.05 ± 0.9, R = 0.835, and S_0 = 1.4.

As noted above, coefficients $d\mathrm{RI}/dC$ do not correlate with the absolute values of log$P$. However, the figures suggest these correlations are strong for combinations of $d\mathrm{RI}/dC$ with homologous increments of hydrophobicity. Correlation coefficient (R) of the $d\mathrm{RI}/dC(i_{\log P})$ dependence for water–methanol eluents is 0.835.

Ninhydrin is the only substance whose $i_{\log P}$ value is blatantly at odds with the correlation that has been found. The explanation appears to be that the presence of methanol in the eluent efficiently inhibits the production of polar compound hydrates, leading to a clear correlation between the structures and the log*P* values. It can be presumed that ninhydrin (CAS № 938-24-9) is stable in aqueous-methanol solutions since it produces an unusually stable monohydrate (CAS № 485-47-2 and 2462-59-1) with a pK_a of 8.47. The log*P* value for this monohydrate should deviate significantly from the value corresponding to the non-hydrated structure.

3.4.3. Homologous increments of retention indices

Homologous increments of retention indices are another factor that can correlate with coefficients *d*RI/*dC*. Additionally, they show how the general relation (3.16) has been modified in this form [152].

$$i_{RI} = RI - x\Delta RI(CH_2) \qquad (3.16)$$

Since by definition, $\Delta RI(CH_2) \equiv 100$ for chromatographic RIs of reference components, and we may assume that $\Delta RI(CH_2) \approx 100$ for homologs of other series, relation (3.16) is simplified:

$$i_{RI} \approx RI - 100x \qquad (3.17)$$

In RP HPLC, it is presumably logical to consider the condition $\Delta RI(CH_2) \neq 100$ (in analogy with our above estimate of $\Delta \log P = 0.54 \pm 0.07$). But a closer look at this matter is necessary. Initially, homologous increments of RIs were solely suggested for identification [152]. It was later demonstrated, nevertheless, that they can be considered a feature of the polarity of organic molecules. Table 3.8 and Table 3.11 contain a list of the i_{RI} values for each sorbate that was studied. The relationship plots (3.18) for the various eluents are displayed in Figure 3.13. The parameters of the linear regression equations are indicated in the caption to the figure:

$$dRI/dC = ai_{RI} + b \qquad (3.18)$$

The correlation coefficient of the $d\mathrm{RI}/dC(i_{\mathrm{RI}})$ dependence, like that of the $d\mathrm{RI}/dC(i_{\log P})$ the linear dependence (3.18) is higher $R = 0.922$, with water–methanol eluents, which is slightly higher than the correlation coefficient $R = 0.847$ for the dependence RI(log$P$) (Figure 3.10).

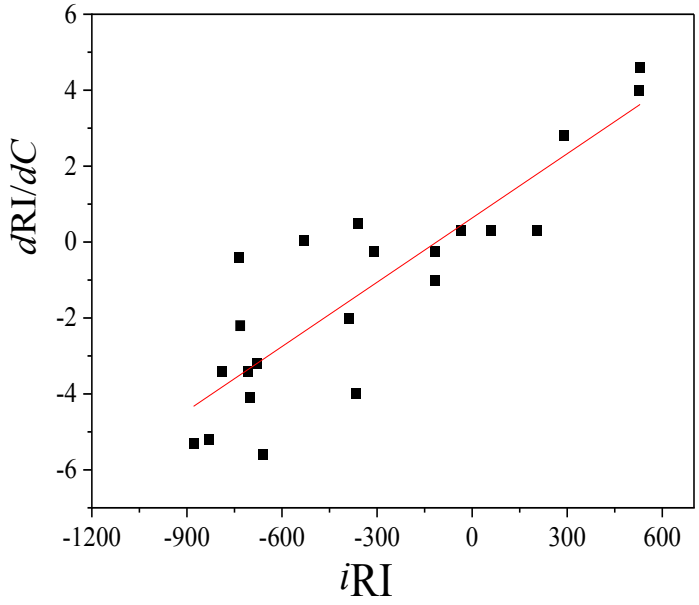


Figure 3.13. Dependence of coefficients $d\mathrm{RI}/dC$ of the considered sorbates (water–methanol eluents) on the values of the homologous increments of retention indices i_{RI} (70% CH_3OH); linear regression equation parameters: $a = (6.3 \pm 0.5) \times 10^{-3}$, $b = 0.6 \pm 0.3$, $R = 0.922$, and $S_0 = 1.1$.

Since coefficients $d\mathrm{RI}/dC$ of the same sorbates differ considerably in terms of statistics for eluents of different compositions, it is logical to analyze their relationship. Figure 3.14 shows a plot that characterizes the correlation between coefficients $d\mathrm{RI}/dC$ of the same sorbates for water–acetonitrile and water– methanol eluents.

The parameters of the linear regression equation are given in the caption to the Figure 3.14; the coefficient of correlation is $R = 0.774$, which is quite acceptable compared to the standard correlation (Figure 3.10). This plot also suggests that coefficients $d\mathrm{RI}/dC$ for nonpolar compounds are typically lower than those for water–methanol eluents when using water–acetonitrile eluents. The opposite is true for polar compounds [153,154].

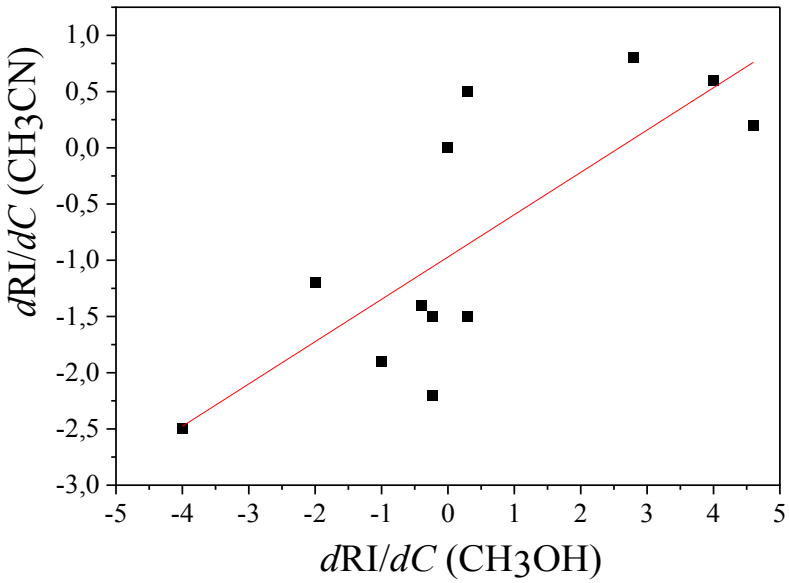


Figure 3.14. Plot for characterizing the cross-correlation of coefficients *d*RI/*dC* of the same sorbates for water–acetonitrile and water–methanol eluents. Linear regression equation parameters: $a = 0.38 \pm 0.09$, $b = -1.0 \pm 0.2$, $R = 0.774$, and $S_0 = 0.8$.

3.5. Sharing the retention parameters of analytes with their spectral characteristics. Relative optical densities $A(\lambda_1)/A(\lambda_2)$.

Absorption spectroscopy in the near UV and visible regions of the spectrum (190–800 nm) is one of the most "popular" physico-chemical methods for the characterization of organic and inorganic compounds [155–158] and one of the most efficient methods of detection in a high-performance liquid chromatography [159].

The fundamental principle of spectrophotometric analysis is the regularities of the absorption of monochromatic radiation by a layer of substance; it is described by so-called Booger-Lambert-Ber law:

$$A(\lambda) = -\log T = \log I_0/I_1 = \varepsilon c l \qquad (3.19)$$

The transmittance (T) I_1/I_0 or the percent transmittance $100 \times I_1/I_0$ is also commonly used [156], where I_0 and I_1 are the intensities of the parent and transmitted lights

respectively, c is the concentration of the light-absorbing substance, l is the length of the layer, ε (λ) is the characteristic of the intensity of light absorption by the sample.

Various techniques of quantitative spectrophotometric analysis are essentially based on the utilization of different modifications of the ratio (3.19). It is important to understand that there are several techniques involving duplication of registration of spectra for different samples (differential spectrophotometry) or at different full lengths (two-wave spectrophotometry) of both cases of difference A–values.

$$A_x = \varepsilon c_x l - A_0 \ (\lambda=\text{const}) \qquad (3.20)$$

where A_o is the optical density of the sample to compensate for errors.

The relative optical densities were recommended as an additional criterion for the identification of the analytes using RP HPLC in combination with the chromatographic parameters [160], including the level of the so-called group identification (attribution to the corresponding homologous series with the same chromophores).

$$A_{rel} = A(\lambda_1)/A(\lambda_2) \qquad (3.21)$$

The registration of the absolute UV spectroscopic parameters in HPLC is not reliable enough; hence, the determination of the so-called relative optical densities (A_{rel}) seems to be preferable: $A_{rel} = A(\lambda_1)/A(\lambda_2) \approx S_1/S_2$ (3.22)

where $S(\lambda_1)$ and $S(\lambda_2)$ are the areas of the same chromatographic peak at different wavelengths.

The relative errors in determining A_{rel} were estimated by the coefficients of variation in peak areas. $\delta(A_{rel}) = \text{sqr}[(\delta S_1)^2 + (\delta S_2)^2]$ (3.23)

Table 3.12 contain examples of compounds with both ascending and descending dependencies $A_{rel}(C)$ measured with the methanol–water eluents (the range of the methanol content is (50–85% *v/v*), as well as with almost no clearly pronounced dependencies. For instance, the aromatic hydrocarbons (toluene, *o*-xylene) in the methanol–water eluents demonstrate the ascending dependence $A_{rel}(C)$.

Table 3.12. Relative optical densities *A*(254)/*A*(220) of six N-substituted *p*-toluenesulfonamides and some other organic compounds, depending on the methanol content in the eluent.

Analyte	MW	*N*{H}	Content of methanol in the eluent, vol %							
			50	55	60	65	70	75	80	85
Toluene	92	0	0.14	0.21	0.24	0.26	0.30	0.34	0.37	—
o-Xylene	106	0	0.08	0.08	0.09	0.10	0.11	0.13	0.14	—
Chlorobenzene	112	0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Benzotriazole	119	1	3.40	3.01	2.36	2.30	4.23	4.88	6.00	—
Nitrobenzene	123	0	1.49	1.56	1.64	1.60	1.64	1.68	1.79	—
2,3,5-Trimethylphenol	136	1	1.41	2.16	1.19	0.98	0.31	1.61	1.84	—
3-Nitrophenol	139	1	0.39	0.33	0.38	0.40	0.41	0.45	0.45	—
Phthalimide	147	1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	—
1-Phenylpyrazolidin-3-one	162	1	2.59	2.73	1.29	1.58	2.21	1.68	2.33	—
4-Methoxybenzoylhydrazide	166	3	—	—	—	0.46	0.45	0.44	0.48	—
Ninhydrin (hydrate)	178	2	—	0.42	0.48	0.46	0.47	0.44	0.44	—
Diethyl-*m*-toluamide	191	0	0.13	0.13	0.12	0.12	0.11	0.11	0.12	—
4-Nitro-N-(2-hydroxypropyl) aniline	196	2	0.41	0.57	0.44	0.46	0.55	0.50	0.61	—
N-allyl-*p*-toluenesulfonamide	211	1	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.05
4-Nitro-2-chloro-N-(1-pyrrolidinyl) benzene	226	0	—	—	1.78	1.89	1.66	1.82	1.79	—
N,N-diethyl-*p*-toluenesulfonamide	227	0	0.28	0.28	0.28	0.27	0.21	0.26	0.24	0.26
N-*tert*-butyl-*p*-toluenesulfonamide	227	1	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.10
2,4-Dinitro-N-(2-hydroxypropyl) aniline	228	2	0.60	0.73	0.59	0.41	0.70	0.72	0.77	—
N-phenyl-*p*-toluenesulfonamide	247	1	0.26	0.26	0.26	0.26	0.25	0.29	0.27	0.25
Sulfamethoxazole (hydrate)	253	3	0.89	0.91	0.88	—	—	—	—	—
N-hexyl-*p*-toluenesulfonamide	255	1	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.06
1,2-*bis*(4-methoxybenzoyl) hydrazine	300	2	—	—	—	0.20	0.22	0.19	0.19	—
N-benzyl-*p*-toluenesulfonamide	261	1	0.08	0.08	0.07	0.07	0.07	0.07	0.07	0.07

The reference compounds in RP HPLC, *n*-alkyl phenyl ketones, are characterized by $dA_{rel}/dC < 0$ in all eluents. The most interesting objects, the N-substituted *p*-toluenesulfonamides, demonstrate practically no dependence regarding their relative optical densities on eluent composition.

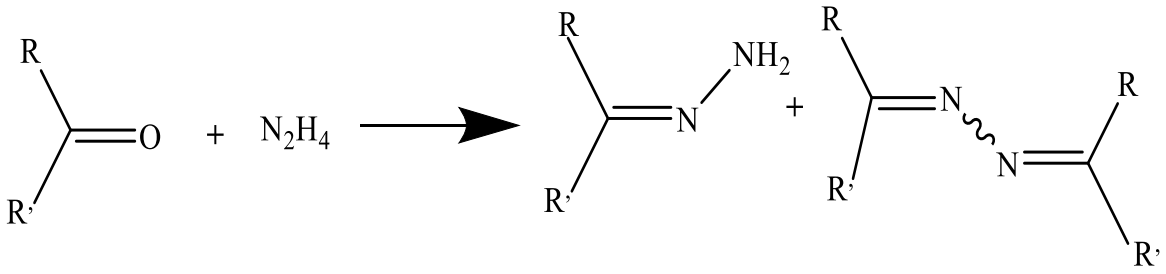
The variations of these parameters, depending on the organic modifier concentration, are not directly related to hydrate formation. Despite the negative character of this conclusion, it seems rather important because it prevents further attempts to use spectral parameters for detecting the formation of hydrates [145].

For some polar organic compounds, we can guess the reversible formation of their hydrates during reversed-phase HPLC separation. However, hydration confirmation seems to be a complex problem. The testing of the so-called relative optical densities, $A_{rel} = A(\lambda_1)/A(\lambda_2)$, shows their dependence on the composition of eluents in some cases, but, in general, they exhibit inapplicability to the detection of hydrate formation [145].

3.6. Characterization of compounds of different classes

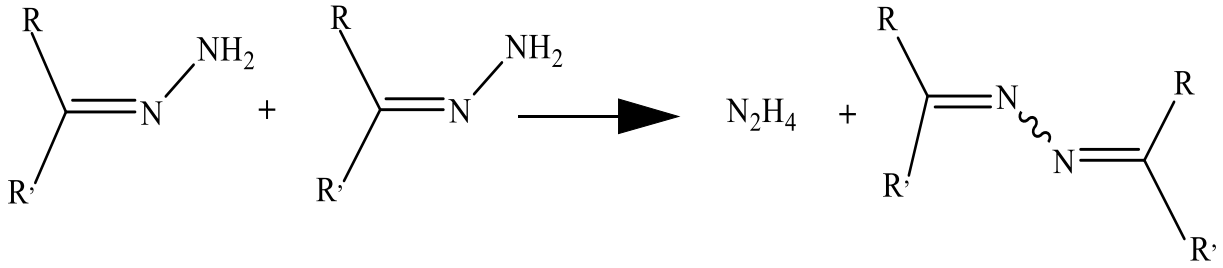
3.6.1. Unsubstituted hydrazones of aromatic carbonyl compounds

Unsubstituted hydrazones of aliphatic and aromatic aldehydes and ketones ($RR'C=N-NH_2$) are examples of very simple chemicals that are intrinsically unstable. The hydrolysis of unsubstituted hydrazones is easy; estimations currently available [161] indicate that it is 10^2–10^3 times easier than those of isostructural oximes RR'C=NOH. The hydrazine (or hydrazine hydrate) reacts with carbonyl compounds to produce these readily synthesized hydrazones. When there is an excess of carbonyl compounds, there is a significant increase in the parallel synthesis of azines (Reaction 3.1).



Reaction 3.1. Interaction of carbonyl compounds with hydrazine.

Compounds of this class are well known and they are, for example, intermediate products in the reduction of carbonyl compounds with hydrazine hydrate to isostructural hydrocarbons (the Kizhner–Wolff reaction) [162–164]. However, the NIST database [165], surprisingly, contains information about only one of the simplest member of this class, acetone hydrazone. Only one value of its gas chromatographic retention index on standard nonpolar polydimethylsiloxane stationary phases is known, determined with a large error (700 ± 24). The available reference values of the normal boiling point of this hydrazone scatter significantly (according to various sources, 124–125, 114–116, 122–126, 128–131, 110.5°C, etc.), indicating the thermal instability of this compound. Even storage at room temperature causes a gradual conversion of unsubstituted hydrazones into more stable azines. Therefore, testing the possibilities of the gas chromatographic separation of both aliphatic and aromatic compounds of this class expectedly confirmed their decomposition in a chromatographic column [166]. This process is a second-order bimolecular reaction, which sets it apart from most other instances of the thermal degradation of analytes (Reaction 3.2). The hydrazine that results can interact secondarily with other elements of the test samples.



Reaction 3.2. Decomposition of unsubstituted hydrazones to form azines.

Chromatographic peaks are distorted as a result of these processes, and chromatograms have abnormal forms. As conditionally shown in Figure 3.15, if component A becomes component B (or vice versa), a broadened zone Z (also called a "tail," "plateau," etc.) can be recorded between their peaks. Chromatograms of compounds that can undergo tautomeric transformations during chromatographic separation have a similar shape [167]. The profiles of the chromatograms of analytes unstable under separation conditions are similar both in gas chromatography and in reversed-phase HPLC. As a rule, decomposition products A → B have lower retention parameters, $t_R(B) < t_R(A)$. However, more complex products of bimolecular reactions B → A the retention parameters for compounds formed in the results of Reaction 3.2 may be greater than those of initial ones. The plateau between the peaks may belong to both components A and B in varied ratios, or only to decomposition products B, according to the mass spectra collected at various locations inside the diffuse zone Z (Figure 3.15) [168]. Based on their boiling points at atmospheric pressure without decomposition, the temperature ranges of analytes thermal stability during chromatographic separation are evaluated [169], and it appears that this requirement is not met even for the most basic unsubstituted hydrazones.

Due to the inherent inability of the gas chromatographic separation of unsubstituted hydrazones (*a*) makes it necessary to check the applicability of reversed-phase high-performance liquid chromatography (RP HPLC). A necessary condition for the UV detection of analytes in this version of separation is the presence of a chromophore in the molecule; therefore, we limited the consideration only to hydrazones of aromatic carbonyl compounds.

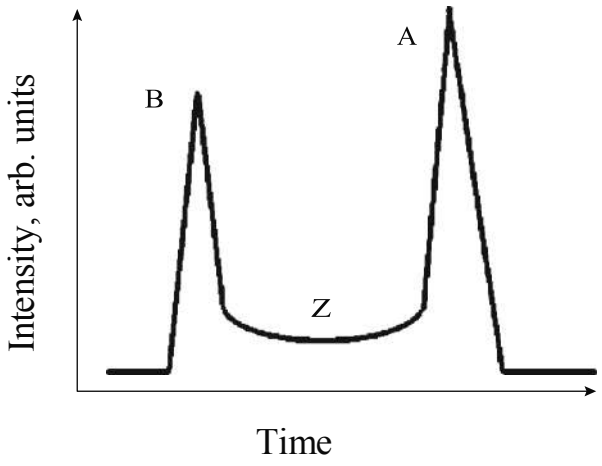


Figure 3.15. Schematic representation of the chromatogram of analytes unstable under chromatographic separation conditions. The diffuse zone Z (named as "trail region", "plateau", etc.) between the peaks indicates that component A is converted into component (or *vice versa*) in the chromatographic column.

The instability of unsubstituted hydrazones during storage determined the nature of the test samples in this work: these were reaction mixtures of the corresponding carbonyl compounds containing a large excess of hydrazine hydrate. The objective of this part of work was to elucidate the stability of the simplest unsubstituted hydrazones of aromatic carbonyl compounds under RP HPLC conditions rather than characterize a large number of analytes.

Hydrazones of aromatic ketones. Table 3.13 presents the analytical data for the products of the interaction of four aromatic ketones with hydrazine hydrate, first of all, the retention indices of the products determined in isocratic modes at a methanol concentration between $50 \leq C \leq 85$ vol % in the eluent. The m/z ranges corresponding to the molecular weights of the initial compounds and expected hydrazones, necessary for their selective mass spectrometric detection, and the relative optical densities $A_{rel} = A(254/220)$ are also presented. For comparison, the corresponding relative absorbance values of the initial carbonyl compounds and their retention indices are presented. The use of such spectral parameters increases the reliability and uniqueness of the identification of analytes in RP

HPLC. In some cases, they can be assigned to the corresponding homologous series [160,170].

Table 3.13. Analytical data (retention indices, *m*/*z* values, and relative absorbance *A*(254/220) values) for hydrazones.

Analyte	*m/z* range of $[M+H]^+$	Content of methanol in the eluent, vol %								Average A_{rel}
		50	55	60	65	70	75	80	85	
Acetophenone hydrazone (I)										
RI	135.0896 – 135.0937	—	738	737	738	734	737	736	734	—
$A_{rel} = A(254)/A(220)$		—	1.23	1.22	1.20	1.13	1.05	1.04	1.23	1.16 ± 0.07
$\Delta RI = RI_{hydrazone} - RI_{ketone}$		—	-62	-63	-62	-66	-63	-64	-66	—
4-Methylacetophenone hydrazone (II)										
RI	149.1051 – 149.1096	784	799	803	830	816	871	934	—	—
$A_{rel} = A(254)/A(220)$		1.18	1.22	1.18	1.16	1.21	1.19	1.12	—	1.18 ± 0.03
$\Delta RI = RI_{hydrazone} - RI_{ketone}$		-100	-85	-81	-54	-68	-13	+40	—	—
Propiophenone hydrazone (III)										
RI	149.1051 – 149.1095	795	808	819	830	821	840	879	—	—
$A_{rel} = A(254)/A(220)$		1.33	1.16	1.13	1.03	1.10	1.07	1.09	—	1.13 ± 0.10
$\Delta RI = RI_{hydrazone} - RI_{ketone}$		-105	-92	-81	-70	-79	-60	-21	—	—
Butyrophenone hydrazone (IV)										
RI	163.1222 – 163.1238	875	885	894	901	890	905	939	—	—
$A_{rel} = A(254)/A(220)$		1.30	1.13	1.12	1.08	1.11	1.07	1.01	—	1.12 ± 0.09
$\Delta RI = RI_{hydrazone} - RI_{ketone}$		-125	-115	-106	-99	-110	-95	-61		—
Average A_{rel}	—									1.15 ± 0.03
$\Delta RI = RI_{hydrazone} - RI_{ketone}$	-81 ± 21									—
A_{rel} of initial ketone	—									2.6 ± 0.2

Information presented in Table 3.13 enabled the following conclusions:

✓ The mass numbers of the $[M + H]^+$ ions of the main components of the reaction mixtures in all cases correspond to the mass numbers of such ions for the expected aromatic ketone hydrazones I–IV;

✓ The mass chromatograms of reaction mixtures recorded by mass numbers of $[M + H]^+$ ions of initial carbonyl compounds showed their absence. The total ion current chromatograms in the *m*/*z* range of 50−750 and the results of HPLC analysis also demonstrate the absence of detectable amounts of azines in such reaction mixtures;

✓ The retention indices of the detected components are always smaller than the retention indices of the initial ketones and depend on the concentration of methanol in the eluent. The average difference between the retention indices of hydrazones and initial ketones ($\Delta RI = RI_{hydrazone} - RI_{ketone}$) is −81 ± 21. Since the retention indices in RP HPLC depend on the concentration of the organic component of the eluent [155], the coefficients dRI/dC were additionally estimated to be –0.11 ± 0.05 (for hydrazone I), 1.9 ± 0.6 (II), 1.6 ± 0.3 (III), and 1.6 ± 0.4 (IV);

✓ The values of relative optical densities A_{rel} of the main components of the reaction mixtures are 1.15 ± 0.03 and differ statistically significantly from the A_{rel} of initial carbonyl compounds.

Differences in the retention indices and values of relative optical densities A_{rel} of the initial ketones and the expected hydrazones confirm the completeness of the interaction of ketones with hydrazine hydrate. The absence of chromatographic anomalies of the interaction products also indicates that such hydrazones are stable to hydrolysis under the RP HPLC conditions even at a weakly acid reaction of the water–acetonitrile eluent (0.1% of formic acid).

Another argument in favor of the formation of alkylaryl ketone hydrazones is the presence of two peaks with the same molecular masses corresponding to the *syn*- (minor component with a shorter retention time) and *anti*-hydrazone isomers in the mass chromatograms of the reaction mixtures by the masses of $[M + H]^+$ hydrazones of

unsymmetrical carbonyl compounds (all alkylaryl ketones). A similar separation of *syn*- and *anti*-isomers was previously was noted for 2,4-dinitrophenylhydrazones of unsymmetrical carbonyl compounds [171].

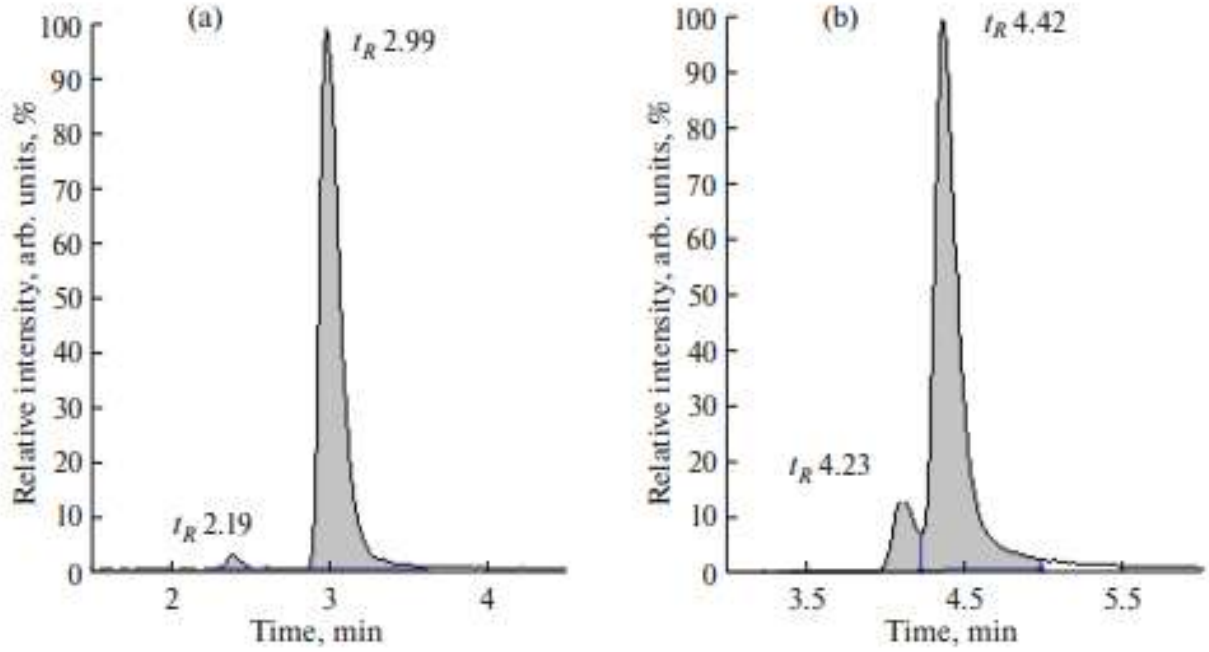


Figure 3.16. Fragments of the mass chromatograms of (a) acetophenone hydrazone and (b) propiophenone hydrazone by mass numbers of $[M + H]^+$ ions. Minor peaks with shorter retention times correspond to *syn*-isomers, while the main peaks correspond to *anti*-isomers.

The behavior of hydrazones of substituted benzaldehydes under HPLC conditions. The analytical data for the reaction products of three aromatic aldehydes are given in Table 3.14 under RP HPLC settings, it can be difficult to understand the chromatograms of these reaction mixtures. First, it is well known [172] that aldehydes react more quickly with hydrazines comparing with ketones, which imply that reaction 3.1, can produce both the equivalent azines and unsubstituted hydrazones.

One example that may be used to get a more thorough evaluation of the general characteristics of the behavior of hydrazones of aldehydes under RP HPLC settings is the examination of the reaction mixture of *p*-methylbenzaldehyde (V) with hydrazine hydrate.

A fragment of its total ion current chromatogram in the *m*/*z* range of 50−300 is shown in Figure 3.17a. There are no noticeable chromatographic peaks in the retention time region of the initial carbonyl compound; the only signal is a peak at a retention time of 12.39 min, corresponding to *p*-methylbenzaldehyde azine. A mass chromatogram was acquired in the *m*/*z* range of 121.0630–121.0666 in order to specifically identify potential traces of the original carbonyl molecule (Figure 3.17b) [124].

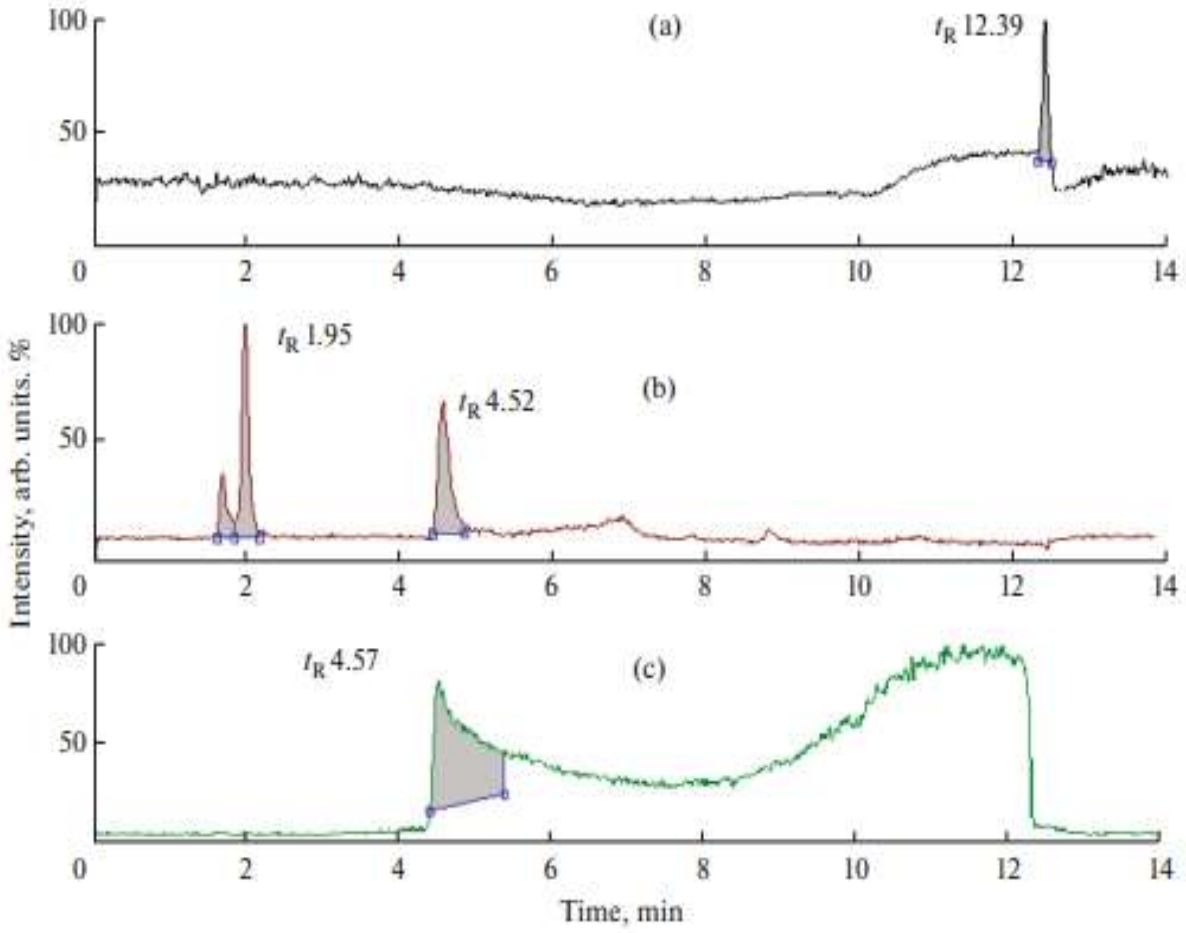


Figure 3.17. Mass chromatograms of the reaction mixture of *p*-methylbenzaldehyde with an excess of hydrazine hydrate (a) by total ion current in the *m*/*z* range of 50–300, (b) by the mass number of $[M + H]^+$ ions of the initial aldehyde (in the *m*/*z* range of 121.0630–121.0666), and (c) by the mass number of $[M + H]^+$ ions of unsubstituted hydrazone (in the *m*/*z* range of 135.0896–135.0037).

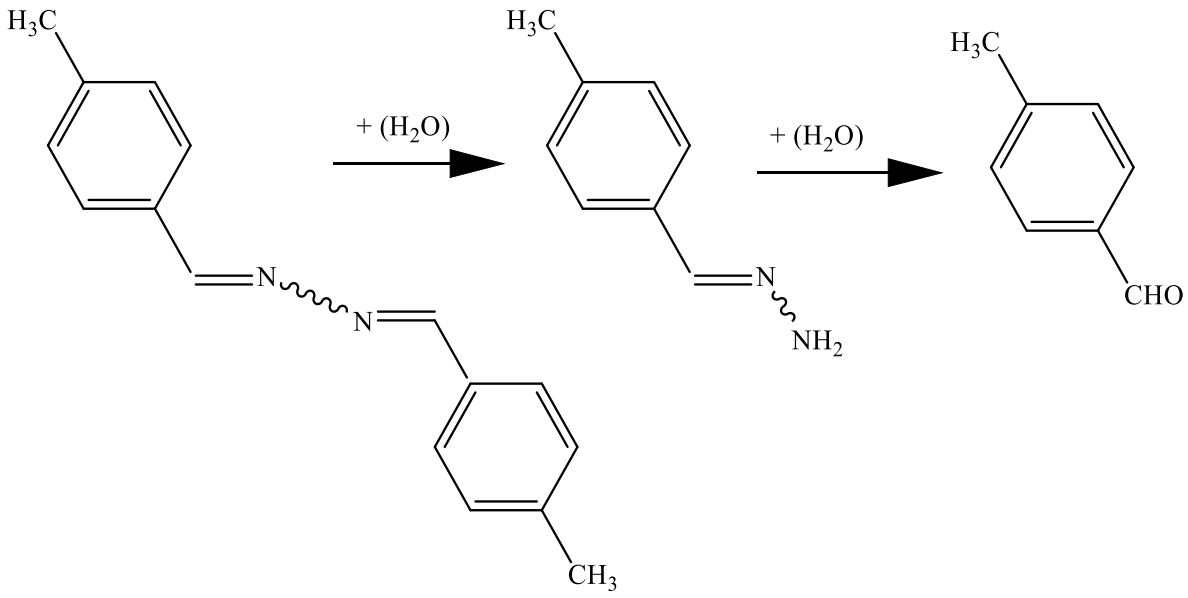
Table 3.14. Analytical data (retention indices and relative optical densities A_{rel}) of the components of the reaction mixtures of some substituted benzaldehydes with hydrazine hydrate [124].

Analyte	Content of methanol in the eluent, vol %								Average A_{rel}
	50	55	60	65	70	75	80	85	
RI 4-Methylbenzaldehyde hydrazone* V (A)**	719	720	720	721	724	720	723	—	—
$A_{rel} = A(254)/A(220)$	1.04	0.94	0.95	1.09	0.99	0.93	0.91	—	0.98 ± 0.06
RI 4-Methylbenzaldehyde (B)	870	—	874	—	879	—	884	—	—
$A_{rel} = A(254)/A(220)$	2.36	—	2.01	—	2.02	—	1.97	—	2.09 ± 0.18
$\Delta RI = RI_{hydrazone} - RI_{aldehyde}$	-151	—	-154	—	-155	—	-161	—	—
RI 2-Methylbenzaldehyde hydrazone VI (A)	—	728	724	721	717	716	712	710	—
$A_{rel} = A(254)/A(220)$	—	0.63	0.63	0.59	0.62	0.60	0.53	0.63	0.60 ± 0.03
RI 2-Methylbenzaldehyde (B)	860	—	860	—	860	—	861	—	—
$A_{rel} = A(254)/A(220)$	2.51	—	2.00	—	1.71	—	1.52	—	1.9 ± 0.4
$\Delta RI = RI_{hydrazone} - RI_{aldehyde}$	—	—	-136	—	-143	—	-149	—	—
Average A_{rel} for hydrazones	—								0.79 ± 0.0
Average A_{rel} for aldehyde	—								2.0 ± 0.1
$\Delta RI = RI_{hydrazone} - RI_{aldehyde}$	-150 ± 9								—
RI 2-Hydroxybenzaldehyde hydrazone VII (A)	—	722	719	715	713	711	708	702	—
$A_{rel} = A(254)/A(220)$	—	0.48	0.47	0.45	0.46	0.41	0.40	0.40	0.44
RI 2-Hydroxybenzaldehyde (B)	795	—	804	—	811	—	819	—	—
$\Delta RI = RI_{hydrazone} - RI_{aldehyde}$	—	—	-85	—	-98	—	-111	—	—

* The *m/z* range for $[M + H]^+$ ions are 121.0630–121.066.

** Results of (A) chromatographic analysis (eluents: methanol–water) and (B) chromatography–mass-spectrometric analysis (eluent: acetonitrile–water).

The signal detected at 4.52 min of retention time is identified as *p*-methylbenzaldehyde; on the other hand, the second signal, which was recorded at t_R 1.95 min, is inherently associated with the mixture of *p*-methylbenzoic acid, which is the result of oxidizing this kind of aldehyde with air oxygen. A mass chromatogram in the *m*/*z* range of 135.0896–135.0037 was recorded for a similar reason, namely to find potential evidence of unsubstituted hydrazone (Figure 3.17c). Nevertheless, a diffuse, wide signal is obtained rather than distinct chromatographic peaks. The right boundary of this trail region (approximately 12.4 min) corresponds to the retention time of *p*-methylbenzaldehyde azine, while the left boundary (4.57 min) practically coincides with the retention time of the initial aldehyde [124].



Reaction 3.3. Hydrolysis of *p*-methylbenzaldehyde azine to form hydrazone and (finally) aldehyde.

The chromatographic profile depicted in Figure 3.15, which describes the instability of analytes in the chromatographic column during separation, is comparable to the shape of this kind of signal. In this instance, the profile shows that *p*-methylbenzaldehyde azine was hydrolyzed during analysis (Reaction 3.3).

The signal broadening shown in Figure 3.17 c is primarily associated with $[C_8H_{10}N_2 + H]^+$ ions, which are compositionally similar to the unsubstituted hydrazone of *p*-methylbenzaldehyde. Nevertheless, this component is created by the hydrolysis of azine

and is not a part of the reaction mixture. Reaction 3 suggests that there should be two steps in this process. As far as information goes, this is the first instance of two-stage analyte hydrolysis under RP HPLC conditions and only the second instance of two-stage processes in a chromatographic column. Previously, reactions of this kind were thought to be responsible for the thermal breakdown of unsubstituted hydrazones of carbonyl compounds during gas chromatographic examination [166,167].

The results of the chromatographic analysis of the same mixtures of aromatic aldehydes with hydrazine hydrate, obtained using a Shimadzu LC-20 Prominence chromatograph and using an UltiMate 3000 chromatograph with mass spectrometric detection, differ significantly. Initially, the retention indices of the components that were found do not match up; for the Shimadzu LC-20 Prominence, they are –150 ± 9 index units smaller. When acetonitrile is used in place of methanol in the eluent, these differences outweigh any potential fluctuations in the retention indices. To put it another way, the pattern is the same as when it comes to carbonyl compounds and aromatic ketone hydrazones, for which the difference is –81 ± 21 (Table 3.13).

Unsubstituted hydrazones containing two active hydrogen atoms in the molecule are eluted earlier than their precursors – corresponding aldehydes. Secondly, the values of relative optical densities A_{rel} of 0.79 ± 0.00 and 2.0 ± 0.1 do not match. We note here an analogy with the A_{rel} values for ketone hydrazones and ketones themselves (1.15 ± 0.03 and 2.6 ± 0.2, Table 3.13). The combination of two independent analytical parameters (retention indices + spectral ratios) enables an unequivocal conclusion that under the conditions of chromatographic separation, we detect only hydrazones, while under the conditions of chromatography–mass spectrometry, aldehyde hydrazones are completely hydrolyzed. The reason for this is the slightly acidic reaction (pH ≈ 2.7) of the eluent containing 0.1% of formic acid [124].

3.6.2. Oximes of aromatic carbonyl compound

The chromatographic characteristics of several representatives of oximes of aromatic carbonyl compounds in reversed-phase HPLC have been determined, including determination of their retention indices and recurrence control of the dependences of retention times of sorbates on the concentration of organic compounds ($30 \leq C \leq 80$ %, *v/v*). Such control allows the detection of a significantly larger number of irregularities retention time anomalies than it is possible using other techniques. Chromatographic information is supplemented with spectral parameters, namely relative optical densities A_{rel}.

Anomalies in the chromatographic retention of sorbates in RP HPLC are often attributed to variations in their sorbate retention methods. However, an equally important reason appears to be a change in the chemical nature of sorbates due to interaction with eluent components. Taking into account the chromatographic properties of oximes allowed us to compounds that are stable under separation conditions, as well as to identify examples of reversible hydration (oximes).

Examples of both reversible hydration (oximes of 2-methoxy- and 3,4-dimethoxybenzaldehydes) and irreversible hydrolysis (oximes of 2- and 4-hydroxybenzaldehydes, acetophenone) with the formation of the corresponding aldehydes. It is shown that the coefficients of dependence of retention indices from the concentration of the organic component of the eluent for aldehydes predominantly satisfy the inequality $d\mathrm{RI}/dC > 0$, and for their oximes are usually negative. Consequently, the differences of retention indices $\Delta\mathrm{RI} = \mathrm{RI}_{(\mathrm{oxime})} - \mathrm{RI}_{(\mathrm{aldehyde})}$ in the RP HPLC are not constant. However, they diminish as the methanol content of the eluent increases.

Table 3.15 shows the substituents in aryl fragments of molecules, the values of retention indices (RI) of the oximes of nine aromatic aldehydes (*a–i*) and three aromatic ketones (*j–l*) were characterized and their precursors (aldehydes) using eluents with different methanol concentrations (*C*) from ($30 \leq C \leq 80$ %, *v/v*) in increments of 10 %, as

well as the values of the relative optical densities $A_{rel} = A(\lambda_1)/A(\lambda_2) = A(254)/A(220)$ [160,170].

Table 3.15. Retention indices of aromatic carbonyl compounds and their oximes at different concentrations of methanol in the eluent (RI), relative optical densities A_{rel}, dRI/dC values and the difference of retention indices " between oximes and substituted benzaldehydes" (ΔRI). Anomalous values are bolded with the "direction" of deviations (↑ or ↓).

Substituent in the aromatic fragment		Content of methanol in the eluent, vol %						Average A_{rel}	dRI/dC (R)
		30	40	50	60	70	80		
2-Methyl	RI aldehyde	—	—	870	878	885	876	—	—
	A_{rel}	—	—	4.60	4.77	4.79	4.27	4.6 ± 0.2	
a	RI oxime	796	826	846	837	825	799	—	-1.5 ± 0.3 (-0.968)
	A_{rel}	—	0.93	1.18	1.23	0.96	1.17	1.09 ± 0.14	—
	ΔRI	-9	25	-24	-41	-60	-77	—	—
4-Methyl	RI aldehyde	—	—	857	857	858	846	—	—
	A_{rel}	—	—	3.63	3.93	3.39	3.20	3.5 ±0.3	—
b	RI oxime	—	**806↓**	855	855	842	813	—	-1.4 ± 0.5 (-0.906)
	A_{rel}	—	**0.83↓**	1.82	1.78	1.07	1.86	1.6 ± 0.4	A(40) is less than others
	ΔRI	—	—	-2	-2	-16	-33	—	—
2-Hydroxy	RI aldehyde	782	785	790	798	802	798	—	RI and A_{rel} are almost identical for the aldehyde
	A_{rel}	0.92	0.91	0.84	0.61	0.60	0.59	0.74 ± 0.16	
c	RI oxime	780	785	792	799	808	805	—	
	A_{rel}	0.88	0.86	0.80	0.72	0.82	0.77	0.80 ± 0.06	
	ΔRI	-2	0	2	1	6	7	—	—

Table 3.15. (Contd.)

4-Hydroxy	RI aldehyde	706	694	751	796	846	886	—	4.6 ± 0.1 (0.9992)
	A_{rel}	—	—	0.04	0.04	0.04	0.04	0.04 ± 0.00	—
d	RI oxime	747	798	792	800	846	886	—	3.3 ± 0.6** (0.969)
	A_{rel}	—	—	0.02	0.07	0.07	0.04	0.04 ± 0.02	—
	ΔRI	—	—	41	4	0	0	—	RI and A_{rel} are almost identical to data for aldehyde
2-methoxy	RI aldehyde	—	—	902	907	908	892	—	-0.29±0.34 (-0.51)
	A_{rel}	—	—	0.08	0.08	0.08	0.05	0.07 ± 0.02	—
e	RI oxime	822	805	794	788	772	746	—	-1.4 ± 0.1 (-0.978)
	A_{rel}	0.75	0.71	0.76	0.67	0.76	0.54	0.07 ± 0.08	—
	ΔRI	—	—	-108	-119	-136	-146	—	—
4-methoxy	RI aldehyde	802	793	790	789	789	782	—	-0.32±0.07 (-0.92)
f	A_{rel}	0.52	0.49	0.52	0.49	0.54	0.63	0.53 ± 0.05	—
	RI oxime	794	778	765	753	741	708	—	-1.31±0.04 (-0.998)
	A_{rel}	1.23	1.24	1.34	1.29	1.28	**0.52**↓	1.28 ± 0.04	—
	ΔRI	-8	-15	-25	-36	-48	-74	—	—
4-hydroxy-3-methoxy	RI aldehyde	699	707	749	797	846	887	—	4.6 ± 0.1 (0.9995)
	A_{rel}	**0.33**↑	0.04	0.05	0.04	0.04	0.04	0.04 ± 0.01	—
g	RI oxime	672	655	643	635	623	611	—	-1.17 ± 0.06 (-0.9995)
	A_{rel}	0.52	0.54	0.54	0.53	0.55	0.54	0.54 ± 0.01	—
	ΔRI	-27	-52	-106	-162	-223	-276	—	—

Table 3.15. (Contd.)

3-hydroxy-4-methoxy	RI aldehyde	746	706	750	796	846	886	—	4.6 ± 0.1 (0.9995)
	A_{rel}	0.05	0.04	0.05	0.05	0.05	0.04	0.05 ± 0.01	—
h	RI oxime	691	669	653	642	630	614	—	-1.47 ± 0.09 (-0.993)
	A_{rel}	0.54	0.55	0.53	0.53	0.53	0.52	0.53 ± 0.01	—
	ΔRI	-55	-47	-97	-154	-216	-272	—	—
3,4-Dimethoxy	RI aldehyde	760	739	750	797	845	886	—	4.6 ± 0.1 (0.9996)
	A_{rel}	—	—	0.04	0.04	0.04	0.04	0.04 ± 0.00	—
i	RI oxime	753	724	707	694	680	668	—	-1.63 ± 0.14 (-0.985)
	A_{rel}	0.58	0.34	0.59	0.62	0.60	0.57	0.55 ± 0.1	—
	ΔRI	-7	-15	-43	-103	-165	-218	—	—
Acetophenone	A_{rel}	—	—	3.34	2.85	2.78	2.53	2.8 ± 0.3	RI and A_{rel} are almost identical for the aldehyde
	RI oxime	816	802	803	803	810	800	—	
j	A_{rel}	3.70	3.51	3.31	2.12	2.21	1.97	2.8 ± 0.3	
	ΔRI	16	2	3	3	10	0	—	
Propiophenone	A_{rel}	—	—	1.98	2.56	2.29	1.82	2.2 ± 0.3	—
	RI oxime	887	872	915	902	893	862	—	-1.7 ± 0.3 (- 0.962)
k	A_{rel}	**0.34↓**	**0.4↓**	1.14	1.16	1.18	0.99	1.12 ± 0.09	A(30), A(40) less than others
	ΔRI	-13	-28	15	2	-7	-38	—	—
Butyrophenone	A_{rel}	—	—	2.78	2.76	2.52	2.37	2.6 ± 0.2	—
	RI oxime	—	968	1015	1015	987	948	—	-2.3 ± 0.6 (- 0.932)
l	A_{rel}	—	**0.29↓**	1.09	1.12	1.14	1.13	1.12 ± 0.02	A(40) less than others
	ΔRI	—	-32	15	15	-13	-52	—	—

Considerable variations in the values of A_{rel} serve as evidence for the transformation of aromatic carbonyl compounds into corresponding oximes as a result of treatment with hydroxylamine. In some cases, the retention times and, hence, retention indices of these compounds are close, but if the A_{rel} values are different, this is a definite and unequivocal indication of a reaction. From the RI(C) values we calculated the coefficients dRI/dC dependence of RI on the methanol content in the eluent and their corresponding correlation coefficients of linear regressions (R).

The comparison of several RI(C) values determined for eluents with $30 \leq C \leq 80$ volume %, as well as several values of A_{rel}, makes it possible to identification of anomalous values (highlighted in bold in Table 3.15). If they are larger than the average values for other methanol concentrations or their corresponding points are located above the regression lines, they are marked with the symbol "↑", if less – with the symbol "↓". However, the number of such anomalies detected when considering the indices is relatively small. For example, for 2-methylbenzaldehyde the RI values at C = 30 and 40% (805 and 806) are noticeably smaller than the others (870–885). The same applies to the values of A_{rel} at C = 30–40% (0.11–0.12) and the rest (4.27–4.79).

In reversed-phase HPLC, retention anomalies are often attributed to changes in sorbate-sorbent interaction mechanisms. However, it is important to note that if such anomalies are simultaneously observed not only for the retention parameters, but also for A_{rel} values, this clearly confirms, that their cause is not a change in the sorption mechanism, but in the chemical nature of the sorbates. Changes in A_{rel} mean that chemical transformations affect the chromophores in the molecules. At high water content in the eluent, one of the reasons for the A_{rel} variations could be the reversible formation of hydrated forms of sorbates, which is in agreement with the literature data known for many compounds [108,173,174].

For aldehydes, however, their reversible methanolysis, equivalent to the formation of covalent hydrates or semi-acetals, cannot be excluded:

$$Ar–CHO + H_2O \rightleftharpoons Ar–CH(OH)_2 \qquad (3.4)$$

$$Ar–CHO + CH_3OH \rightleftharpoons Ar–CH(OH)OCH_3$$

The peculiarities of A_{rel} values were noted both for 2-methylbenzaldehyde itself and for its oxime at C = 30 % (0.07 versus 0.96–1.23). Similar anomalies values of RI and A_{rel} are also observed for oxime of 4-methylbenzaldehyde at concentrations of methanol in the eluent 30 and 40%. There are examples of overestimating A_{rel} values at low methanol concentrations compared to the others: 4-hydroxybenzaldehyde, vanillin and 3,4-dimethoxybenzaldehyde. However, it can be noticed that, in general, the reproducibility of the relative optical densities A_{rel} = A(254)/A(220) is not high, which depends on the absolute intensity of the chromatographic peaks, which may be due to the presence of UV–absorbing impurities in the eluent.

Finally, rather unexpected examples of chromatographic behavior were revealed by comparing the retention indices of 2– and 4–hydroxybenzaldehydes and the main components of their reaction mixtures with hydroxylamine. At all concentrations of methanol in the eluent, the values of retention indices of 2–hydroxybenzaldehyde and its corresponding reaction product differ only by (–2) ± (+7) units, and A_{rel} are 0.74 ± 0.16 and 0.80 ± 0.06, respectively, i.e., practically coincide with each other. At the content of methanol in the eluent C = 50%, the retention indices of 4–hydroxybenzaldehyde are statistically significantly different from the RI of the reaction mixture component (751 and 792 respectively), as are the retention times (8.80 and 10.82 min, see below). However, when C is increased to 60–80%, they become almost identical. If this fact is compared with the equality of the values of A_{rel} (Table 3.15), it should be concluded that they belong to the same compounds, namely aldehydes. The same similarity of RI and A_{rel} values was observed for acetophenone and the main component of its reaction mixture with hydroxylamine. For the following homologues (propiophenone and butyrophenone) such coincidences are not revealed.

Several reasons for this are possible. First, acetophenone, 2– and 4–hydroxybenzaldehydes theoretically may not interact with hydroxylamine under selected

conditions. However, this seems unlikely, since for the other aromatic aldehydes (including, for example, 4-hydroxy-3-methoxy- and 3-hydroxy-4-methoxybenzaldehydes) and other ketones anomalies are not observed. The second possibility is the rapid hydrolysis of oximes when the reaction mixtures are dissolved in a large amount of eluent containing water at the stage of sample preparation. And thirdly, hydrolysis of oximes is possible in the process of separation during the movement of their chromatographic zones on the column, which manifests itself in distortion of the shapes of chromatographic peaks and was observed in the case of unsubstituted hydrazones of aromatic aldehydes. Consequently, the second reason is the most probable. If so, then the values of *d*RI/*dC* coefficients calculated for such "problematic" oximes are incorrect and are excluded from further consideration.

Thus, the conclusion made in [161] about the greater stability of oximes to hydrolysis compared to unsubstituted hydrazones is most likely valid only for some representatives of these classes. Taking into account the stability of unsubstituted hydrazones noted in [124] stability of unsubstituted hydrazones of aromatic ketones in RP HPLC in contrast to hydrazones of aromatic aldehydes, we can conclude that in both classes of compounds there are examples of different behavior under such conditions.

The series of characterized oximes (*a*–*i*) includes substituents differing in nature (methyl, hydroxy, and methoxy groups) and, consequently, of chromophores, derivatives of the simplest aromatic aldehydes. Therefore, it is not surprising that their characteristics, including *d*RI/*dC* values, differ markedly. Concerning the *d*RI/*dC* ratios, a conclusion was made in [175]: the more polar (hydrophilic) is the sorbate, the smaller is this coefficient. However different characteristics of polarity (according to [176] there are more than a hundred of them) of aromatic carbonyl compounds and their corresponding oximes do not make it impossible to choose the most polar ones.

Judging by the presence of an active hydrogen atom in the molecules, it can be assumed that oximes are more hydrophilic. However, if we compare the "classical" characteristics of polarity of organic compounds (dipole moments (μ) and dielectric permeabilities (ε)), they are larger for carbonyl compounds. For example, 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 log*P* hydrophobicity factors known even for a limited number of structural analogs show that aromatic carbonyl compounds and their oximes are comparable in this parameter:

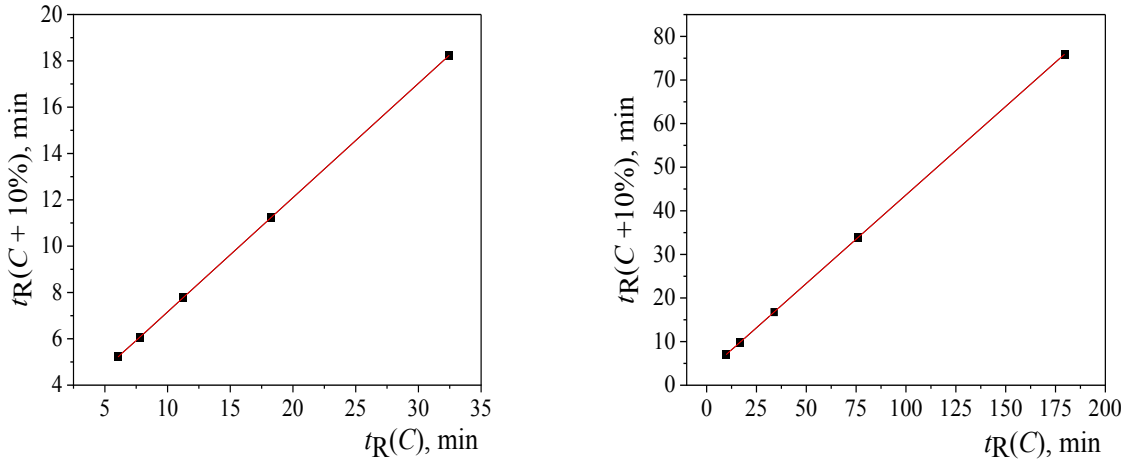
Carbonyl compound	log*P*	log*P* for oximes
Benzaldehyde	1.46 ± 0.02	1.49
2-Hydroxybenzaldehyde	1.83 ± 0.19	1.88
4-Hydroxybenzaldehyde	1.3	1.2
4-Methoxybenzaldehyde	1.7	1.5
Acetophenone	1.70 ± 0.09	1.88
Propiophenone	2.23 ± 0.05	2.27

At the same time, the ratio of *d*RI/*dC* values, which can also be considered as another characteristic of polarity, is reversed for aromatic carbonyl compounds and their oximes. While for the *d*RI/*dC* values for aldehydes themselves vary in the range (–0.3) ÷ (4.6), while for the corresponding oximes they are smaller and belong to a "narrower" range (–1.2) ÷ (–1.6).

This regularity explains an important chromatographic feature of oximes of aromatic aldehydes in RP HPLC. If for aldehydes predominantly *d*RI/*dC* > 0, and for their oximes *d*RI/*dC* < 0, then the retention indices differences $\Delta RI = RI_{(oxime)} - RI_{(aldehyde)}$ are not constant, but decrease to different degrees with increasing concentration of methanol in the eluent. This is confirmed by the data in Table 3.15 for all substituted benzaldehydes. Such examples significantly complicate the application of additive schemes for estimating the retention indices in RP HPLC and in some cases make it impossible.

The premise of the identification of factors affecting the retention times of sorbates is that, in the absence of any anomalies, the recurrence dependences $t_R(C + \Delta C) = at_R(C) + b$, ΔC = const (3.5), where ΔC = is the constant increment of methanol concentration (10 % in our cases), *a* and *b* – coefficients calculated by LSM are linear (correlation

coefficients exceed 0.999). This fact was repeatedly noted earlier [145], but given its particular importance, it seems appropriate to illustrate it further. Examples of such compounds are, for example, *n*-alkyl phenyl ketones – reference components for the determination of retention indices in RP HPLC. Figure 3.18 shows the corresponding dependences for acetophenone (*a*) and butyrophenone (*b*).



(*a*) (*b*)

Figure 3.18. Recurrence approximation of retention times of *a*) acetophenone and *b*) butyrophenone, illustrating the absence of anomalies of their retention times. Parameters of linear regression equations: *a*) $a = 0.4931 \pm 0.0005$, $b = 2.233 \pm 0.008$, $R = 1.000$, $S_0 = 0.02$; *b*) $a = 0.4059 \pm 0.0003$, $b = 3.05 \pm 0.02$, $R = 1.000$, $S_0 = 0.06$.

Similar linear dependences characterize the behavior of oximes of 4- methoxy-, 4-hydroxy-3-methoxy-, and 3-hydroxy-4-methoxybenzaldehydes. The recurrence approximations of retention times of other oximes are characterized by certain anomalies. Thus, on the graphs of dependence (3.5) for the retention times of 2-methoxy- and 3,4-dimethoxybenzaldehyde oximes, the points $t_R(30) - t_R(40)$, corresponding to the highest water content in the eluent, deviate "downward" from the regression lines. This type of recurrence dependences testifies to the reversible hydration of sorbates (equation 1.31) [125,145]. Experimental evidence of hydrate formation was obtained for oxime 4-hydroxybenzaldehyde [177,178].

Table 3.16. Results of recurrence control of retention times of aromatic carbonyl compounds and their oximes combined with relative optical densities A_{rel}. Anomalous values are highlighted in bold with the indicating the "direction" of the deviations.

Substituent in the aromatic fragment		Content of methanol in the eluent, vol %						*R*
		30	40	50	60	70	80	
2-Methyl	t_R aldehyde	33.82	**19.02↑**	16.25	10.32	7.35	5.84	0.99999
	A_{rel}	**0.12↓**	**0.11↓**	4.60	4.77	4.79	4.27	—
a	t_R oxime	31.26	21.74	14.26	8.87	**6.40↑**	**5.22↑**	0.9997
	A_{rel}	**0.07↓**	0.93	1.18	1.23	0.96	1.17	—
4-Methyl	t_R aldehyde	33.37	**17.48↑**	15.12	9.54	6.90	5.59	0.9998
	A_{rel}	**0.48↓**	**0.50↓**	3.63	3.93	3.39	3.20	—
b	t_R oxime	—	**18.95↑**	15.00	9.46	6.64	5.32	0.9998
	A_{rel}	—	**0.83↓**	1.82	1.78	1.07	1.86	—
2-Hydroxy	t_R aldehyde	27.59	16.43	10.80	7.74	6.17	5.27	0.9998
	A_{rel}	0.92	0.91	0.84	0.61	0.60	0.59	—
c	t_R oxime	27.59	16.43	10.80	7.74	6.17	5.27	0.9998
	A_{rel}	0.88	0.86	0.80	0.72	0.82	0.77	—
4-Hydroxy	t_R aldehyde	**14.98↓**	**9.16↑**	8.80	7.68	6.71	5.93	0.9996
	A_{rel}	**0.78↑**	**0.74↑**	0.04	0.04	0.04	0.04	—
d	t_R oxime	**21.85↑**	**18.91**	10.82	7.77	6.70	5.98	1
	A_{rel}	**1.74↑**	**1.05↑**	0.02	0.07	0.07	0.04	—
2-methoxy	t_R aldehyde	—	—	19.34	11.56	7.77	5.98	0.99998
	A_{rel}	—	—	0.08	0.08	0.08	0.05	—
e	t_R oxime	**39.03↓**	18.92	11.15	7.46	5.70	4.85	1
	A_{rel}	0.75	0.71	0.76	0.67	0.76	0.54	—

Table 3.16. (Contd.)

4-methoxy	t_R aldehyde	33.10	17.46	10.68	7.50	5.91	5.09	0.9996
	A_{rel}	0.52	0.49	0.52	0.49	0.54	0.63	—
f	t_R oxime	31.00	15.67	9.45	6.65	5.34	4.61	0.9993
	A_{rel}	1.23	1.24	1.34	1.29	1.28	**0.52↓**	—
4-hydroxy-3-methoxy	t_R aldehyde	**14.17↓**	9.90	8.72	7.69	6.71	5.93	0.9993
	A_{rel}	**0.33↑**	0.04	0.05	0.04	0.04	0.04	—
g	t_R oxime	11.44	7.25	5.48	4.70	4.29	4.08	0.9994
	A_{rel}	0.52	0.54	0.54	0.53	0.55	0.54	—
3-hydroxy-4-methoxy	t_R aldehyde	**20.82↓**	9.86	8.78	768	6.70	20.82	0.9993
	A_{rel}	0.05	0.04	0.05	0.05	0.05	0.04	—
h	t_R oxime	13.34	7.88	5.70	4.79	4.33	4.09	0.9993
	A_{rel}	0.54	0.55	0.53	0.53	0.53	0.52	—
3,4-Dimethoxy	t_R aldehyde	**23.25↓**	**12.15↓**	8.80	7.68	6.70	5.93	0.998
	A_{rel}	**0.31↑**	**0.34↑**	0.04	0.04	0.04	0.04	—
i	t_R oxime	**21.93↓**	11.07	7.17	5.53	4.75	4.37	0.9995
	A_{rel}	0.58	0.34	0.59	0.62	0.60	0.57	—
Acetophenone	t_R ketone	32.46	18.24	11.23	7.77	6.05	5.23	
	A_{rel}	—	—	3.34	2.85	2.78	2.53	—
j	t_R oxime	**37.19↓**	18.49	11.43	7.85	6.18	5.26	
	A_{rel}	3.70	3.51	3.31	2.12	2.21	1.97	—
Propiophenone	t_R ketone	75.82	36.76	19.15	11.23	7.62	6.95	
	A_{rel}	—	—	1.98	2.56	2.29	1.82	—
k	t_R oxime	**67.65↓**	**30.02↑**	20.86	11.31	7.50	5.72	
	A_{rel}	**0.34↓**	**0.4↓**	1.14	1.16	1.18	0.99	—
Butyrophenone	t_R ketone	179.52	75.91	33.86	16.80	9.82	7.07	
	A_{rel}	—	—	2.78	2.76	2.52	2.37	—
l	t_R oxime	—	**60.15↑**	36.86	17.89	9.49	6.52	
	A_{rel}	—	**0.29↓**	1.09	1.12	1.14	1.13	—

To graphically illustrate such anomaly, one can choose, for example, the data for oxime 2-methoxybenzaldehyde (Figure 3.19). Similar deviations were also recorded for the oximes of aceto- and propiophenones (Table 3.16). It is interesting to note that similar anomalies were previously observed only when using eluents containing acetonitrile rather than methanol [131]. This is due to the fact that methanol forms hydrates, the stability of which is higher than that of many organic compounds.

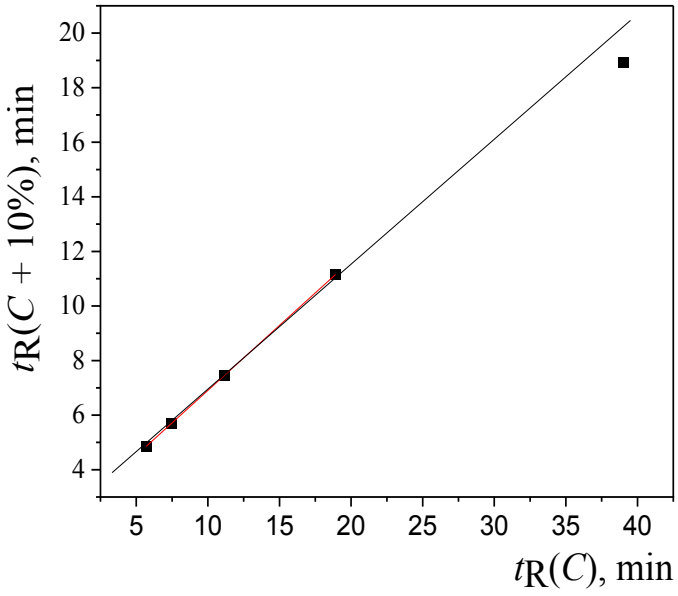


Figure 3.19. Illustration of the anomaly of recurrence approximation of retention times using oxime 2-methoxybenzaldehyde as an example. Parameters of linear regression equations (without taking into account the data for anomalous point): $a = 0.4763 \pm 0.0006$, $b = 2.144 \pm 0.007$, $R = 1.000$, $S_0 = 0.008$.

If the deviations of points "downward" at the highest water content in the eluent are consistent with the formation of more hydrophilic hydrated forms of sorbates [145], then, following the same logic, the deviation of points "upwards" should indicate the transformation of sorbates into more hydrophobic forms. This question certainly deserves a more detailed consideration, and therefore now we can limit ourselves to a mere assumption: the cause of such t_R anomalies may be not hydration, but partial hydrolysis of

oximes with the formation of the corresponding aldehydes. If such hydrolysis occurs during the movement of chromatographic zones of sorbates on the column, it may not result in the registration of hydrolysis products in as separate peaks. An alternative process may be the methanolysis of oximes with formation of semi-acetals or -ketals, which cannot be isolated from the solutions preparatively due to their instability, but, compared to both the oximes and the corresponding carbonyl compounds are more hydrophobic:

$$Ar–CH=NOH + CH_3OH \rightleftharpoons Ar–CH(OH)–OCH_3 \rightleftharpoons Ar–CHO \qquad (3.5)$$

If we return to the anomalies in the conclusion of the discussion recurrence approximation of the retention times of the initial aldehydes, there are examples of their absence (2-hydroxy-, 2-methoxy-, and 4-methoxy and 4-methoxybenzaldehydes, as well as, as noted above, all alkyl phenyl ketones), and deviation of the points "downward" due to hydration (4-hydroxy-3-methoxy-, 3-hydroxy-4-methoxy-, and 3,4 dimethoxybenzaldehydes). However, along with this, for 2-methyl-, 4-methyl-, and 4-hydroxybenzaldehydes, more complex cases of deviations, which, if necessary, may require more detailed consideration.

It can be assumed that in these cases, too, the cause may be hydration or methanolysis of aldehydes (Reaction 3.4). Figure 3.20 illustrates such a case on the example of 4-hydroxybenzaldehyde.

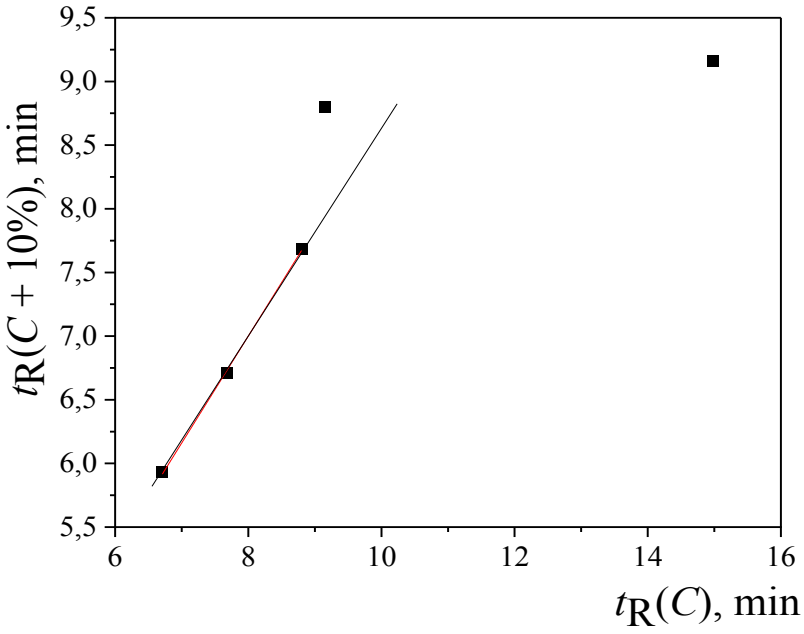


Figure 3.20. Illustration of anomalies of recurrence approximation of retention times on the example of 4-hydroxybenzaldehyde. Parameters of linear regression equations (excluding the two anomalous points): $a = 0.84 \pm 0.02$, $b = 0.28 \pm 0.20$, $R = 0.9997$, $S_0 = 0.03$.

The anomalies of relative optical densities A_{rel} also speak in favor of this assumption A_{rel} optical densities: at methanol concentrations in the eluent of 30 and 40 %, these values are significantly different from other A_{rel} values. Thus, for example, for 2–methylbenzaldehyde, RI(30) and RI(40) values are significantly lower than the other values, as well as $A(30)$ and $A(40)$ values. The decrease of A_{rel} is quite explainable by the destruction of the Ar–CO chromophore of aromatic carbonyl compounds during the processes (3.4) or (3.5).

Thus, on the example of oximes of aromatic carbonyl compounds it is shown that a detailed characterization of chromatographic properties of sorbates in the reversed-phase HPLC should include both determination of their retention indices and recurrence control of retention times, or rather retention times dependence on the concentration of the organic component of the eluent. This kind of control can detect a significantly larger number of

retention time anomalies than other methods. The cause of most of the observed anomalies is not variations in retention mechanisms, but rather changes in the chemical nature of the sorbates due to their interactions with components of the eluent. To confirm this, it is advisable to supplement chromatographic information with spectral parameters, namely relative optical densities $A_{rel} = A(\lambda_1)/A(\lambda_2)$. Variations in the values of A_{rel} indicate changes in the nature of chromophores in the molecules and, consequently, in the chemical transformations of the sorbates.

The joint consideration of the both mentioned above characteristics of oximes allowed us to among them the compounds stable under the conditions of separation by RP HPLC, as well as to reveal examples of their reversible hydration (oximes of 2-methoxy- and 3,4-dimethoxybenzaldehydes) and irreversible hydrolysis (oximes of 2- and 4-hydroxybenzaldehydes, acetophenone oxime) with the formation of the corresponding aldehydes. It was found that the coefficients of dependence of retention indices on the concentration of the organic component of the eluent $d\mathrm{RI}/dC$ for aldehydes are most often greater than zero, whereas for their oximes $d\mathrm{RI}/dC < 0$. Consequently, the differences of retention indices $\Delta\mathrm{RI} = \mathrm{RI}_{(oxime)} - \mathrm{RI}_{(aldehyde)}$ are not constant but decrease with increasing methanol concentration in the eluent, which limits the applicability of additive schemes for estimation of retention indices in RP HPLC.

Conclusion

The results of the work allowed us to formulate the following:

1. The recurrence approximation of analyte retention times from the concentration of the organic component of the eluent in reversed-phase HPLC, $t_R(C+\Delta C) = at_R(C) + b$ (ΔC = const), provides the highest accuracy compared to other retention models. Such approximation is applicable not only for corrected ($t_R' = t_R - t_0$) retention times, but also for directly measured net (t_R) values, in the latter case the accuracy of approximation is even slightly higher than that at the using of adjusted retention times. This is explained by the unique properties of recurrence functions.

2. It is shown that in the absence of retention anomalies of analytes in reversed-phase HPLC, the recurrence approximation of their retention parameters, $t_R(C+\Delta C) = at_R(C) + b$ (ΔC = const), is characterized by linearity, or, otherwise, by correlation coefficients above 0.999. Examples of such compounds are *n*–alkyl phenyl ketones used as reference components for the determination of retention indices.

3. Deviations of points on the graphs of analyte retention time dependences on the concentration of the organic component of the eluent "downward" from the regression line in the areas corresponding to high water content in the eluent, testify to the reversible formation of hydrates of the analyzed compounds in reverse-phase HPLC. Detection of such processes by other methods is impossible.

4. On the example of N-substituted *p*-toluenesulfonamides it is shown that deviations of the recurrence approximation of the retention time dependence on the methanol concentration in the eluent due to the reversible formation of their hydrates are expressed to a lesser extent than for eluents containing acetonitrile. The most probable reason for this is the large free energy of methanol hydration, exceeding the hydration energies of other organic compounds. The largest deviations for eluents containing methanol were registered in the case of oximes of some aromatic carbonyl compounds.

5. As a result of consideration of data for compounds of different chemical nature, it was found that the coefficients of dependence of their retention indices in reversed-phase HPLC on the content of organic solvent in the eluent (dRI/dC) do not correlate with retention indices (RI) and hydrophobicity factors of these analytes (log$P$). Correlations are revealed with homologous increments of these values ($i_{\log P}$, and i_{RI}), which are polarity characteristics, weakly depending on the position of homologues in the corresponding series.

6. Signs and absolute values of dRI/dC coefficients are determined by polarity of organic compounds. Positive values of $d\mathrm{RI}/dC > 0$ are characteristic for relatively nonpolar analytes, and negative values ($d\mathrm{RI}/dC < 0$) – for more polar ones. Therefore, the determination of even the sign of this coefficient provides important information about the chemical nature of the analytes.

7. It is shown that the determination of relative optical densities, $A_{\mathrm{rel}} = A(\lambda_1)/A(\lambda_2)$, in addition to the chromatographic retention parameters allows to detect changes in the chemical nature of analytes in the process of their separation due to the formation of hydrates or other interactions with the components of the mobile phase.

8. The groups of organic compounds unstable under conditions of reversed-phase HPLC have been identified. They include, for example, unsubstituted hydrazones of aromatic aldehydes and some oximes of aromatic carbonyl compounds.

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