Scientific Papers of the University of Pardubice, Series A; Faculty of Chemical Technology
26 (2020) 5–66.

UNIVERSITY OF PARDUBICE
FACULTY OF CHEMICAL TECHNOLOGY

50 years of high performance liquid chromatography: The contribution of the Department of Analytical Chemistry in Pardubice

Pavel Jandera*

Department of Analytical Chemistry, The University of Pardubice
CZ–532 10 Pardubice, Czech Republic

Received: June 2, 2020; Accepted: August 3, 2020

This article surveys the research activities and contributions of the Department of Analytical Chemistry (first at the Institute of Chemical Technology, later at the Faculty of Chemical Technology of the University of Pardubice), to the field of High Performance Liquid Chromatography in the half a century period between 1969 and 2018. The research topics investigated include various theoretical and practical analytical or preparative techniques, in agreement with the general development trends in the field. The attention is focused on the mechanism and predictive modelling of the retention and separation selectivity in the reversed-phase, organic and aqueous normal phase, ion-pairing, salting-out, micellar and mixed-mode liquid chromatography modes. The effects of the stationary phase, of the mobile phases, and of the sample structure on the retention were investigated in detail. The principal point of the research was the development and verification of the new theory of gradient elution. An important part of the recent research activities has been oriented on the theory and practical applications of the new two-dimensional liquid chromatography technique. During the past half a century, new applications of liquid chromatography were developed in the environmental analysis, in the analysis of drugs and their metabolite products, industrial dyes, intermediates, surfactants, of the phenolic antioxidants and plant oils in food and beverages; some attention being also paid to the separation of chiral compounds.

Keywords: Phase systems; Gradient elution; Two-dimensional chromatography

* Corresponding author, ✉ pavel.jandera@upce.cz

Introduction

The Russian botanist M.S. Tsvet discovered liquid chromatography (of course, low-pressure one) in the first years of the twentieth century [1]. He separated green leaf pigments on a column of powdered calcium carbonate in a solvent percolating through the column. The chromatographic separation results from the different affinities of sample components towards the stationary (solid) material placed in a tube (column chromatography) or on a plane (paper and thin layer chromatography) on one side and a moving liquid or gas flowing through the solid bed (the mobile phase) on the other. According to the mobile phase, we distinguish gas chromatography (GC) and liquid chromatography (LC). Since the Tsvet era, the chromatographic techniques marked an amazing progress and nowadays provide invaluable information on the composition of complex multi-component samples in the environmental, industrial, biomedical, clinical, forensic analysis, as well as in many other fields.

Czech scientists contributed significantly to the development of chromatography. Jaroslav Janák developed the technique of gas adsorption chromatography for the analysis of gaseous samples and founded a laboratory for further development of chromatographic techniques, which later became the Institute of Analytical Chemistry of the Czechoslovak (Czech) Academy of Sciences in Brno, which he headed — with short intermissions — until 1993. Many prominent scientists were working under his leadership, developing gas chromatography (GC), high performance liquid chromatography (HPLC), isotachophoresis (ITP), capillary electrophoresis (CZE), field flow fractionation (FFF), supercritical fluid chromatography (SFC) and supercritical fluid extraction (SFE) techniques.

Karel Macek and Ivo Hais introduced planar chromatographic techniques to Czechoslovakia in late 1940s and further developed paper, and thin-layer chromatography techniques and applications. In 1954, they wrote together a comprehensive monograph on paper chromatography, translated since into several European languages. In 1960, Karel Macek had founded the Chromatography Section (later renamed as Chromatography and Electrophoresis Group) of the Czechoslovak (Czech) Chemical Society, which he chaired up to 1991.

Throughout the past half a century, chromatographic, electrophoretic, and related techniques developed mainly at the universities and research institutes in Prague, Brno, Hradec Králové, Pardubice, Olomouc, Bratislava, and in several industrial plants. Before the advent of HPLC, low-pressure liquid column chromatography on polar adsorbents, such as silica gel or alumina served for purification or preparative fractionation of natural or synthetic products rather than for the final analysis [2].

Prof. Miroslav Jureček, the founder and for long years the head of the Department of Analytical Chemistry at the Institute of Chemical Technology (present Faculty of Chemical Technology of the University of Pardubice) oriented

the department more towards the analytical chemistry of organic compounds, in comparison with the other universities and colleges in former Czechoslovakia. The students learned the elementary and functional microanalysis of organic compounds. Prof. Jaroslav Churáček started in 1966, as the very first in Czechoslovakia, the regular training course on chromatographic separation techniques.

I finished my master studies in chemistry in 1967, the year of publication of the milestone work by Horvath et al. on the separation of nucleotides on small spherical particles with solid core and a thin superficial ion-exchange film, which is by many considered the first publication on a practical application of high performance liquid chromatography (HPLC) [3]. I continued my formation as a PhD. student of Jaroslav Churáček, who and his small group were practising planar chromatography and gas chromatography by that time. Jaroslav suggested me to work on dissertation in the emerging new field of HPLC. Since the very beginning, I have devoted my research work to this technique and have stuck with it for the next more than 50 years, first under the Jaroslav's supervision, later with my HPLC group of co-workers, undergraduate and graduate students.

The liquid chromatographic modes cover various separation mechanisms, combination of stationary and mobile phases and column formats. Table 1 surveys the individual HPLC modes and techniques (the fields to which we more or less contributed in our research projects are marked in bold).

I am deeply indebted to my undergraduate students for the years of pleasant and fruitful co-operation. They did all the experimental work and most result evaluation: Josef Čáslavský, Miroslava Vojáčková, Dezider Szabó, Boleslav Taraba, Jaroslav Kubát, Jiří Schvantner, Jitka Rozkošná, Miroslav Špaček, Petr Butzke, Martina Kučerová, Hana Blažková-Brúnová, Petr Zvoníček, Jana Holíková, Libor Petránek, David Komers, Hynek Effenberger, Lukáš Anděl, Lubomír Prokeš, Jitka Jebavá, Karolína Klemmová, Veronika Bačkovská, Petr Řezáč, Lucie Baldrianová (Soukupová), Gabriela Škopová, Hana Lahovská, Jiří Hrabica, Gregor Vohralík, Petr Langmaier, Romana Kubíčková, Zuzana Šromová, Marie Růžičková. Many of them later pursued their PhD dissertation in our team: Ladislav Svoboda, Jaromír Kubát, Josef Urbánek, Jan Fischer, Bořivoj Prokeš, Michal Holčapek, Simona Bunčeková, Václav Staněk, Michal Škavrada, David Komers, Lenka Kolářová (Česlová), Lucie Grynová (Řehová), Kateřina Novotná (Vyňuchalová), Petr Česla, Michal Halama, Dana Moravcová, Jiří Urban, Dana Ansorgová (Vaněrková), Martina Lasáková, Martina Molíková, Veronika Škeříková, Tomáš Hájek, Zdeňka Kučerová, Jan Soukup, Magda Staňková, Petr Janás.

Table 1 HPLC modes and techniques

LC mode	Separation principle	Stationary phase	Mobile phase	Analytes	Comment
Organic normal phase (adsorption) NPLC	Polar interactions	Silica gel, alumina, polar bonded	Mixed organic solvents	Nonionic, weakly polar	
Reversed phase RPLC	Hydrophobic interactions	Nonpolar bonded, organic polymer	Mixed aqueous-organic	Nonionic	
Non-aqueous reversed phase NARPLC	Hydrophobic interactions	Nonpolar bonded, organic polymer	Mixed organic solvents	Nonpolar	
Aqueous normal phase (HILIC, ANP)	Polar interactions	Silica gel, polar bonded	Mixed aqueous-organic	Polar nonionic, ionizable	High organic concentration
Non-aqueous HILIC	Polar interactions	Silica gel, polar bonded	Mixed organic	Polar nonionic, ionizable	Acetonitrile + alcohol (methyl, ethyl)
Micellar (MLC)	Partition to micelles	Nonpolar bonded, organic polymer	Aqueous + surfactant	Small polar	> critical micellar concentration
Ion exchange	Coulombic interactions	Organic polymer, or silica ion exchanger	Electrolyte solution	Small ions, ionic bio-polymers	Buffered mobile phase
Ion pairing	Formation of ionic associates	Nonpolar bonded, organic polymer	Aqueous-organic + ionic surfactant	Organic cations and anions	Buffered mobile phase
Reversed phase ionic suppression	Suppression of dissociation	Nonpolar bonded, organic polymer	Buffered aqueous-organic	Ionizable weak acids and bases	pH<7 acids, pH 7–8.5 bases
Salting-out	Donnan, ionic exclusion + hydrophobic interactions	Nonpolar bonded, organic polymer	Aqueous-organic solution of strong electrolytes	Organic acids with bulky hydrocarbon moiety	0.05–0.4M salt
Size exclusion	Molecular volume	Wide-pore polymers	Organic solvents-aqueous	Synthetic and bio-polymers	Pore size distribution
Affinity biochromatography	Molecular-shape complexes	Tailor-made polymers	Aqueous, buffered	Biopolymers-enzymes, antigens, etc.	Complementary "key-lock" interactions
Molecular imprinting	Molecular-shape complexes	Polymers with imprinted template	Aqueous-organic	Organic pollutants, drugs, etc.	Sample pre-treatment
Micellar electrokinetic chromatography (MEKC)	Partition to micelles	No	Aqueous + surfactant	Small weakly polar organics	>critical micellar concentration, migration in the electric field

Modes investigated at the Department of Analytical Chemistry are in **bold**

Some of them stayed for further time at the department as the post-doctoral fellows: Václav Staněk, Kateřina Novotná (Vyňuchalová), Dana Moravcová, Jiří Urban, Jan Soukup, Magda Staňková; a few joined the staff of the department and have been participating at their new HPLC and related projects: Jan Fischer, Bořivoj Prokeš, Michal Holčapek, Lenka Kolářová (Česlová); some moved into other research groups – Dana Moravcová, Jiří Urban in Brno, Veronika Škeříková in Prague. Petr Česla and Tomáš Hájek are continuing in our co-operation in Pardubice.

The present review summarizes the results of their master and PhD dissertations and the later participation on the grant research HPLC projects at the Department of Analytical Chemistry in Pardubice, in relation to the development of the technique abroad. During the almost 50-years long research activities in HPLC, we investigated various aspects of the technique, which are treated here. I participated in many of these projects, which formed a part of my life, that is why I am sometimes too much personal. Our research focused mainly on the characterization, prediction, and separation methods development in various HPLC separation modes (Table 1), with specific impact on the long underestimated role of the mobile phase; especially, the development of the universal theory of gradient elution; in the new millennium combined with multidimensional liquid separations and dual-mode retention systems.

We developed new application methods for separations of technically, environmentally, biologically, and pharmaceutically important types of compounds, such as synthetic oligomers, co-polymers, surfactants, synthetic dyes and intermediates, pesticides, vegetable oils, antioxidants, poly-phenolic compounds and chiral isomers.

The dawn of HPLC

The late 1960s had witnessed a breakthrough advance in understanding the fundamental theory of chromatography. C. Giddings, J. Huber, J. Knox, C. Horvath, V. Pretorius, R.P.W. Scott, and other excellent scientists clearly proved the necessity of using columns packed with small particles and a high-pressure operation technique for increasing the performance of liquid chromatography.

For fundamental reasons, the efficiency of liquid chromatography (LC) separations cannot compete with the gas chromatography (GC), because in the liquid mobile phase, the diffusion of sample molecules is by four orders of magnitude slower than that in the gas phase. However, the LC outperforms the GC in the selectivity of separation of non-volatile or thermally unstable biologically and industrially important polar, ionic, and macromolecular compounds. The LC enables optimizing the differences in the retention of the sample components; i.e., the selectivity of separation, by adjusting not only the chemistry of the stationary phase, but also by selecting the composition of the

mobile phase. This is an additional separation dimension not available in GC, where the mobile phase, i.e., the carrier gas (hydrogen, helium or nitrogen) does not show any affinity to the sample compounds. The ultimate target of High Performance Liquid Chromatography (HPLC) is the fast resolution and determination of sample components. The retention, the separation selectivity and the chromatographic efficiency control the resolution and the separation quality. The development of efficient chromatographic methods has experienced a remarkable success in the last two decades.

The development of the instrumentation was lagging behind the theoretical advances in HPLC. As the HPLC technique must operate at high pressures (up to 40 MPa) to push the liquid mobile phase through the efficient HPLC columns packed with small-size particles (10 μm or less), the acronym is sometimes read as High Pressure Liquid Chromatography (or, by some bad guys) as High Price Liquid Chromatography). By the end of 1960s, there was no commercial liquid chromatograph available in Czechoslovakia and few abroad. So far published work in the field employed homemade relatively low-pressure instruments. The modern types of efficient stationary phases with sub-2μm-particles employed in ultra-high performance liquid chromatography (UHPLC) enable fast and efficient separations in less than one minute, but there was a long way to this target.

The first task we had met was building a liquid chromatograph from various parts available at that time. This was a challenging and often frustrating work, but with the assistance of my senior colleague, Václav Říha, and of the Institute's mechanical workshop, we succeeded in constructing an instrument, working up to 50 bar and equipped with a photometric detector operating only in the visible spectral range (later adapted to enable the fluorimetric detection). For some instrumental components we had obtained Czechoslovak patents (AO) [4–8]. We displayed our instrument at the exposition of technical innovations EXPO Brno 1970 and I, myself, used the instrument for the experimental work on my dissertation. Figure 1 shows our home-made liquid chromatograph assembled from a glass column packed with 20 micrometer silica gel particles, an injector, a Spekol photometer with a micro-flow-through cell adaptor, and a pump reproducing a two-component mobile phase gradient profile, hand-drawn on a chart fastened by a slowly rotating drum.

In the literature, we found many interesting examples of relatively low-efficient separations of various classes of organic compounds on organic ion exchangers, which we overviewed in a series of articles in *Journal of Chromatography* [9–12]. Our reviews received several hundred postcard reprint applications. (By that time, the internet and the Web of Science were yet unknown). This encouraged us to focus the future research on the possibilities of novel separations of small non-ionic organic molecules on the columns packed with fine organic polymer ion-exchange particles, when using the non-ionic hydrophobic, and polar interactions of the samples with the matrices. The first practical application of our liquid chromatograph was for the separation of colored

derivatives of aliphatic amines and alcohols. The derivatives were prepared using the reagents developed at the Departments of Analytical and of Organic Chemistry at our institute. We presented the principal results at the 3rd Analytical Conference in Budapest in 1970 and published in the *Journal of Chromatography* [13].

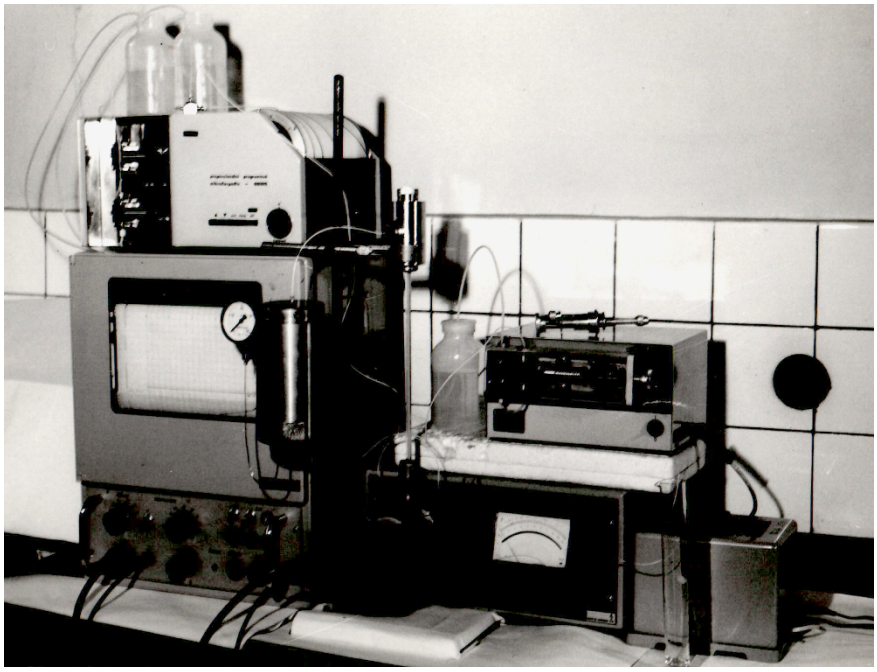

Fig. 1 Home-made liquid chromatograph

In the early 1970s, J. Čoupek, O. Mikeš and co-workers developed original organic polymers, Czech (poly)hydroxymethacrylate gels and ion exchangers. In the late 1940s, O. Mikeš et al. and several other Czech scientists started practicing low-pressure ion-exchange column liquid chromatography of amino acids, peptides, proteins, nucleotides and oligonucleotides, as well as other biopolymers in Prague [14]. Lachema Brno started the commercial production of the Spheron organic separation media. The hydrophilic matrix materials offer interesting interactions useful for chromatographic separations. In the following decade, we co-operated with Lachema on the investigation of the properties of the neutral Spheron gels and Spheron-based anion- and cation- exchangers, when developing novel LC separation methods for carboxylic acids, phenols, nucleosides, barbiturates, sulphonamides, and some other types of compounds [15–17].

In 1977, I visited the Prof. Isztván Halász research group in Saarbrücken (Germany) on a DAAD fellowship. Prof. Halász was one of the inventors of the chemically bonded stationary phases for chromatography, so that I could learn from his co-workers the synthesis of chemically bonded alkyl siloxane stationary phases and the technique of packing efficient HPLC columns with fine-particle materials. The three-month stay had had a great impact on my future formation.

After my return, we constructed a high-pressure column-packing device, with a kind help of Dr. Zbyněk Plzák from the Czechoslovak Academy of Sciences (Řež nearby Prague). The column packer was quite noisy and rather unpopular with my colleagues, but it enabled preparing our own cheap columns packed with bulk hard silica-based chemically bonded small-particle (5–10 μm) sorbents. Such packed HPLC columns which our group used for the next 10 years, have shown the separation efficiency comparable to the expensive commercial columns marketed by Merck.

The phase systems – the stationary and liquid phases in HPLC

Even on the most efficient LC columns, the separation results from the interactions between the solute, the stationary and the mobile phases. We investigated various stationary and mobile phase systems in different LC modes on a plethora of commercially available HPLC columns. Later, I wrote a chapter on the separation systems published at *Elsevier* [18]. (The second edition of the book appeared in June 2020.)

In an ideal chromatographic process, the equilibrium distribution of the sample compounds between the stationary and the mobile phase is disturbed and re-establishes at any instant during the chromatographic process in the chromatographic bed. The changes in the partial molar Gibbs energy, ΔG, of the solute transfer from the mobile to the stationary phase control the thermodynamics of the chromatographic process [19]. For strongly diluted samples, it applies:

$$\Delta G = -RT \ln K_D = -RT \ln \frac{c_s}{c_m} \tag{1}$$

where R is the gas constant, T is the temperature (in Kelvins) and K_D is the distribution (partition) constant, controlling the equilibrium ratio of the concentrations of the solute in the stationary, c_s, and in the mobile, c_m, phases, respectively. The velocity of a solute moving along the column is controlled by the ratio of the time spent by a solute in the stationary phase, t_s, to the time spent in the mobile phase, t_m. This ratio, the retention factor, k, and the most important quantity in chromatography, is equal to the ratio of the masses of the solute in the column in the stationary, m_s, and in the mobile, m_m, phases, respectively. The retention factor k is directly proportional to the distribution constant of the solute, K_D, between the stationary and the mobile phase:

$$k = \frac{t_s}{t_m} = \frac{t_R - t_m}{t_m} = \frac{V_R - V_m}{V_m} = \frac{m_s}{m_m} = \frac{c_s}{c_m} \cdot \frac{V_s}{V_m} = K_D \cdot \frac{V_s}{V_m} = K_D \cdot \Phi \tag{2}$$

The proportionality constant Φ in eq. (2) is the phase ratio, i.e., the ratio of the volumes of the stationary, V_s, and of the mobile, V_m, phases in the column. From the Eq. (2), it follows:

$$t_R = t_m \left(1 + k\right) \tag{3}$$

$$V_R = V_m \cdot \left(1 + k\right) = t_m \cdot F \cdot \left(1 + k\right) \tag{4}$$

where t_m (sometimes marked as t_0) and V_m are also known as the column hold-up time and hold-up volume, respectively; F being the flow rate of the mobile phase through the column.

The retention factors depend on the ratio of the volumes of the stationary and mobile phases in the column, i.e., on the column phase ratio, $\Phi = V_S / V_m$. The volumes of the stationary and of the bulk mobile phase in the column are often difficult to measure exactly because of the uncertainty of the boundary – the position of the dividing plane between the phases [20].

Normal-phase (adsorption) chromatography in organic mobile phases – NPLC

In principle, the differences in polarities are the main factor controlling the separation of non-ionic compounds in LC. In the normal-phase separation systems (NPLC), the polarity of the stationary phase is stronger than that of the mobile phase; the retention increases proportionally to the solute polarity and decreases with the increasing polarity of the mobile phase. Fig. 2 shows schematically the effects of the polarity of the stationary phase, mobile phase and analytes in normal-phase (NPLC) and reversed-phase (RPLC) liquid chromatography.

Normal-phase chromatography (NPLC), known also as adsorption liquid chromatography, is the oldest liquid chromatographic mode [21,22]. The stationary phase in the organic NPLC is a polar adsorbent, most often silica gel, either bare or chemically modified by bonding amino, diol, etc. groups; the mobile phase being usually a mixture of two or more organic solvents. The adsorption sites occupy the fixed positions on the surface of a polar adsorbent. The sample affinity to the polar stationary phases increases proportionally to the polarity of the analytes. The NPLC usually shows excellent isomer-separation selectivity. The preferential adsorption of polar solvents, such as even traces of water, may cause irreproducible retention and long equilibration times, when changing the separation conditions. However, the reproducibility improves significantly working with dried solvents and at a controlled temperature [23]. Unfortunately, poor solubility of polar samples in non-polar solvents limits the application possibilities of NPLC [24].

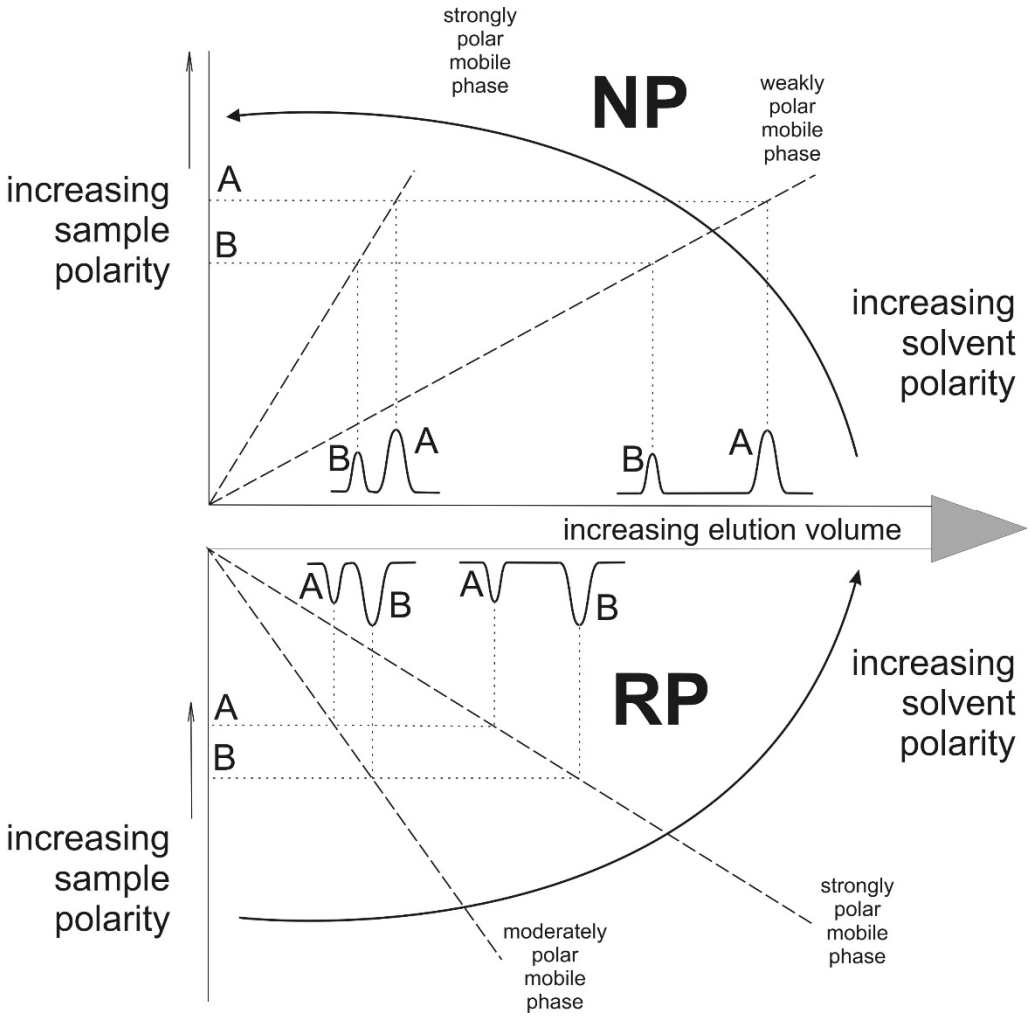

Fig. 2 Effects of the polarity of the stationary phase, mobile phase and sample compound on the retention in the NP and RP LC phase systems

Reversed-phase liquid chromatography (RPLC)

A major breakthrough in the development of HPLC was the introduction of the non-polar alkylsiloxane stationary phases chemically bonded on the surface of the silica gel support by J.J. Kirkland, I. Halász, and C. Horvath [25–27]. The non-polar stationary phases, combined with polar aqueous-organic mobile phases (methanol, acetonitrile) provide excellent conditions for reversed-phase liquid chromatography (RPLC). In reversed-phase (RP) systems employing low-polarity columns and polar aqueous-organic mobile phases, non-polar (solvophobic) interactions are the main, but not the only forces controlling the separation mechanism [28]. In spite of the widespread applications, the exact mechanism of retention in reversed-phase chromatography is still controversial.

The retention generally increases with the decreasing solute polarity. Increasing amount of the bonded hydrocarbon phase and longer bonded alkyls increase the retention. Non-polar or weakly polar compounds show significantly higher retention on the columns with bonded $C_{30}H_{61}$- alkyls in comparison to the shorter-alkyl bonded phases [29].

Some very hydrophobic samples, e.g., lipids, do not elute in an acceptable time even when using pure methanol or acetonitrile as the mobile phase. Such samples are often adequately separated by non-aqueous reversed-phase (NARPC) chromatography on bonded alkylsiloxane columns in mixed mobile phases containing a polar (e.g., acetonitrile or methanol) and a less polar (e.g., tetrahydrofuran, dichloromethane, methyl-*tert*-butyl ether) organic solvent, often modified by an alkane.

Organic solvents preferentially adsorbed from the mixed aqueous-organic mobile phases on the surface of non-polar bonded phases may affect the retention behavior [30]. The main reason for why RPLC is the first choice among the LC separation modes is that the separation utilizes even minute structural differences in the non-polar hydrocarbon part of the molecules; hence, RPLC allows one the separations of a variety of non-polar and weakly polar compounds.

The structure of the bonded phase significantly affects the results of the reversed-phase separation process. The retention on phenyl- or cyanopropyl-bonded phases is weaker than that on an alkylsilica phase (C_{18} or C_8); the main advantage is a lower concentration of the organic solvent required to elute the weakly polar samples, reducing the separation time, and adjusting the polar selectivity of separation.

We have reviewed various techniques for characterization of the RPLC stationary phases: nuclear magnetic resonance, infrared, fluorescence and photoacoustic spectroscopic methods, surface area measurements, elemental analysis, thermal analysis, micro calorimetric and chromatographic techniques. The latter do not require the destruction of an expensive column to get the information about its properties [31].

The test probe compounds, such as toluene, ethylbenzene, aniline, phenol, isomeric *o*-, *m*-, *p*-toluidine, and others [32], characterize the column hydrophobicity, shape selectivity, residual silanol activity, and peak asymmetry. The tests usually employ a single aqueous-organic mobile phase composition, however the mobile phase influences the retention behavior by controlling the interactions of the solvents with the test compounds and also by solvating and modifying the properties of the stationary phase.

Isomeric naphthalene sulfonic acids are fully ionized strong acids comparable with the mineral acids. The isomeric naphthalene di-sulfonic acids do not retain on reversed-phase columns in purely aqueous-organic mobile phases. However, the addition of a strong electrolyte to the mobile phase enhances their retention proportionally to the increasing dipole moment of the molecules, i.e., with increasing size of the hydrocarbon part of the molecules exposed to the direct

contact with the bonded alkyls in the stationary phase [33]. In agreement with this rule, we found the same elution order of isomeric acids on 25 different columns: 1,5 < 1,6 < 1,3 < 1,7 naphthalene di-sulfonic acids, but with very significant differences between their relative retention (isomeric selectivity), depending on the properties of the stationary phases. Hence, we found the relative retention (selectivity factor) of isomeric naphthalene-di-sulfonic acids in 0.4 M sodium sulphate aqueous phase a sensitive test indicator of the properties of reversed-phase LC columns unaffected by the organic solvents in the mobile phase [34].

Reversed-phase separations in micellar mobile phases

Many polar and ionic compounds are difficult to separate in the RPLC systems because of too low a retention. The retention and separation of small non-ionic polar organic molecules sometimes improves by substituting organic solvents in the mobile phase by ionic surfactants with long alkyl chains such as sodium dodecyl sulfate (SDS), which — if present in a sufficiently high concentration above the critical micellar concentration, CMC —, form micellar aggregates with polar (ionic) outer surface and non-polar inner cavity. In the micellar liquid chromatography (MLC), the sample retention results from the distribution between the non-polar stationary phase, the aqueous mobile phase and the micellar pseudo-phase [35]. We found anionic surfactants, even if occurring at low concentrations or in presence of organic solvents, where the micelles cannot form, to affect the retention of small organic compounds [36]. The micellar electrokinetic chromatography (MEKC) is the capillary electromigration arrangement employing the formation of surfactant micelles in the working electrolytes. We utilized the differences in the separation selectivity in MLC, MEKC and conventional RP HPLC for separations of small polar compounds [37–38].

Ion-pairing and salting-out separations of strong organic acids

Weakly acidic or weakly basic additives into the mobile phase suppress the ionization of weakly basic or weakly acidic compounds and increase their retention in RPLC. However, this approach does not work in the LC of fully ionized strong acids, such as naphthalene sulfonic acids (NSA), used as the intermediates in the dye production. In ion-pairing chromatography, quaternary tetralkylammonium surfactants added into the mobile phase form relatively non-polar ion associates with organic anions retained on chemically bonded non-polar columns [39]. With these additives, we could separate the NSAs into groups containing 1–4 sulfonic acid functionalities ($-SO_3^-$), but distinguishing all the individual NSA isomers was not possible [40]. In the aqueous–organic mobile phases containing relatively high concentrations of salts, such as sodium or

ammonium sulfate, the separation of strong anions containing bulky hydrocarbon structural elements often improves, due to the combined hydrophobic and ion-exclusion interactions under the RP conditions (the salting-out effect) [41]. We developed a theoretical model describing the effects of ion-pairing and concentrated neutral salts on the retention [40].

Aqueous normal-phase (hydrophilic) liquid chromatography

In the classical normal-phase chromatography on polar adsorbents, polar sample compounds often elute too late, if at all, in purely organic mobile phases. Hydrophilic Interaction Liquid Chromatography (HILIC), which is essentially an aqueous normal-phase (ANP) mode employing a polar column in aqueous-organic mobile phases with high concentration of the organic solvent, often provides a significant improvement in the separation of polar organic compounds [42].

HILIC has become increasingly popular and tends to supplant the traditional ion exchange LC (IEC) as the separation method for low-molecular organic ionic compounds. Because of a relatively low separation efficiency for small organic molecules, IEC serves nowadays mainly for the analysis of small inorganic ions and ionic biopolymers.

There is a wide selection of polar columns suitable for HILIC separations, including silica gel, either bare or with various bonded polar ligands, and polar organic polymers [43], showing significant differences in the chromatographic selectivity. The HILIC retention mechanism is complex, including partition equilibria between the adsorbed water and the bulk aqueous-organic mobile phase. The adsorption of polar compounds, mainly water, on the solid phase obviously more or less contributes to the HILIC retention, due to hydrogen-bonding, ionic and other interactions with the bonded polar functional groups, or with residual silanols on the silica-based polar bonded phases. Ion exchange or ion repulsion may be also involved in the distribution of ionized analytes [44].

Polar stationary phases strongly attract water and the adsorbed water forms a part of a polar HILIC stationary phase as a diffuse liquid layer subject to a dynamic exchange with the bulk organic-rich mobile phase – Fig. 3.

Using the frontal analysis of aqueous-organic solvent mixtures combined with Karl-Fischer titration of the separated fractions, we compared water adsorption on a variety of polar stationary phases potentially suitable for HILIC (aqueous normal phase, ANP) separations (bare silica gel, hydrosilated or hybrid silica, inorganic oxides or organic polymers carrying various polar and ion-exchange groups or zwitterionic bonded ligands). There are large differences in water uptake between the individual stationary phases – Fig. 4. Columns with the bonded polar ligands (hydroxyl, diol, nitrile, pentafluorophenylpropyl, zwitterionic sulphobetaine and phosphoryl choline), show stronger water adsorption in comparison to bare silica.

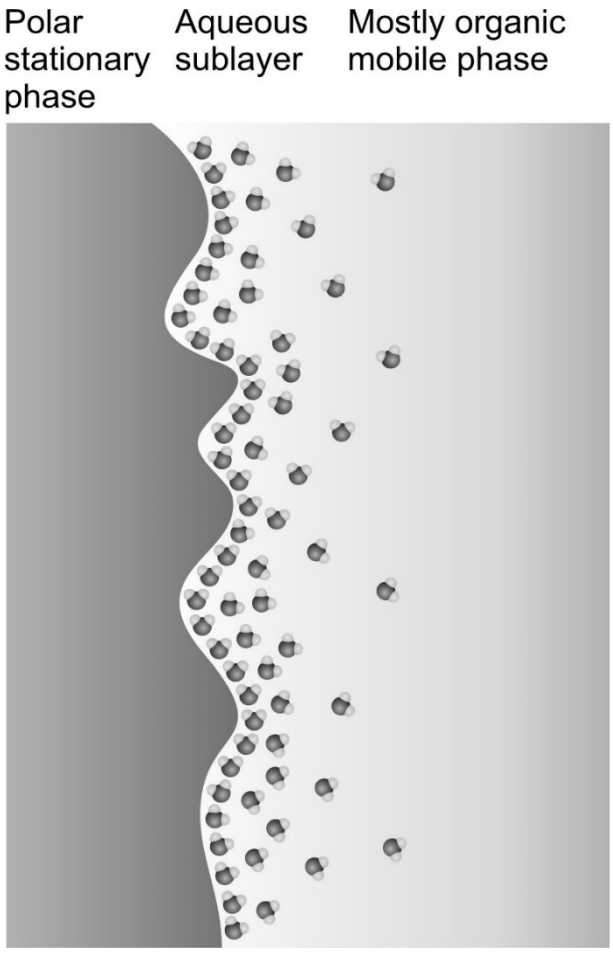

Fig. 3 Schematic picture of a HILIC stationary phase with an adsorbed diffuse water layer at the surface of a polar stationary phase in highly organic environment

At full column saturation, the excess adsorbed water, V_{ex}, may fill up to 45 % of the pore volume of silica-based columns. Due to a low affinity to water, silica hydride-based stationary phases adsorb less water than one monomolecular layer equivalent (full horizontal line), corresponding to 0.2–0.4 % water in the 2.6–5.5 % of the inner pore volume [45].

A sub-monomolecular layer of water adsorbed on silica hydride (silica gel type C) and some other column types does not provide enough space for sample partition. Hence, a competition between the surface adsorbed water and the polar solutes controls the retention [46]. A low number of the adsorbed monomolecular equivalents may distinguish the aqueous normal-phase (ANP) from the traditional HILIC systems [47]. Electrolytes in the mobile phase usually increase the adsorption of water and consequently the retention of non-polar compounds [48].

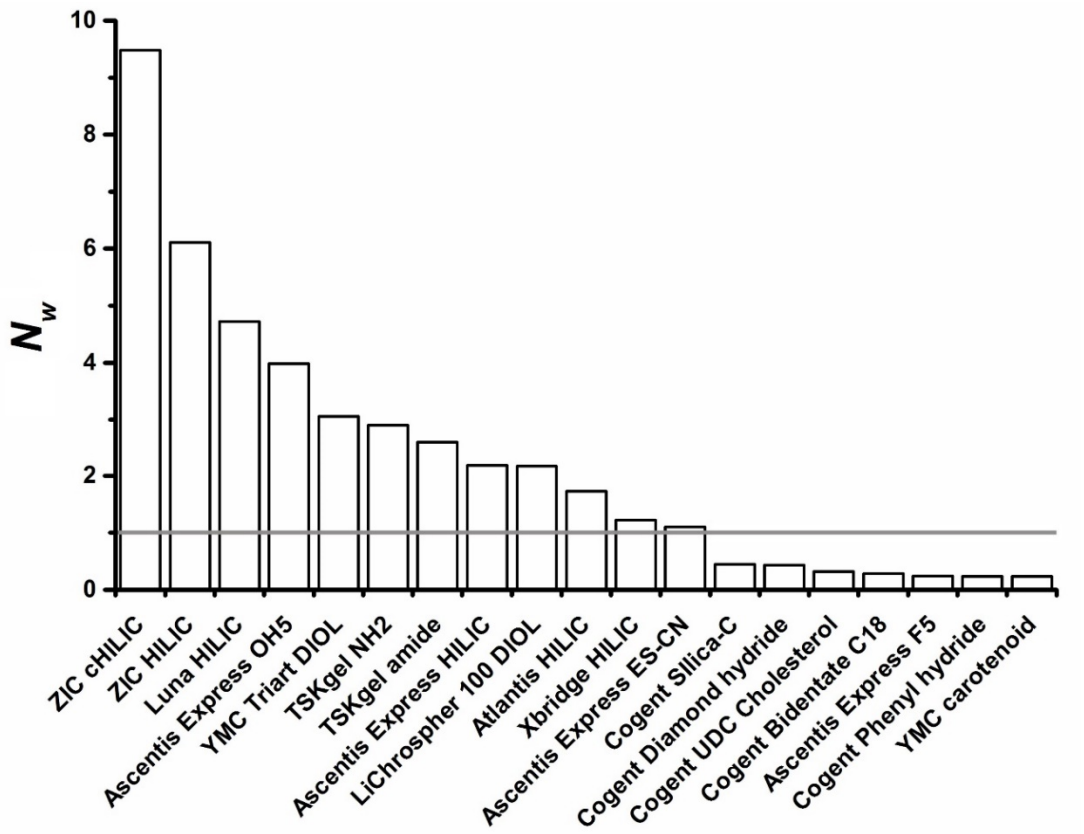

Fig. 4 Water adsorption onto HPLC columns: The equivalent number of adsorbed monomolecular water layers, N_w, inside the pores at full saturation capacity of the columns

The horizontal line denotes the equivalent of a compact monomolecular adsorbed water layer. HILIC > 1 layer; NARP < 1 layer

We employed normal phase chromatography on a chemically bonded amino stationary phase in the organic mobile phases consisting of cetyl-trimethylammonium bromide, 2-propanol and *n*-heptane for the separation of sulfated oligoethyleneglycol nonylphenyl-ether surfactant oligomers [49]. The addition of water to the mobile phase enables simultaneous separation of the individual oligomers of sulfated oligoethyleneglycol nonylphenyl ether surfactants and their unreacted non-sulfated parent compounds [50]. Here, the separation mechanism obviously was the "non-aqueous HILIC chromatography" (NA-HILIC), where a polar organic solvent substitutes water: a "protic modifier", such as methanol, ethanol or propanol (with the elution strength decreasing in this order). NA-HILIC covers the gap between the non-aqueous NPLC and the aqueous-organic HILIC of polar samples. NA-HILIC is helpful in the analysis of some poorly soluble oligomers or weakly polar compounds which may precipitate in water-containing HILIC mobile phases. The differences between the aqueous and non-aqueous modes are due to the absence of the adsorbed water layer in NA-HILIC [51].

Modeling and prediction of retention in LC

Mobile phase effects on the retention

The traditional trial-and-error selection of mobile phases in planar chromatography (paper and TLC) is not appropriate in the modern efficient and sensitive HPLC, requiring the solvents of special purity. On the appropriately selected HPLC column, a rational adjustment of the composition of the mobile phase is the most efficient tool to achieve the desired quality of separation of a particular sample. For the systematic HPLC method development, it is important to understand the effects of the mobile phase on the retention and on the selectivity of separation [52].

At the time of the advent of HPLC, Lloyd Snyder developed a displacement model of the retention in adsorption chromatography with a mobile phase prepared by mixing two organic solvents of different polarities (solvent strengths) [21]. The Snyder quasi-stoichiometric model describes the chromatographic retention as the result of the competition between the molecules of the solute and of the strong solvent for the adsorption sites. The adsorption of a solute molecule causes the displacement (desorption) of one or more strong solvent molecules from the adsorbent surface. The adsorption energy controlling the retention factor, k_{ab}, in a two-component mobile phase depends on the specific adsorbent surface, A_s, adsorbent activity, α', and solvent strength of the binary mobile phase, ε_{ab}, comprised of a stronger (polar) solvent B (solvent strength ε_b) and a less polar solvent A (solvent strength ε_a):

$$\log k_{ab} = \log k_a + \alpha' \cdot A_s \cdot \left(\varepsilon_a - \varepsilon_{ab}\right) \tag{5}$$

E. Soczewiński introduced a model of adsorption in planar chromatography (in the terms of R_M, equivalent to the log k in the column LC) as the formation of a molecular complex of the solute with the active centers on the adsorbent surface, accompanied with the release of an adsorbed molecule of the polar solvent into the mobile phase [53]. The Soczewiński and the Snyder models provide similar description of the adsorption NPLC systems.

Since the beginning of our activities, the rational modelling of HPLC separation methods has strongly attracted our attention. We simplified the Snyder and Soczewinski description of the adsorption to a two-parameter equation, Eq. (6), enabling the prediction of the analyte retention factor, k, in binary mobile phases comprised of two solvents of different polarities [54]:

$$k = k_0 \cdot \varphi^{-m} \tag{6}$$

where φ is the volume fraction of the strong (polar) solvent in the mobile phase. The parameter m is a quasi-stoichiometric coefficient characterizing the number of the molecules of the strong solvent necessary to displace one adsorbed molecule of the analyte, k_0 is k in the pure polar solvent.

The RPLC systems employing non-polar or weakly polar columns (usually silica gel with bonded alkyl siloxane ligands) and aqueous-organic mobile phases represent the standard in the contemporary HPLC. The surface of chemically modified stationary phase does not contain localized adsorption centers (except for a few residual more or less shielded silanol groups), but non-polar chemically bonded alkoxysilane groups, where either non-localized adsorption or partition control the retention. Several semi-empirical models introduced for RPLC presume, to a first approximation, that the interactions in the non-polar stationary phase are less significant than the polar interactions in the mobile phase which are the main factor controlling the retention. Hence, the transition of a solute molecule from the bulk mobile phase to the surface of the stationary phase results from a decrease in the contact area of the solute with the mobile phase, in the absence of strong (polar) interactions with the stationary phase. This is the basic idea of our model of interaction indices [55].

Snyder and co-workers in their linear solvent strength (LSS) model for prediction of retention in the isocratic and RP HPLC presume a simple linear correlation between the logarithm of the distribution constant and the volume fraction of organic solvent in binary aqueous–organic mobile phases. The commercial Dry-Lab software employs nowadays the LSS model. According to the LSS model, log k decreases proportionally to the increasing volume fraction, φ, of the strong eluent (organic solvent) [56]:

$$\log k = \log K_D + \log \frac{V_s}{V_m} = a - m \cdot \varphi \tag{7}$$

where K_D is the distribution constant characterizing the solute non-specific surface adsorption or the partition between the surface layer of the adsorbed solvent and the mobile phase. V_S and V_M are the volumes of the stationary and the mobile phase in the column, respectively.

The two-parameter retention models may fail to describe exactly some systems by simple Eq. (6) or Eq. (7). A simple empirical approach to improve the model fit by formal adding a quadratic φ^2 term into the Eq. (7) often does not allow describing accurately the RP retention over the full composition range of the aqueous-organic mobile phases [57]:

$$\log k = a - m \cdot \varphi + d \cdot \varphi^2 \tag{8}$$

The Eq. (8) often fails at low concentrations of the strong solvent, φ. We compared a variety of three- or more- parameter models including additional empirical terms [58]. Several models provide more or less improved data fit, but the physical meaning of the additional terms often is not clear yet [59].

We developed a three-parameter ABM model, introducing an additional term, *b,* into the Eq. (1) which corrects for possible retention in the pure weak solvent, $k_0 = 1/(b)^m$, in Eq. (4) [60]:

$$k' = \left(b + a \cdot \varphi\right)^{-m'} \tag{9}$$

The parameters *a*, *b* and *m´* are the experimental constants depending on the solute and on the chromatographic system, φ is the volume fraction of the strong solvent in a two-component mobile phase. For $\varphi = 0$, the Eq. (9) simplifies to the Eq. (6). The parameter m' is proportional to the area of the solid surface occupied by one molecule of the strong solvent B. The strong solvent can be either water (aqueous buffer) in HILIC, or a polar organic solvent (acetonitrile) in the RPLC. Rearranging the Eq. (9) to the Eq. (10) allows us estimating the retention factors in the pure weak solvent (e.g., water), k_0, and in pure strong solvent (e.g., acetonitrile) from the experimental elution data [61]:

$$k' = \left(b + a \cdot \varphi\right)^{-m'} = \left[\left(\frac{1}{k_1^{\frac{1}{m'}}} - \frac{1}{k_0^{\frac{1}{m'}}}\right) \cdot \varphi + \frac{1}{k_0^{\frac{1}{m'}}}\right]^{-m'} \tag{10}$$

$k_0 = 1/b^{m'}$, $k_1 = (b + a)^{-m'}$. The ABM model does not distinguish between the adsorption and the partition retention mechanisms. We found an improved retention data fit for the three-parameter equation (9) in the RPLC on core-shell columns compared to the two-parameter equations, Eq. (6) and Eq. (7) [61,62].

Modelling is an extremely useful tool for the method development and optimization. The retention models use the equations with parameters determined by the regression analysis of the experimental data. Hence, a model can only be as reliable as the experimental data obtained. The mobile phases employed for the acquisition of the parameters retention data should not lead to too weak a retention; otherwise, the model can be a subject to gross errors. A good fit of the experimental data to the regression equation alone does not prove the physical correctness of the model. From a mathematical point of view, the fit of experimental data to a model regression equation always improves with the increasing number of model parameters; however, this does not necessarily mean that the additional parameters do have any physical meaning. Prof. Pat Sandra said: "Models should be used, but not believed." Only a series of positive tests with a variety of samples and separation conditions verifies the practical utility of a model.

Models characterizing the structural effects on the retention in liquid chromatography

In the RPLC systems, the retention increases proportionally to the solute hydrophobicity, i.e., it decreases with the increasing polarity, contrary to the normal-phase LC. RP on the C18 bonded columns also provides a fast method for the determination of a compound's lipophility and is a substitute for the tedious classical shake-flask equilibrium method for the determination of octanol-water partition coefficients [63].

The models of retention suggested for RPLC depart from the Hildebrand solubility parameter theory [64], from the concept of molecular connectivity [65], from the solvophobic theory [66], or from the molecular statistical theory [67]. Unfortunately, sophisticated models introduce a number of physicochemical constants which are often difficult and time-consuming to determine.

Structural models provide a useful tool for verifying the expected effects on the retention of various chemical substituents bonded on the silica surface and for method optimization [34]. Generally, any test based on chromatographic retention does not characterise the chromatographic column alone, but the chromatographic system comprising the stationary and mobile phase. The tests of the HPLC column properties can be normalized to cover a more-or-less wide range of the mobile phase composition.

In spite of its inherent limitations, the solvophobic theory of retention is advantageous as the starting point in the derivation of a simplified semi-empirical description of reversed-phase systems to characterize and predict the retention and the selectivity of separation in reversed-phase chromatography in the model of interaction indices.

RPLC is especially useful for separations in homologous or oligomeric series with different numbers of non-polar or weakly polar structural units. The structural contributions to the free energy of retention are additive, which means that the logarithm of the retention factor ($\log k$) is directly proportional to the number of the methylene groups n_C, in a homologous series:

$$\log k = \log \alpha \, n_C + \beta \tag{11}$$

where α is a measure of the methylene group (hydrophobic) selectivity and β represents the contribution of the end group in the series to the retention. Hence, the parameters of the Eq. (10) characterize the lipophility of the stationary phase. Fig. 5 illustrates increasing retention factors, k, of alkylbenzenes on the columns with longer bonded alkyls (from benzene to hexyl benzene) and in more water-rich mobile phases, φ [the volume fraction of the strong (organic) solvent, in % (v/v) · 10^{-2}], on ACE 3 Ph (A), ACE 3 C4 (B), ACE 3 C8 (C) and ACE 3 C18 (D) columns.

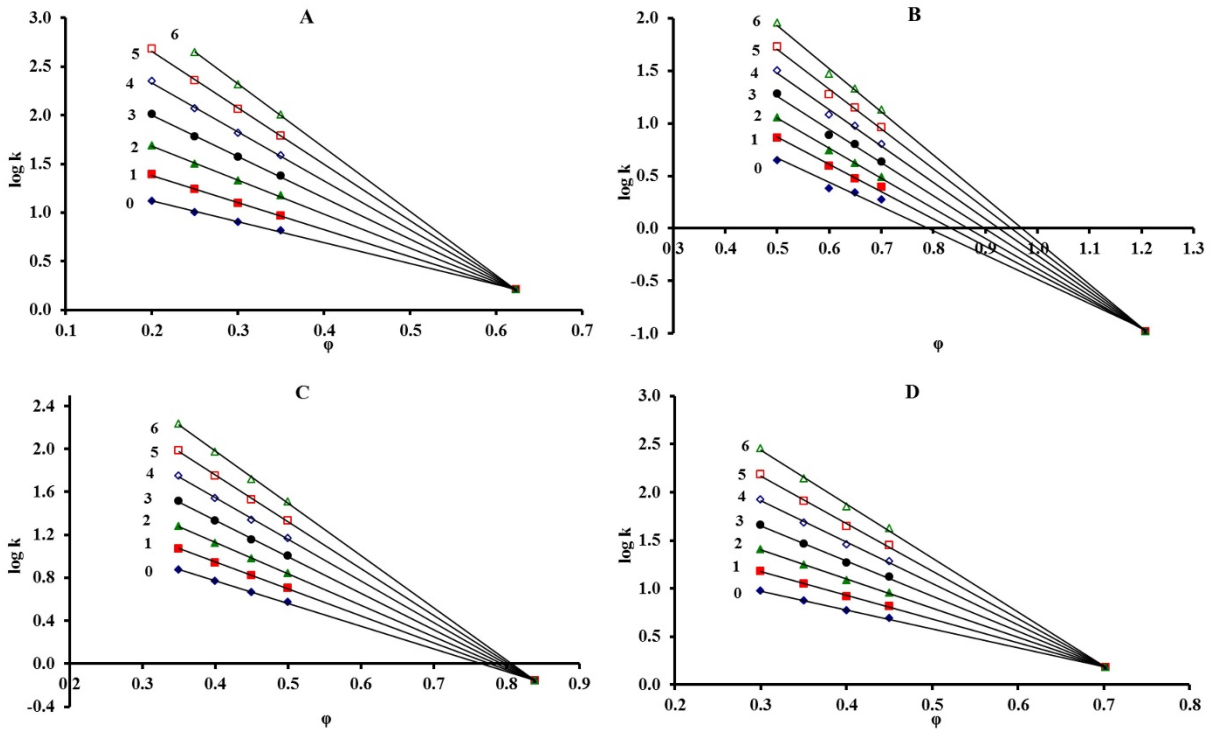

Fig. 5 Retention factors, k, of homologous n-alkylbenzenes (from benzene – 0 to hexylbenzene – 6) on ACE 3 Phenyl (A), ACE 3 C4 (B), ACE 3 C8 (C) and ACE 3 C18 (D) columns

φ [% (v/v) · 10^{-2}] – volume fraction of acetonitrile

In all LC separation systems, the mobile phase is a very active — but often underestimated — factor affecting the retention, separation selectivity, and ultimately the sample resolution. The following Eq. (12) describes the simultaneous effects of the number of repeated structural elements, n and of the volume fraction of the strong solvent in the mobile phase, φ, on the retention factor, k:

$$\log k = a_0 + a_1 \cdot n - \left(m_0 + m_1 \cdot n\right) \cdot \varphi \qquad (12)$$

The parameters of Eq. (12) depend on the structure of the homologous series, on the column and on the type of the strong (organic) solvent. The constants a_1 and m_1 characterize the selectivity of RPLC separations for the repeat structural groups, whereas the constants a_0 and m_0 characterize the contributions of the end-group (non-repeating structural moiety) to the retention [68]. The Eq. (12) applies not only in various homologous series, but also in oligomeric series with other nonpolar repeat structural groups [69].

We developed a new method for characterization of the separation selectivity in reversed-phase liquid chromatography, based on a selectivity scale, calibrated by a homologous series (usually n-alkylbenzenes) with toluene as the reference standard. The selectivity is comprised of lipophilic and polar

contributions, quantitatively defined by the constants related to the solute structure. The indices Δq characterize the polar contribution to selectivity and depend on the type of the solvent in the aqueous – organic mobile phase. Δq in one binary solvent system (such as acetonitrile–water) plotted *versus* Δq. In another binary solvent system (such as methanol–water) provides a graph where the compounds with a specific functional group occupy narrow, limited areas and can be distinguished from the compounds with other functional groups. This is a helpful tool for the identification of the functional groups in simple organic compounds from reversed-phase chromatographic data. [70,71].

Stationary phases chemically bonded on silica gel used in reversed-phase and HILIC separations contain significant amounts of the residual silanol groups and some contain polar groups in bonded ligands. These groups provide more or less significant selective dipole-dipole, proton-donor, proton-acceptor, π-π electron and electrostatic interactions, in addition to the lipophilic interactions of the bonded moieties (such as the alkyl chains). The retention decreases with the increasing polar interactions, but decreases with the increasing molar volume of the solute, V_X [55]:

$$\log k^* = \frac{\log k - \log \varphi}{V_X} = A - BI_X \tag{13}$$

The parameters A and B depend on the column type and on temperature. The parameter A is a measure of the column hydrophobity; the parameter B is the response of the retention to a change in the sample polarity (including all selective contributions) and can be used to characterize the column selectivity for polar compounds. Determining the parameters A and B of Eq. (13) employs a set of suitable calibration standards covering the interaction indices scale. On the basis of comparative studies, we selected the following scale calibration standard set: benzene (I_x = 2.76), toluene (I_x = 2.46), nitrobenzene (I_x = 4.49), acetophenone (I_x = 5.60) and anisole (I_x = 3.85), 1,4-dichlorobenzene (I_x = 1.05), 3-chlorotoluene (I_x = 2.20) 3-bromonitrobenzene (I_x = 3.44), nitrobenzene (I_x = 4.49) and benzonitrile (I_x = 5.32) [72–74]. Eq. (13) applies not only to bonded alkylsilica stationary phases, but also to non-polar organic polymer stationary phases such as polymethacrylate monolithic columns and a stationary phase with carbon deposited on the surface of zirconium oxide support, Discovery ZR-CARBON.

The Quantitative Structure – Retention Relationships (QSRR) approach describes quantitatively the structural contributions of the selective polar interactions to the retention in liquid chromatography. It forms the basis of the Linear Solvation Energy Relationships (LSER) model employing multiple linear correlations between the retention and the molecular structural descriptors of a solute [75]:

$$\log k = c + v \cdot V + s \cdot S + a \cdot A + b \cdot B + d^- \cdot D^- + d^+ \cdot D^+ \tag{14}$$

V, S, A, B, D^- and D^+ are the characteristic solute structural descriptors: the molar volume of the solute, *V*, the dipole-dipole activity, *S*, the hydrogen bonding acidity, *A*, and the hydrogen bonding basicity, *B*. D^- represents the negative charge carried by anionic species, and D^+ defines the positive charge carried by cationic species. The coefficients *v*, *s*, *a, b,* d^- and d^+ of Eq. (14) obtained by multivariate simultaneous least-squares regression of experimental retention data provide a measure of the response of the separation system (the stationary and the mobile phase) to the structural properties of the analytes. The products of the system parameters and the corresponding molecular structural descriptors in Eq. (14) characterize the contributions of the selective interactions to the retention in particular separation systems. We used the LSER model as an efficient tool for characterizing a suitability of RPLC columns for specific sample separations [76,77].

The LSER model characterizes the structural effects on the retention also in HILIC separation systems with silica hydride, bonded polyethylene glycol, diol, and zwitterionic sulfobetaine columns, in mobile phases with high concentrations of acetonitrile in water [78,79].

Mixed-mode retention and dual-mode stationary phases

The mixed-mode retention mechanism is due to a cocktail of different solute — stationary phase — mobile phase interactions, such as solvophobic, polar, attractive or repulsive electrostatic effects [80]. The LSER model (Eq. 14), in fact, characterizes a mixed mode reversed-phase – normal-phase behavior, of course, with a dominant RPLC contribution.

The mixed-mode HILIC/ion-exchange stationary phases with long-alkyl chains and hydrophilic polar terminal ion-exchange groups bonded on the silica gel support improve the separation selectivity for a wide range of polar and non-polar compounds in the organic-rich mobile phases. This is due to the combination of the HILIC partition and ion-exchange mechanisms [81]. The Acclaim mixed-mode WAX-1 and WCX-1 columns and the weak anion-exchange Poly WAX LP column offer complementary application possibilities to the typical HILIC columns. The zwitterionic stationary phases such as the TSK Gel Amide-80, ZIC-HILIC or Polysulfoethyl A show similar behavior. [82]. Many strong anion-exchange (SAX) or weak anion-exchange (WAX) columns exhibit mixed reversed-phase – HILIC retention mechanisms [83].

The mixed-mode columns show the cocktail of interactions with the analytes essentially over the full mobile phase range. We can distinguish them from the dual-mode columns which provide different predominating HILIC and RPLC retention mechanisms over limited mobile phase composition ranges. We focused our attention on the dual-mode columns as they provide possibilities of controlling the separation mechanism on a single column by selecting the mobile phase composition.

At high concentrations of organic solvent, the sample polar interactions with the chromatographic system control essentially the retention. A gradual increase of the water concentration over a more-or-less limited range decreases the sample retention in the HILIC mode. At high concentrations of water, the retention is due to the solvophobic interactions with non-polar moieties contained in the structure of stationary phase. This means that the compounds essentially show the reversed-phase behavior, where the interactions of polar solutes with water may be very strong, so that the polar sample compounds elute close to the column hold-up (dead) volume.

The probability of the dual HILIC-RPLC mechanism increases for polar stationary phases containing a large non-polar moiety. The useful mobile phase range is limited and depends both on the stationary phase and on the analytes. For example, aglycones are subject to the HILIC mechanism in 70–90% acetonitrile in water and to the RPLC mechanism in highly aqueous mobile phases on bonded PEG and diol columns, whereas the HILIC range is shifted to cover more water-rich mobile phase ranges for phenolic glycosides [84]. Adjusted composition of the mobile phase controls not only the separation selectivity, but also the retention mechanism on such dual-mode column [85]. As the separation selectivity on dual-mechanism columns is usually highly complementary (orthogonal) in the HILIC and in the RP mobile phase ranges, a single column can often be used to obtain useful information on the sample injected subsequently in acetonitrile-rich (HILIC) and in highly aqueous (RPLC) mobile phases.

In the presence of a dual HILIC-RPLC mechanism, the graphs of the sample retention factors, k, versus the volume fraction of water, φ_{H_2O}, show characteristic U-shape profiles over a broad range of binary aqueous-organic mobile phases and can be often described by Eq. (15) (at $\varphi_{H_2O} > 0.02$) [84]:

$$\log k = a + m_{RP} \cdot \varphi_{H_2O} - m_{HILIC} \cdot \log\left(1 + b \cdot \varphi_{H_2O}\right) \tag{15}$$

The parameter m_{RP} characterizes the effect of the increasing concentration of water in the mobile phase on the retention due to the reversed-phase mechanism in water-rich mobile phases, whereas the parameter m_{HILIC} is a measure of the water contribution to the decrease of retention in highly organic mobile phases (HILIC range). The system constant a depends on the solute and on the type of organic solvent. The parameter b in Eq. (15) accounts for a low, but finite retention at very low water concentrations [85].

The major part of the medium mobile phase composition range manifests too low a retention and is not suitable for practical separations, where the "U-turn" transition from the HILIC to the RPLC mechanism occurs at the minimum retention at the φ_{min} concentration of water in the mobile phase:

$$\varphi_{min} = 0.434 \cdot \frac{m_{HILIC}}{m_{RP}} \tag{16}$$

The dual-mode HILIC-RPLC behavior depends both on the column and on the sample and the probability of the dual HILIC-RPLC mechanism increases for polar stationary phases containing a significantly non-polar moiety. The useful mobile phase range is limited and depends both on the stationary phase and on the analytes [84].

The hydrosilation process replaces up to 95 % of the original silanol surface groups on the silica gel surface by the non-polar silicon hydride Si-H groups. The Si-H groups do not attract significantly water and the hydrosilated silica gel is almost non-polar [86]. Silica hydride materials chemically modified by low-polarity bonded groups, such as the UDC cholesterol and the C_{18} bidentate columns, show dual-mode HILIC-RPLC properties and provide suitable selectivity for HILIC separations of polar compounds such as phenolic acids. The columns work in the RPLC mode in highly aqueous mobile phases, whereas in buffered mobile phases containing more than 50–70% acetonitrile, the aqueous normal-phase (HILIC) mode applies. On the other hand, the bare silica hydride and Diamond hydride columns (containing approximately 2.5 % carbon) show very weak retention in the reversed-phase mode [87] – Fig. 6 [88].

Zwitterionic columns based on the silica gel or on the organic polymer support show dual HILIC-RPLC mechanism, with orthogonal selectivity in the HILIC and the RPLC ranges. We synthesized new organic polymer capillary monolithic columns by co-polymerization of the zwitterionic sulfobetaine (MEDSA) [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide] functional monomer and dioxyethylene dimethacrylate (DiEDMA) or bisphenol A glycerolate dimethacrylate (BIGDMA) cross-linking agents. The co-polymerization reaction was thermally initiated using 1% 2,2´-azo-bis-isobutyronitrile (AIBN) at 60 °C [89,90]. The organic polymer zwitterionic polymethacrylate monolithic columns give rise to dual retention mechanism with a relatively broad mobile phase range both for HILIC and reversed-phase separation modes. We investigated their applications in the separation of nucleic bases and other neutral, basic, and acidic polar analytes in aqueous-organic mobile phases [91].

The dual HILIC-RPLC mechanism enables separation of various classes of polar compounds, namely flavonoids, phenolic acids, barbiturates, sulfonamides, nucleic bases, and nucleosides on a single DiEDMA or BIGDMA column. Increasing (HILIC) or decreasing (RPLC) concentration gradients of water in buffered mobile phase at pH = 3.1 provide good resolution of polar samples both in the one-dimensional and two-dimensional setups [92].

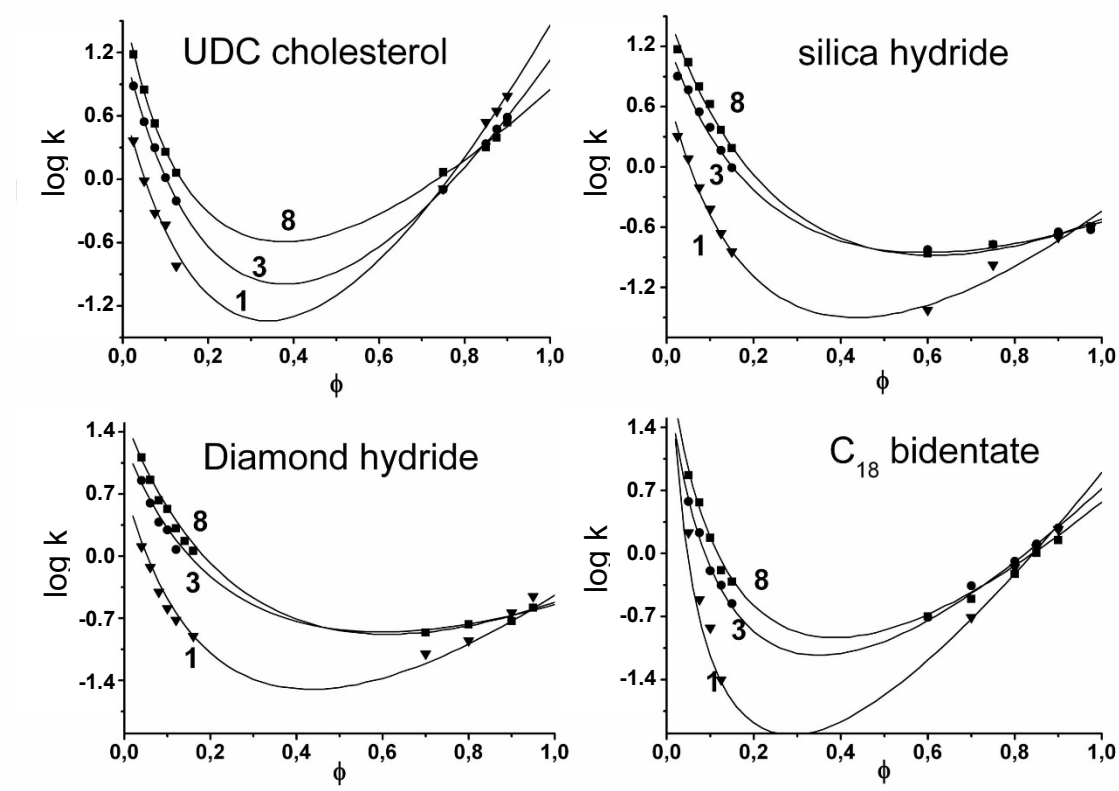

Fig. 6 Effects of the volume fraction of aqueous buffer (10 mM ammonium acetate, pH 3.26), Φ (% (v/v) · 10^{-2}), on the retention factors, *k*, of salicylic (1), *p*-hydroxybenzoic (3), and protocatechic (8) acids on the UDC cholesterol, C_{18} bidentate, silica hydride and Diamond hydride columns [88].
Temperature 40 °C; flow rate, F_m= 0.5 mL min^{-1}; sample volume 10 μL
Points – experimental data, lines – best fit the plots of Eq. (15).

Prediction of retention and optimization of the gradient elution

When separating samples containing more than 4–5 compounds with significant structural differences, we often meet the so-called "general elution problem": a mobile phase composition adjusted for optimum resolution of a few sample compounds may result in an incomplete separation of some earlier eluting compounds and (or) too strong a retention and long elution times of some others. Gradually decreasing the retention during the chromatographic run offers an efficient solution [93]. A program for increasing the temperature used in gas chromatography is less suitable in LC (except for separations of polymers), as the available temperature range is limited due to a generally low HPLC column stability at the temperatures higher than 60–70 °C [94].

Gradient elution with increasing solvent strength is the most useful programming technique in liquid chromatography. In the late 1960s, several reports had presented equations for the calculation of retention times in specific gradient applications. Later, L.R. Snyder developed the theory of linear solvent

strength (LSS) gradient elution, where the solute retention in the terms of the logarithm of retention factors, log k, is presumed to decrease proportionally to increasing time since the start of the gradient (the volume fraction of the strong solvent that passed through the column, V_t) [93]:

$$\log k = \log k_a - S \cdot V_t \tag{17}$$

where k_a is the retention factor at the start of the gradient elution and S is the "solvent strength" of the strong solvent in the mobile phase. In reversed-phase chromatography with linear gradients of the volume fraction of the organic solvent in water, the LSS theory is widely used; however the attempts at the applications of the LSS model Eq. (17) in normal phase, ion-exchange, or HILIC gradient systems often show significant prediction errors.

Since 1973, we investigated in detail various theoretical and practical aspects of gradient-elution liquid chromatography. This topic has been our major research interest for more than 15 years. We developed a new, general approach for the prediction of retention in gradient chromatography when having tested various aspects of the theory in a variety of practical LC cases. Gradually, we published a series of the articles, mainly in *Journal of Chromato-graphy*. We summarized the results of this research in the book *Gradient elution in column liquid chromatography*, published at Elsevier in 1985 [95]. Lloyd Snyder positively quoted our long-year activity in the field of gradient elution LC in the preface to his recent book published in 2007 [93].

The theory takes into account that the retention factors of sample compounds, k_i, during the gradient elution are constant only within a very small interval dt, corresponding to an increase in the final net retention time, t'_R, by the differential increment in the column hold-up time, d(t_m):

$$\mathrm{d}\left(t'_R\right) = k_i \cdot \mathrm{d}\left(t_m\right) \tag{18}$$

We suggested an approach for the prediction of the gradient retention data in various LC systems based on the integration of the Eq. (18) after introducing two equations, the first describing an appropriate retention model (such as Eq. (6), Eq. (7) or Eq. (9)) applicable in the concrete chromatographic system. The second equation defines the gradient program set by the operator. Most often, a linear gradient increases the volume fraction of the strong solvent, φ, proportionally to the volume of the mobile phase passed through the column, V, from the initial concentration A, to the φ_G at the end of the gradient ($V = V_G$) [96]:

$$\varphi = A + B \cdot V = A + \left(\varphi_G - A\right) \cdot \frac{V}{V_G} \tag{19}$$

The regression analysis of the experimental isocratic or independent gradient retention data provides the necessary parameters, which allows one introducing any retention model equation into the Eq. (18), together with the Eq. (19) and integration. The solution leads to the final relationship describing the effects of the gradient ramp (B) and the gradient range (φ_G–A) on the elution volumes, $V_{R(g)}$. The advantage of this approach is that the parameters of the isocratic model equations allow us the direct predicting of the gradient retention data for various gradient profiles, assuming that the column hold-up volume, V_m, does not change significantly in the gradient range [97]. Generally, the solution is possible for any combination of the retention model and gradient program using numerical integration. A few retention equations controlling practically useful separation systems (such as Eq. (6), Eq. (7) and Eq. (9)) allow the direct analytical integration. We compared the equations for the prediction of the gradient data for various combinations of separation modes and gradient profiles generated with various older devices and modern instruments forming the mobile phase gradients [98].

All the predictive equations for the gradient retention times (volumes) show strong effects of not only the gradient ramp (steepness B in Eq. (19)), but also of the initial volume fraction of the strong solvent, A, which is often an underestimated operation parameter – Fig. 7. The reversed-phase Eq. (7) provides the Eq, (20), being close to the solution of the Snyder LSS gradient model [99,100]

$$V_{R(g)} = \frac{1}{m \cdot B} \log\left[2.31 \cdot m \cdot B \cdot \left(V_m \cdot 10^{a - m \cdot A} - V_D\right) + 1\right] + V_m + V_D \qquad (20)$$

A similar solution published later Snyder et al. [101] and Schoenmakers et al. [102] and Eq. (20) can thus be marked as the LSS gradient equation.

The isocratic normal-phase retention equation, Eq. (6) results in the displacement model gradient equation, Eq. (21) [99,103]:

$$V_{R(g)} = \frac{1}{B}\left[(m+1) \cdot B \cdot \left(k_0 \cdot V_m - V_D \cdot A^m\right) + A^{(m+1)}\right]^{\frac{1}{1+m}} - \frac{A}{B} + V_m + V_D \qquad (21)$$

The generally valid three-parameter isocratic Eq. (9) applied to the gradient elution yields the ABM gradient Eq. (22) [98,104]:

$$V_{R(g)} = \frac{1}{a \cdot B}\left\{(m'+1) \cdot b \cdot B \cdot \left[V_m - V_D \cdot (b + A \cdot a)^m\right] + (b + A \cdot a)^{m'+1}\right\}^{\frac{1}{m'+1}} - \frac{b + A \cdot b}{b \cdot B} + V_m + V_D \qquad (22)$$

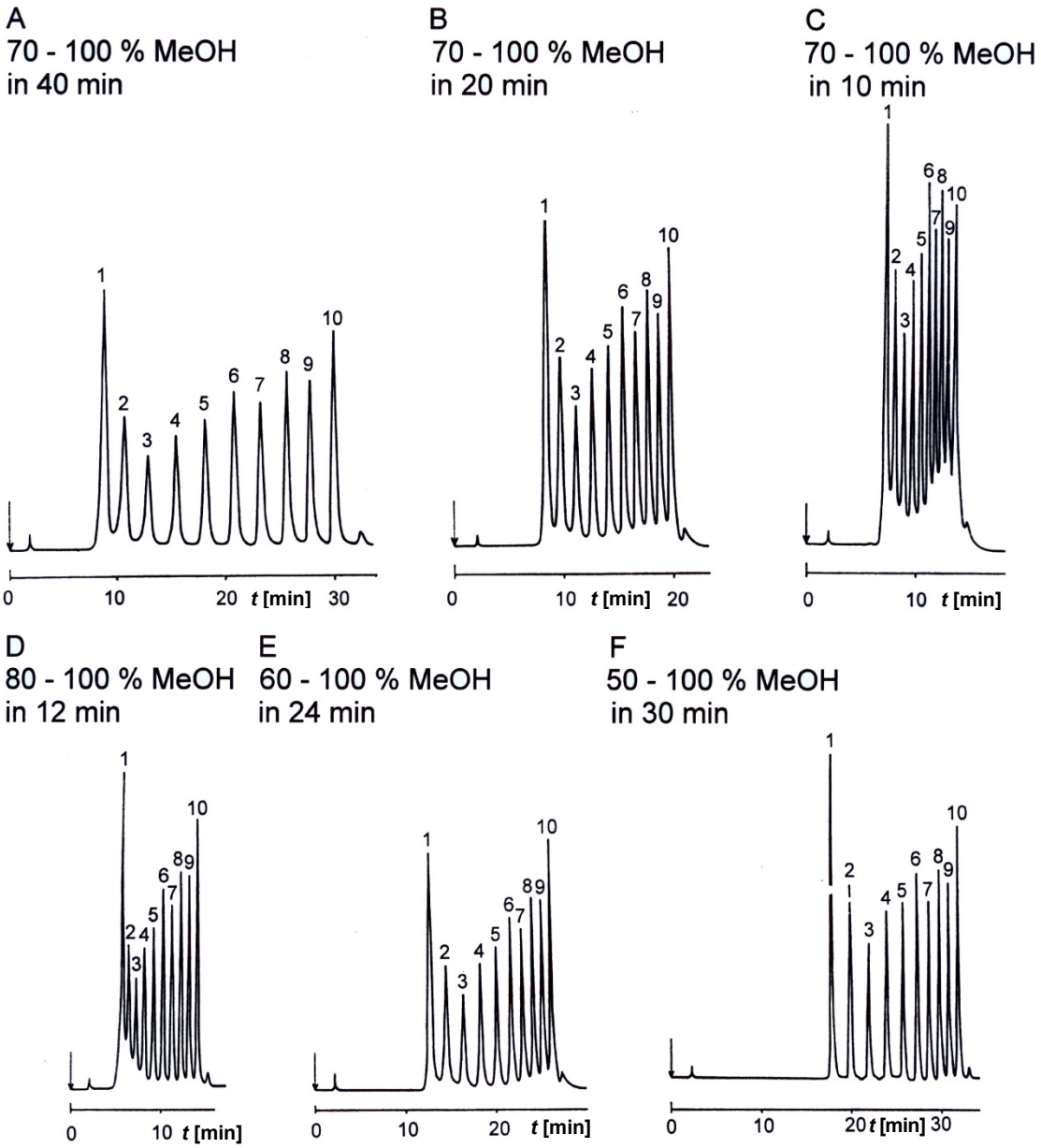

Fig. 7 The effects of the gradient profile on the reversed-phase gradient-elution separation of ten fluorescent derivatives of homologous *n*-alkylamines (methyl- to *n*-decyl-)
The three top chromatograms – gradient steepness (gradient time);
the three bottom chromatograms – initial volume fraction of methanol in water

In the gradient volume equations (20)–(22), V_m is the column hold-up volume. The symbols A and B are the parameters of the linear gradient ($\varphi = A + B \cdot V$), $V = t \cdot F_m$ is the volume of the mobile phase passed through the column since the start of gradient elution. Finally, F_m is the mobile phase flow rate. a, b, k_0, m and m' are the best-fit regression parameters of Eq. (6), Eq. (7) and Eq. (9), respectively. Our treatment takes into account the instrumental dwell volume, V_D (i.e., the volume of the gradient mixer and of the connecting tubing between the

mixer and the column inlet) containing the starting mobile phase in which less retained compounds may move some distance along the column under isocratic conditions, before the front of the gradient program. Other gradient theories do not consider this important factor and suggest the experimental compensation of V_D by delaying the start of the gradient after sample injection. This, of course, increases the separation time and is theoretically correct only with strongly retained analytes and the gradients starting at $A = 0$. The predictive computation is relatively easy with generally available statistical software on a common table editor (Excel, Quattro Pro).

In gradient elution, all sample compounds, except for some very early eluting analytes, show very similar instantaneous retention factors, k_f, at the time they leave the column. The k_f is approximately a half the average retention factor (k^*) during the band migration along the column and depends, to some extent, on the gradient profile, so that the bands are generally narrower in gradient elution than those under isocratic conditions, especially for steeper gradients.

So far, there was no reliable approach to modelling the bandwidths of the separated compounds in the gradient LC except for numerical calculations of the whole eluting band profile, which is not practical in analytical HPLC. We proposed estimating the gradient bandwidths as approximately equal to the isocratic bandwidths in the mobile phase at the time of elution of the band maximum [96]:

$$w_t = 4 \cdot t_m \cdot \left(1 + k_f\right) \cdot N^{-1/2} \tag{23}$$

where N is the number of theoretical plates of the column under isocratic conditions. The gradient bandwidths of most sample compounds are approximately equal and narrow in comparison to the isocratic operation mode.

In a series of articles, we verified the validity of the gradient theory in many practical reversed-phase and normal-phase separations employing continuous and step gradients. We investigated the effects of the gradient conditions on the retention volumes, bandwidths, resolution, upon the elimination of instrumental errors, and — finally yet importantly — the approaches for optimizing the gradient programs. In many RPLC systems, the gradient elution volumes predicted from Eq. (20), Eq. (21), and Eq. (22) leads to the prediction errors less than 1–3 %. Using dry solvents and controlled temperature, the Eq. (21) showed less than 3% prediction errors in the retention volumes of phenylurea herbicides on bonded nitrile and amino-silica gel columns with the gradients of 2-propanol or dioxan in *n*-hexane [105].

The prediction accuracy of the gradient elution times of alkylbenzenes and flavones was within 1–3 % in fast (1–2 min) gradients on short packed 5 cm C18 and Phenyl-Hexyl fully porous [106], core-shell [107] or silica gel bonded C_{18} monolithic columns [108]. The prediction errors of the gradient retention volumes in fast acetonitrile – water gradients (1–5 min, run at 2–4.5 mL min^{-1}) are in-

between 0.4 % and 1.2 % with the ABM model Eq.(22) [109]. The fast gradients are typical for the short columns used in the second dimension of two-dimensional on-line HPLC. The predictive optimization approach gives rise to errors comparable with the commercial Dry-Lab G gradient optimization software intended for the actual separation problem, employing the input experimental data acquired in two scouting gradient experiments [110,111].

Ternary gradients may improve the resolution when the selectivity of binary gradients is not sufficient for the separation in a particular case. Gradients employing simultaneous changes in the concentrations of two organic solvents in water are especially useful when different organic solvents provide optimum separation of the early eluting and of the late eluting compounds; see, e.g., Fig. 8 [112]. In ternary solvent gradients where the concentrations of two organic solvents (such as acetonitrile and methanol) in water change simultaneously according to different linear programs, the gradients are described by Eq. (19) with different parameters *A* and *B* [113].

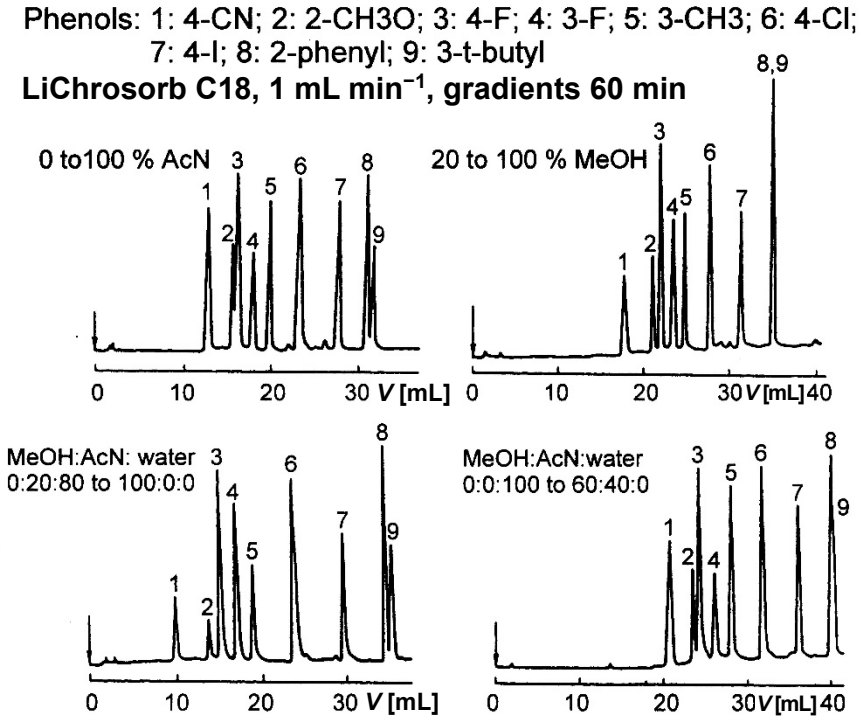

Fig. 8 Reversed-phase gradient-elution separation of a mixture of phenols using binary linear gradients of methanol in water and of acetonitrile in water and a ternary simultaneous gradient of methanol + acetonitrile in water, optimized to attain improved separation of the pairs of compounds 2/3 and 8/9

Column: LiChrosorb RP-C18, 5 μm, 300 × 4 mm I.D., flow rate: 1 mL min^{-1}, detection: UV, 254 nm. Sample compounds: 4-cyanophenol (1), 2-methoxyphenol (2), 4-fluorophenol (3), 3-fluorophenol (4), m-cresol (5), 4-chlorophenol (6), 4-iodophenol (7), 2-phenylphenol (8), and 3-*tert.*-butylphenol (9)

The gradient retention model presumes that the phase ratio remains constant during the gradient elution, but this does not apply in HILIC, where the gradients of the increasing concentration of water continuously increase the thickness of the adsorbed water layer in the stationary phase. The continuously increasing water adsorption may affect the gradient prediction errors even more than the type of the retention model equation employed. The hydrosilated silica gel columns with less than a monomolecular layer of adsorbed water (already 58–78 % saturated at the gradient starting water concentrations as low as 2 %) show excessively high errors in the predicted gradient retention volumes which could be suppressed to 5 % or less by correcting the actual gradient profile for the increasing amount of adsorbed water [114]. On the other hand, the TSK gel Amide-80 and YMC Triart Diol columns strongly adsorbing water are obviously less sensitive to the water uptake during the gradient elution and result in low uncorrected HILIC gradient prediction errors of Eq. (21), 1.2–1.5 % for gradients in the range from 96% to 70% water [78].

The separation of samples containing a wide range of oligomeric and polymeric compounds is generally possible only by using gradient elution conditions [115].

Two-dimensional LC×LC comprehensive separations

The maximum theoretical number of peaks separated in a single run HPLC can hardly exceed one hundred. However, in clinical, pharmaceutical, food, and environmental analysis, molecular biology and elsewhere, we encounter the samples which may contain even millions of compounds, in concentrations possibly spanning over 10 orders of magnitude. The theoretical peak capacity, i.e. the maximum number of peaks separated in a sample during a single chromatographic run, significantly increases by combining two or more different separation mechanisms in two-dimensional (2D) separation systems [116–118]. Two-dimensional gas chromatography, GC×GC has become routine in the analysis of the oil products and elsewhere in the past two decades, but we had to wait until the start of the new millennium which saw the advent of the instrumentation suitable for the real-time practice of two-dimensional comprehensive liquid chromatography (LC×LC).

Two-dimensional LC is a useful tool for separating sample compounds into classes, based on the presence of common structural units, such as methylene ($–CH_2–$) units in the alkyls of carboxylic acids, alcohols, esters, etc., offering excellent separations in the RPLC systems. A sample can be resolved simultaneously according to the distribution of two different repeat structural units in the organic NPLC or HILIC systems. We participated at the European Commission joint project on the comprehensive LC×LC, COM-CHROM 001, together with the groups at the universities of Oslo, Paris, Amsterdam, Karlstadt

(Sweden) and Messina (Italy). COM-CHROM yielded a 5-year period of pleasant and rewarding research activities. Nine young colleagues and PhD students from our group (Petr Česla, Michal Holčapek, Lenka Kolářová (Češlová), Martina Lasáková, Dana Moravcová, Kateřina Novotná (Vyňuchalová), Veronika Škeříková, Václav Staněk, and Jiří Urban) had the opportunity of working each for several months at the renowned partners' laboratories and presenting their work at the first-class world chromatographic meetings. On the other hand, several young PhD students from Italy (Francesco Cacciola), Poland (Kasia Krupczynska, Szymon Bocian, Tomasz Welerowicz), Romania (Simion Beldean-Galea) and Slovakia (Eva Blahová, Zoltán Hajdu) came to Pardubice to work on their dissertations in the frame of the COM-CHROM project. This enjoyable COM-CHROM period resulted not only in a number of common publications in prestigious journals, but also in a long-term co-operation after the end of the project and long-lasting personal friendships.

In Pardubice, we focused the attention on developing two-dimensional LC×LC systems yielding maximum selectivity and peak capacity by optimizing the column dimensions with optimum combinations of the stationary and mobile phases in the first- and in the second separation dimensions. We investigated possibilities of the 2D LC for improving the separation of polar polyphenolic, flavonoids and related compounds in food and beverages (beer, wine, plant extracts) and of surfactants.

The off-line two-dimensional LC approach using two (or more) separate columns is very simple, necessitating no special instrumentation and therefore is suitable for common practice. It allows one independent optimization of the HILIC and RPLC separation systems [119]. However, the on-line two-dimensional separations employing either serial or parallel column setups provide faster results. The serial column coupling does not require complex instrumentation enabling a moderate increase in the peak capacity, because the effects of the individual columns are additive. In the LC×LC on-line technique, two independent LC systems coupled on-line allow subsequently transferring small-volume effluent fractions from the first column onto the second column (Fig. 9). The parallel setup in comprehensive two-dimensional (LC×LC) provides multiplicative effects on the peak capacity and a considerably higher peak production rate (i.e., the number of resolved compounds per the pre-set separation time).

Guiochon et al reviewed the expected increase in the number of separated compounds (the practical peak capacity) in various off-line and on-line multi-dimensional setups in detail, in the terms of performance and of the price to be paid in the analysis time and sample dilution (i.e., sensitivity) [120]. On-line comprehensive LC×LC achieves the peak capacities up to 600. The off-line or stop-flow systems may provide higher peak capacities at the cost of long separation times. The contribution of the stopped flow to the band broadening is negligible [121].

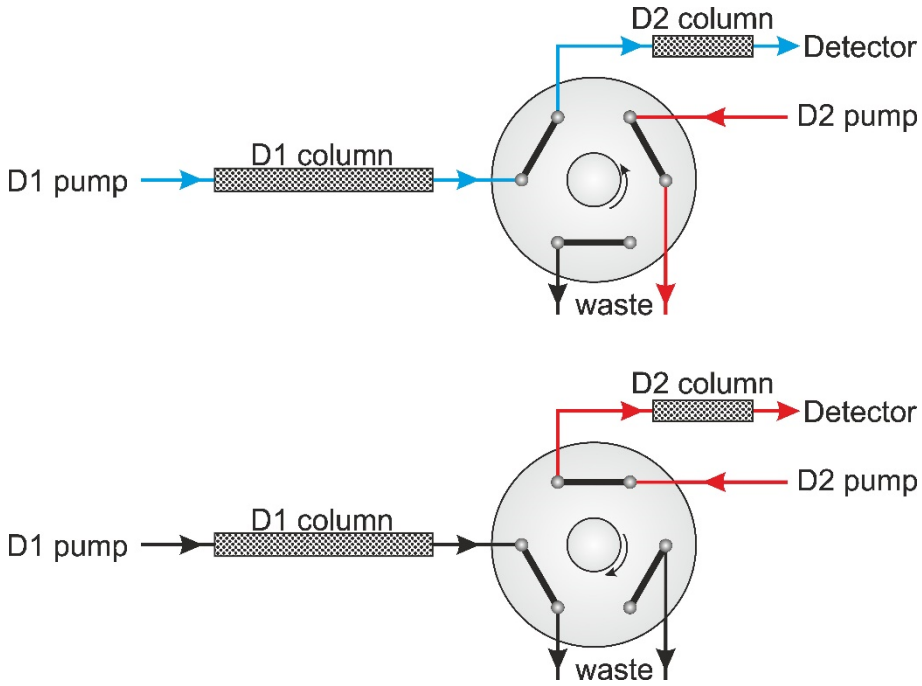

Fig. 9 Comprehensive LC×LC setup of two columns, D1 and D2, with a ten-port valve modulator interface employing continuous operation via two fraction selection sampling loops, A and B, in alternating cycles

We set up a 2D liquid chromatograph by coupling two liquid chromatographs via a ten-port two-way Valco switching valve with two 2 mL or 1 mL sample-collecting loops. In the first dimension, we employed an 1100 Capillary Agilent Liquid Chromatograph with a narrow (0.5 mm I.D., 15–20 cm long) zwitterionic micro-colum. In the second dimension, we used a 1200 Agilent Rapid Resolution Liquid Chromatograph with a short (3–5 cm long) C_{18} silica gel core-shell or monolithic column. This setup allowed the analysis of a second-dimension sample fraction in 1 min or less [122].

The frequency of the valve switching cycle controls the volume of the fractions collected from the first dimension and the time of the second-dimension separation, so that the whole sample passes through the first- and second-dimension columns during the 2D analysis run. To this end, the second-dimension column should be considerably shorter than the first-dimension column, which should have a narrow inner diameter to provide sufficiently low volume of the transferred fraction to avoid the formation of broad peaks and a loss in the eventual sample resolution.

Generally, the peak capacity is higher in the gradient than that in the isocratic mode. We achieved a significant increase of the peak capacity (the number of resolved peaks by using simultaneous gradients in the first dimension and in the second dimension of comprehensive 2D setups [123]. The second-dimension gradient should be steep and able of covering a broad composition range of the mobile phase [124].

We optimized the gradient range and the gradient profile in the second dimension by changing the gradient range during the analysis of the subsequent fractions (shifting gradients and segment-in fraction gradients). Traditionally, linear second-dimension gradients covering the full range of mobile phase composition are used in all consecutive fraction separations, ("Full In Fraction", FIF gradients – Fig. 10a). However, for a more regular coverage of the second-dimension retention space and increased number of the compounds separated in a particular sample, the second-dimension gradient often covers a more or less limited concentration range of the organic solvent.

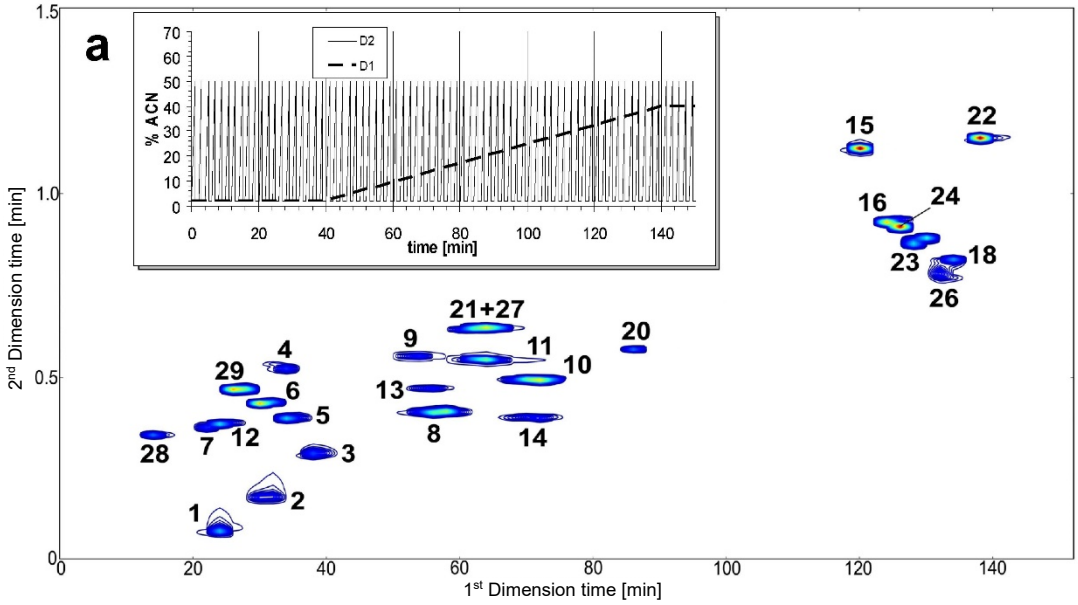

Fig. 10 Profiles of three types of parallel gradients of acetonitrile in 0.05 mM ammonium acetate buffer (pH = 3) run simultaneously in the first and in the second dimension: a) Full in fraction (FIF), b) Segment in fraction (SIF) and c) Continuously shifting (CS) second dimension gradients

Contour plot presentations of the comprehensive 2D separation of 27 phenolic acids and flavones for each gradient type. A bonded polyethylene glycol column (150 × 2.1 mm I.D., 5 µm particles) in the first dimension, a fused-core Ascentis Express C18 column (30 × 3.0 mm I.D., 2.7 µm particles) in the second dimension

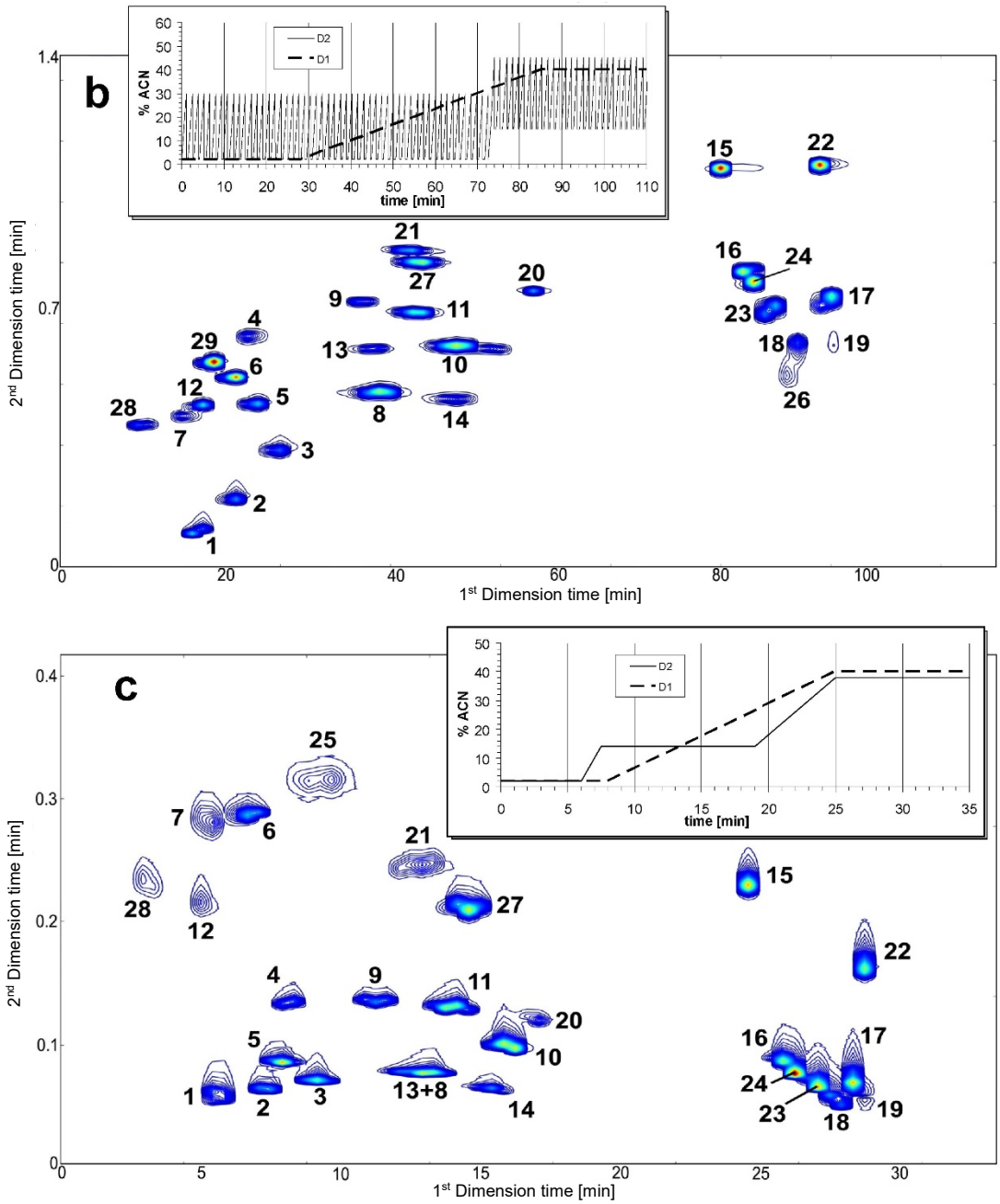

Fig. 10 (continued) Profiles of three types of parallel gradients of acetonitrile in 0.05 mM ammonium acetate buffer (pH = 3) run simultaneously in the first and in the second dimension: a) Full in fraction (FIF), b) Segment in fraction (SIF) and c) Continuously shifting (CS) second dimension gradients

Contour plot presentations of the comprehensive 2D separation of 27 phenolic acids and flavones for each gradient type. A bonded polyethylene glycol column (150 × 2.1 mm I.D., 5 µm particles) in the first dimension, a fused-core Ascentis Express C18 column (30 × 3.0 mm I.D., 2.7 µm particles) in the second dimension

We suggested to gradually increase the optimum second-dimension gradient range in each fraction in two or more steps, proportionally to the progress of the separation in the first dimension in a "Segment In Fraction" (SIF) gradient (Fig. 10b) [124]. Alternatively, a continuous linear second-dimension gradient can span over several or all fractions in the second dimension, in a "Continuously Shifting" (CS) gradient (Fig. 10c) [125]. With the SIF or CS gradients, the orthogonality of a two-dimensional system improves and even strongly retained compounds elute in the second dimension within 1.2 min, corresponding to the gradient time of 1 min + 0.2 min (the gradient delay period. Also the first-dimension gradient time and the total two-dimensional separation time are reduced to approximately 30 min [126].

For the detection of the resolved fractions, we employed a diode-array UV spectrometric, an electrochemical coulo-array or a mass-spectrometric detector [127]. For the evaluation of the detector records, we developed a proprietary program, distinguishing the detector traces corresponding to the individual second-dimension fractions and transforming the signal into a three-dimensional chromatogram or into a two-dimensional planar retention map. The multi-channel UV- or coulometric signal recording of several simultaneous chromatograms at different wavelengths or applied electrochemical potentials significantly improves the identification of the resolved compounds. Figure 11 shows an example of the 2D comprehensive separation of a beer sample with multichannel UV detection at four wavelengths.

The two-dimensional peak capacity can theoretically reach the product of the peak capacities in the individual systems in the orthogonal two-dimensional separations with non-correlated retention. Unfortunately, in practice, most 2D systems show more or less significant retention correlations, which decrease the number of practically resolved peaks [128]. A careful selection of dissimilar (orthogonal) columns and mobile phases in the two dimensions often can mitigate the problem [124]. Coupled HILIC and RPLC separation systems offer two completely different retention mechanisms and a very high degree of orthogonality in comparison to other 2DLC systems, thus providing high number of compounds resolved in the complex samples. Therefore, we focused our attention on the coupled HILIC-RPLC systems [129].

Unfortunately, on-line combinations of HILIC and RPLC present compatibility problems originating from the differences in the elution strengths of the mobile phases in the two dimensions. High concentrations of the organic solvents used for HILIC separations may lead to a weak retention in the RPLC systems, whereas the mobile phases more rich in water used in RPLC are often too strong HILIC eluents. If the mobile phase from the first (HILIC) dimension transfers the fractions to the second dimension, significant decrease in retention, un-symmetrical or even split peaks may appear, with detrimental effects on the separation. There are several ways to mitigate the compatibility problems:

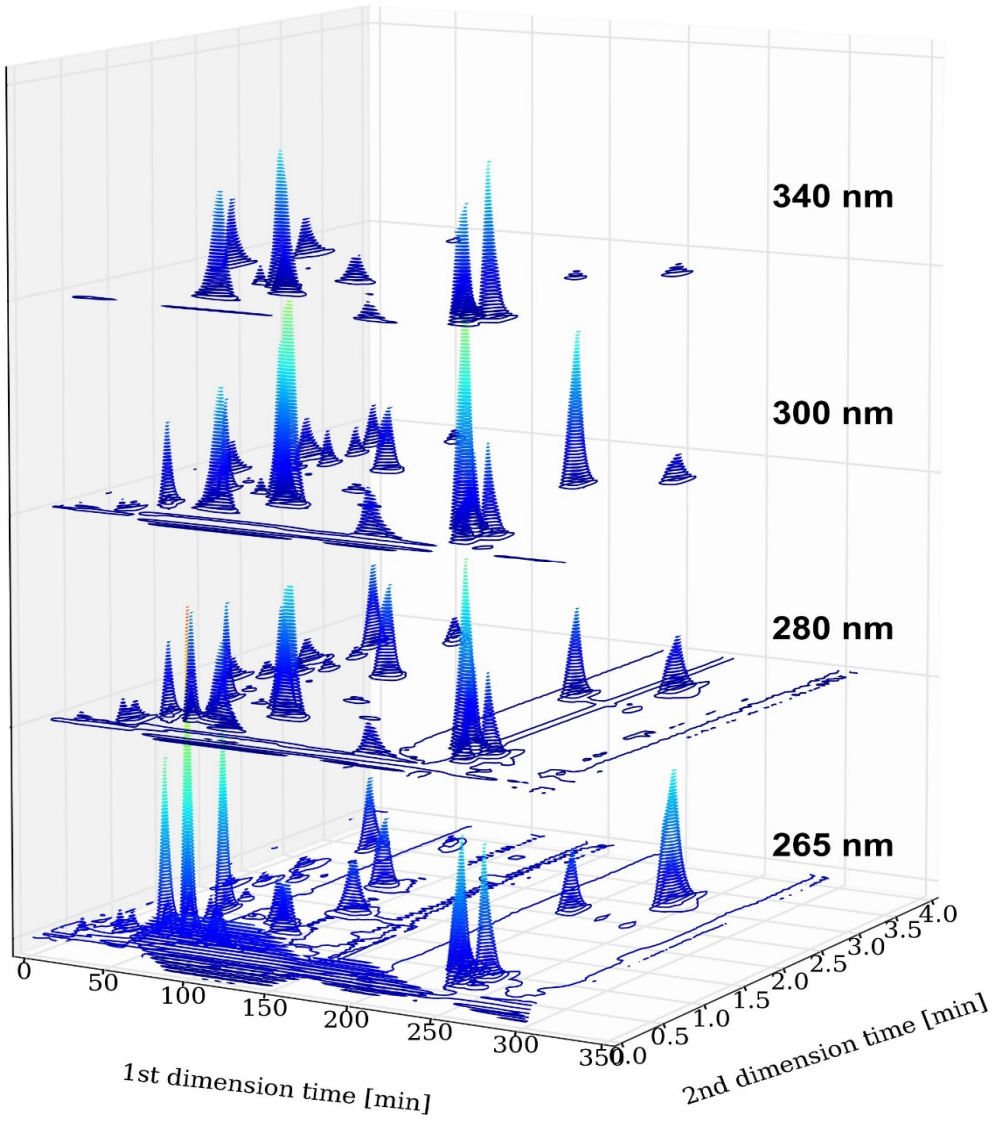

Fig. 11 Three-dimensional chromatogram of a comprehensive 2D separation of phenolic and flavonoid compounds with multi-channel UV-detection at 200–400 nm
The 2D separation system as in Fig. 10

1. A small volume of the sample (for example, 2 μl) injected onto a narrow-bore or a capillary micro-column in the first dimension allows small volume fractions be transferred at a low flow-rate (2–5 μL min^{-1}) at a fast fraction transfer frequency (1 min or less) onto a short regular bore (4 μm I.D.) column in the second dimension. This approach often minimizes the sample solvent effects in the second dimension, however, sometimes with possible negative impacts on the selectivity of two-dimensional separations [130].
2. A make-up flow of water decreases the mobile phase strength of the organic-rich fractions transferred from the HILIC column into a RP column in the second dimension [131].

3. Fractions from the first column can be trapped inside a small column and the solvent removed by evaporation before the transfer to the second dimension [132].

A crucial point affecting the separation time in comprehensive two-dimensional liquid chromatography is the performance of the column used in the second dimension, which should allow highly efficient fast chromatographic separations [133], such as a short column packed with sub-2 μm particles used in an UHPLC instrument, at the cost of a very high operation pressure [134]. A similar result can be obtained with a core-shell column with conventional liquid chromatographic instrumentation [135].

The C_{18} core-shell columns in the second dimension enable fast second-dimension gradient separations at the ambient temperature without excessive backpressure and without compromising the optimal first dimension sampling rates [130]. A lower flow resistance of monolithic columns in the second dimension allows higher flow-rates in comparison to particle-packed columns working at the same operation pressure [127]. The optimized on-line comprehensive HILIC×RPLC provided a very good band symmetry and retention time repeatability of phenolic compounds and flavonoids. The setup employed a 0.5 mm I.D. monolithic sulfobetaine HILIC capillary column at the flow-rate of a few microliters per min in the first dimension, coupled with various 2.5–5 cm long, 3 mm I.D. monolithic and core–shell C_{18} columns, operated at the flow rates of 3–5 mL min^{-1} in the second dimension [130]. Later, we attempted to separate phenolic acids in a coupled two-dimensional RPLC and micellar electrokinetic chromatography system [136].

Our homemade zwitterionic polymethacrylate BIGDMA-MEDSA 0.3–0.5 I.D. micro-bore column offers distinct advantages for the application in the first dimension of comprehensive 2D LC×LC, showing dual RPLC-HILIC mechanism in aqueous-organic mobile phases [91]. This dual-mode column is useful for combined two-dimensional RPLC×RPLC and HILIC×RPLC runs. In the first dimension, the RPLC mode in highly aqueous mobile phase alternates with the HILIC mode running at high acetonitrile concentrations. A short (3–5 cm) core shell or a monolithic silica C18 columns were used in the second dimension for the separation of polyphenolic compounds. The combined HILIC×RPLC and RPLC×RPLC comprehensive two-dimensional separations employ a gradient of decreasing acetonitrile gradient in the first dimension during the HILIC×RPLC period. At the end of the HILIC gradient, the polymeric monolithic micro-column equilibrated with a highly aqueous mobile phase is ready for the repeated sample injection, this time for separation under reversed-phase gradient conditions with increasing concentration of acetonitrile in the first dimension during the RPLC×RPLC period. Fig. 12 shows 2D chromatograms of flavones and related polyphenolic compounds, acquired with a single first-dimension BIGDMA-MEDSA capillary column in two consecutive injections of

the sample, the first one into a decreasing and the second into an increasing acetonitrile gradient. The automated dual LC×LC approach allows obtaining some three-dimensional data in a relatively short time [137,138].

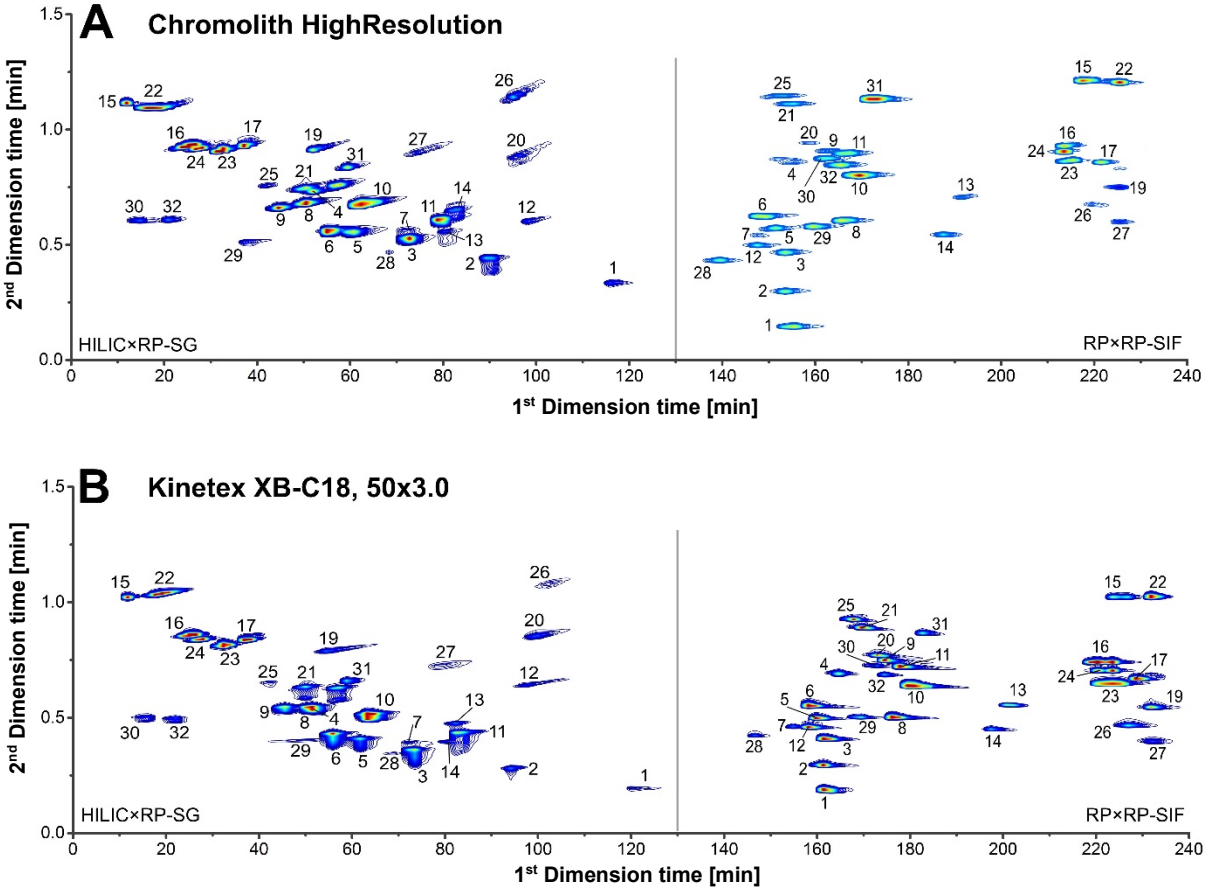

Fig. 12 (Quasi) 3D separation of phenolic acids and flavones on a BiGDMA-MEDSA micro-column in alternating HILIC (left from the arrow indicating the second sample injection) and RP modes in the first dimension and a Chromolith High Resolution column (A) or a Kinetex XB-C18 (B) column in the second dimension.

The environmental applications of HPLC

In the 1980s, we oriented a part of our research on the environmental applications of HPLC, mainly the analysis of water pollutants. Some important compound classes do not provide the response in HPLC with UV spectral detection and need a pre-separation sample treatment to prepare suitable UV-absorbing or fluorescent derivatives. We developed a new reagent for preparation of fluorescent derivatives of aliphatic amines [139]. The sensitivity of the water analysis by HPLC increases significantly when applying sample pre-separation enrichment

by solid-phase extraction (SPE) in a mall column packed with a suitable (usually non-polar) organic polymer. To optimize the SPE – HPLC enrichment methods, we compared the sorption and desorption properties of various sorbents for pesticides [140] and aliphatic amines [141].

The organic polymer sorbents for water-sample enrichment usually employ the surface adsorption of organic pollutants by the solvophobic interactions; some sorbents containing amino-, cyano- or other functional moieties for the enhanced adsorption of polar pollutants [142]. Ionic water contaminants can be pre-concentrated on polymeric ion exchangers and then released using aqueous-organic solutions of electrolytes [143].

We developed new SPE-HPLC methods of the analysis of phenoxyacid- [144] and phenylurea- [145] herbicides, chlorobenzenes, benzene sulfonyl chlorides and benzene sulfonic acids in surface waters [146]. We applied a coupled SPE-HPLC-MS technique in the analysis of azo dyes and degradation products in industrial waste water [147]. Together with the partners from the Aristotle University of Thessaloniki, we examined this method for the analysis of the organic residues after electrochemical degradation of industrial dyes in waste waters from a textile plant [148]. Further, we developed an SPE–HPLC method for the determination of phthalate plasticizers in industrial exhalates [149]. Finally, we tested possibilities of combining SP and supercritical fluid extraction (SFE) pre-concentration methods [150].

The molecular imprinted polymeric sorbents (MIPs) are the SPE materials based on another adsorption principle. The porous sorbent polymerized with the addition of a target compound which is subsequently washed out, holds the shape of the template cavities in the final polymeric structure. In the following analysis of water samples, the MIP sorbent preferentially retains the target pollutant molecules [151,152]. We prepared a home-made MIP for the adsorption of ephedrine, just to give one example of the biomedical application of the approach [153]. We co-operated with the University of Pierre and Marie Curie (Paris) and with the Aristotle University in Thessaloniki. The Greek colleagues spent several months working on MIPs in Pardubice in the frame of a bi-lateral cooperation program (Dr. Georgios Theodoridis, Anastasios Sakalis, and Konstantini Gianitsou); vice-versa, Veronika Škeříková, Tomáš Hájek, and Martina Lasáková, PhD students from Pardubice visited our friends in Thessaloniki to work with them on MIPs.

We tested another, so far unpublished approach for enhancement of the sensitivity of fast HPLC trace analysis by summation of signals recorded in repeated HPLC runs. Here, the accumulated signal is directly proportional to the number of repeated experiments. On the other hand, the accumulated noise signal is proportional only to the square root of the number of repetitions. Consequently, the signal-to-noise ratio increases and, consequently, the limit of determination decreases [154].

Separation of dyes and intermediates

Fully ionized naphthalene sulfonic acids (NSA) are the intermediates in the production of synthetic dyes in the Synthesia Semtín chemical plant, having therefore been interested in their analysis. The retention of NSA decreases with the increasing number of sulfonate groups in the molecule. In ion-pairing chromatography with quaternary tetralkylammonium surfactants additives into the mobile phase, we could separate the relatively non-polar ion associates of NSAs on chemically bonded non-polar columns into groups containing 1–4 sulfonic acid functionalities [155], but we were not able to distinguish all the individual NSA isomers [40]. On a bonded C_{18} column with suitable pore morphology, we resolved all the relevant isomers of naphthalene mono-, di-, tri- and tetra-sulfonic acid dye intermediates by gradient elution in aqueous–organic mobile phases with relatively high concentrations (0.05–0.4 mol L^{-1}) sodium sulfate (Fig. 13) [156].

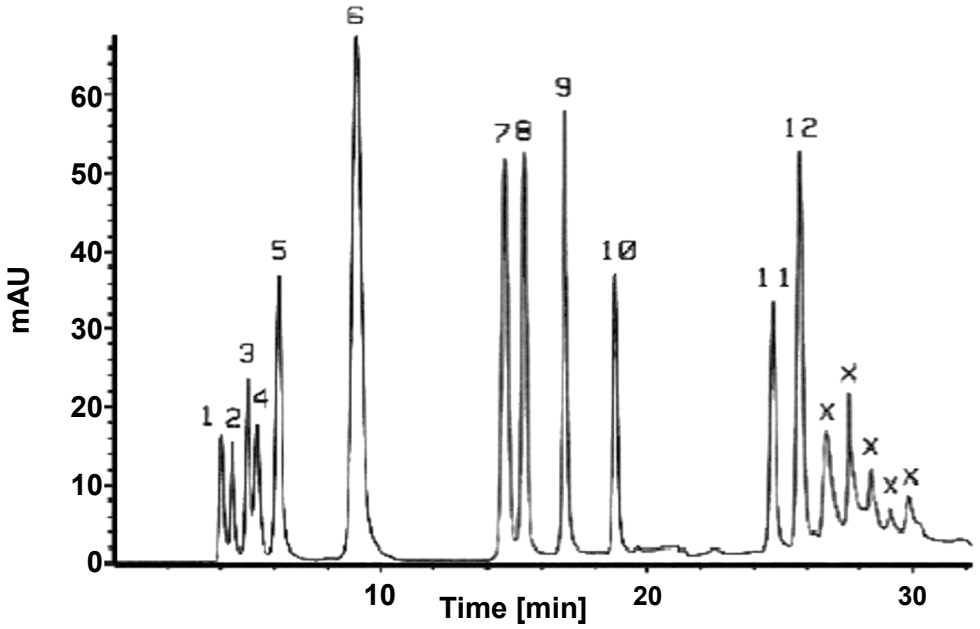

Fig. 13 Separation of twelve naphtalene sulphonic acids by gradient-elution RPC on a Separon SGX RPS column, 7 μm (250 × 4 mm I.D.)

Solvent program: 5 min isocratic, 0.4 mol L^{-1} Na_2SO_4 at 0.5 mL min^{-1}, followed by linear gradient from 0.4 mol/L Na_2SO_4 to 40 % (v/v) methanol in water in 15 min at 1 ml min^{-1}; detection: UV, 230 nm; column temperature 40 °C

Sample compounds: naphthalene-1,3,5,7-tetrasulphonic acid (1), naphthalene-1,3,6-trisulphonic acid (2), naphthalene-1,3,5-trisulphonic acid (3), naphthalene-1,3,7-trisulphonic acid (4), naphthalene-1,5-disulphonic acid (5), naphthalene-2,6-disulphonic acid (6), naphthalene-1,6-disulphonic acid (7), naphthalene-2,7-disulphonic acid (8), naphthalene-1,3-disulphonic acid (9), naphthalene-1,7-disulphonic acid (10), naphthalene-1-sulphonic acid (11), naphthalene-2-sulphonic acid (12), unidentified less polar impurities (X)

With the ion-pairing HPLC, we separated a variety of sulfonated azo dyes [157,158]. Later, we applied mass-spectrometric detection and identification to the dye analysis by ion-pairing LC [147]. The ion-pairing reagents may suppress the intensity of the MS electrospray signal and the sensitivity of determination. We found this negative effect significantly lower with di- and tri-alkyl ammonium acetates in comparison to the tetraalkyl-ammonium ion-pairing reagents. We had to pay for it by a slight impairment of separation selectivity for di- and tri- NSA acids and azo dyes [159]. In cooperation with the Aristotle University in Thessaloniki, we used the ion-pairing method combined with electrochemistry for the analysis of azo dyes and their degradation products in wastewater from a textile factory [148].

We found capillary electrophoresis to provide a valuable complementary selectivity to the HPLC of isomeric naphthalene mono- to tetra-sulfonic acids [160]. Cyclodextrin additives (CD) are not only useful for chiral electrophoretic separations, but their addition to the working electrolyte enables selective separation of NSA isomers by capillary electrophoresis (CE) [161]. In some cases, the separation selectivity can be improved with mixed cyclodextrin additives (β-CD and γ-CD) [162].

HPLC of household and industrial surfactants

(Poly)oxyethylene alkyl- and alkylarylaryl-ethers of alcohols are employed as household and industrial surfactants, exhibiting a dual distribution of oxyethylene (EO) or oxypropylene (PO) repeat structural units, in addition to the distribution of alkyl lengths. The molecules of anionic surfactants also contain carboxylic or sulfonic acid groups. We separated the individual oligomers of sulfated oligoethylene glycol nonylphenyl ether anionic surfactants and their parent non-sulfated analogues in a single run on cyanopropyl and aminopropyl silica gel stationary phases using normal-phase gradient elution with mobile phases containing cetyl trimethylammonium bromide, 2-propanol, and *n*-heptane. For the first time, we observed that not only the type of the organic solvent (methanol, acetonitrile, ethanol,etc.), but also the proportion of the aqueous and organic components in the mobile phase affects the elution order of the oligomers. In highly aqueous mixed mobile phases, the retention increases with the increasing number of EO units, whereas in the mobile phases containing 85 % or more organic solvent, the elution order is reversed and the oligomers with higher numbers of EO units elute before higher oligomers. Hence, the surfactant oligomeric series manifests a mixed RPLC-HILIC behavior [163].

Synthetic (co)polymers or (co)oligomers with two (or more) repeating groups such as, e.g., the oxyethylene-oxypropylene alkylphenyl ether surfactants show not only molar mass distribution, but also the distribution of the composition and sequence of the individual repeat units in the molecules.

To characterize a two- (or more-) dimensional distribution, the 2D LC often allows selecting the conditions that enhance the separation according to one type of repeating units and suppressing the separation according to the second unit in the first dimension and, vice versa, in the second one. Coupling a liquid chromatograph with atmospheric-pressure chemical ionization mass-spectrometric detection (APCI-MS) allowed us to correctly determine the mass of the individual oligomers, enabling also the de-convolution of partially overlapping peaks. Without the MS detection, a study of dual structural distribution would not be possible [164]. We resolved the block EO-PO (co)oligomers by comprehensive two-dimensional LC×LC according to the number of the EO units under HILIC conditions on an aminopropyl column in the first dimension. The distribution of the PO units in a reversed-phase was determined on a C18 column in the second dimension [165].

Mono- and two-dimensional separations of lipids

The gradient retention theory of oligomers is helpful in the HPLC analysis of fats, oils, and related products. The main constituents of lipidic samples are complex mixtures of triacylglycerols (TAG) differing in the length and position of the acyl chains on the glycerol skeleton, the number and the positions of the double bonds.

We employed a non-aqueous reversed-phase (NARP) HPLC-MS method on two Nova-Pak C-18 chromatographic columns connected in series and acetonitrile-2-propanol gradient for unambiguous identification of a high number of TAGs, based on the positive-ion atmospheric pressure chemical ionization (APCI) mass spectra. The retention decreases with the increasing concentration of the less polar solvent in the mobile phase, the behavior opposite to normal-phase chromatography [166].

A new approach to TAG quantization employs the response factors obtained with three typical detection techniques (APCI-MS, evaporative light-scattering detection, and UV at 205 nm). This approach allowed identifying and quantifying 133 TAGs containing 22 fatty acids with 8–25 carbon atoms and 0–3 double bonds in 16 plant oils (walnut, hazelnut, cashew nut, almond, poppy seed, yellow melon, mango, fig, date, hazelnut, pistachio, palm, Brazil nut, rapeseed, macadamia, soyabean, sunflower, linseed, *Dracocephalum moldavica*, evening primrose, corn, amaranth, *Silybum marianum*) using HPLC/APCI-MS [167]. The TAG positional isomers distinguish by different relative abundances of the fragment ions formed by preferred losses of the fatty acid fragments from sn-1(3) positions compared to the sn-2 position. Excellent chromatographic resolution and a broad retention window together with APCI mass spectra enabled positive identification of TAGs containing fatty acids with odd numbers of carbon atoms, such as margaric (C17:0) and heptadecanoic (C17:1) acids [167].

We adapted the NARP gradient method to the analysis of the products of trans-esterification of the rapeseed oil to methyl esters in the production of bio-fuel using coupled HPLC/APCI-MS in the positive-ion mode (Fig. 14). Applying a three-component water – acetonitrile – propanol gradient followed with an acetonitrile – 2-propanol – hexane gradient, we achieved (in 25 min) an efficient separation of the individual C_{12}–C_{24} fatty acid methyl esters, triacyl-glycerols (TAGs), diacylglycerols and monoacylglycerols of oleic, linoleic and linolenic acids and of the free fatty acids according to both the acyl lengths and the number of double bonds. The individual sample compounds could be identified [168].

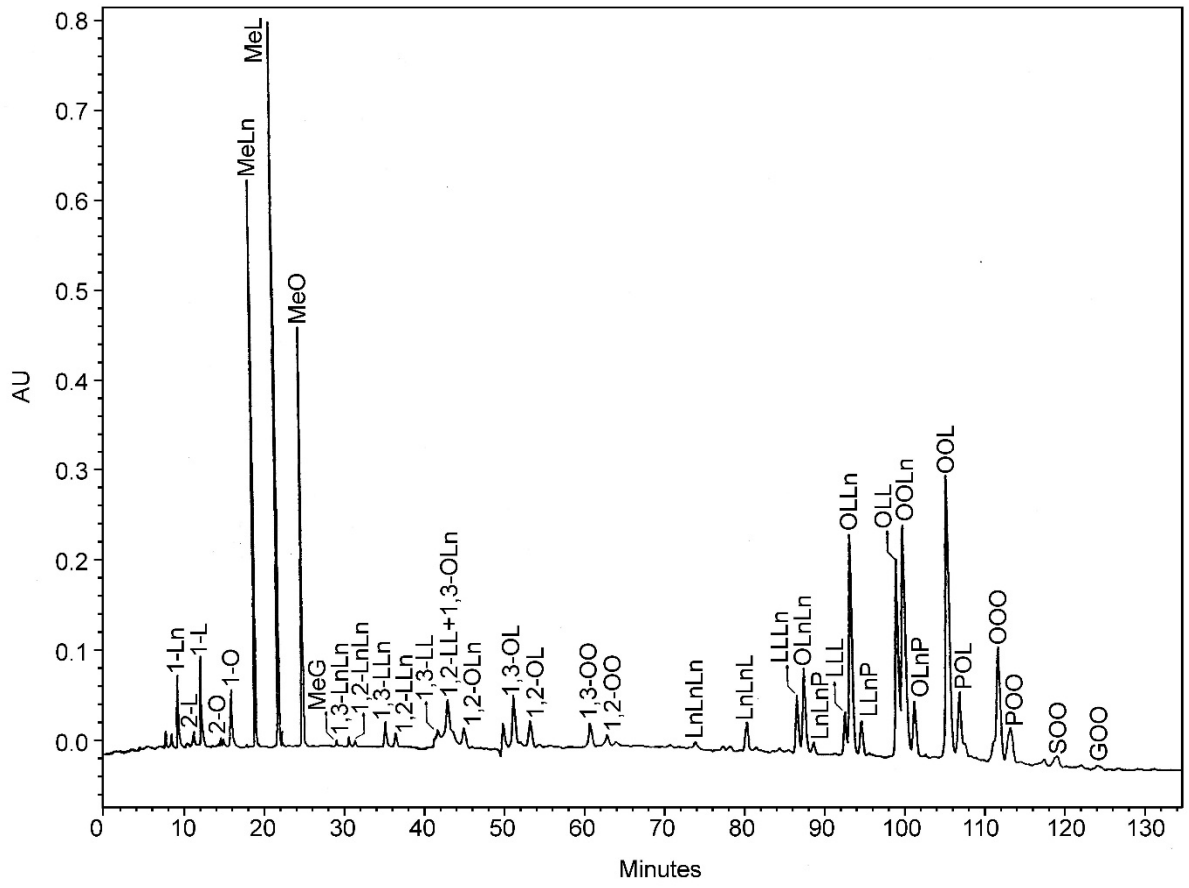

Fig. 14 Separation of a product of partial trans-esterification of rapeseed oil with methanol using combined RPC and NARPC gradient elution

Column: 2× Novapak C18, 7 μm, 150 × 3 mm I.D. Ternary gradient from 30% water + 70% acetonitrile to 100% acetonitrile in 10 min and to 50% acetonitrile + 50% propanol in 20 min, at 1 ml min^{-1}, injection volume 10 μL, UV detection at 205 nm
Notation of sample compounds: Ln, L, O and G are used for linolenic acid, linoleic acid, oleic acid, gadoleic acid, respectively, and for their acid parts in mono-, di- and tri-acylglycerols and methylesters, Me means methyl in methyl esters

Mono-dimensional high-performance liquid chromatographic (HPLC) techniques may be inadequate when challenged with complex lipid matrices. In co-operation with the group of the University of Messina in the frame of the COM-CHROM project, we employed comprehensive HPLC using complementary silver-ion (Ag^+) and non-aqueous (NA) reversed-phase (RP) methods for thorough oil separations by atmospheric pressure chemical ionization mass spectrometry (APCI-MS) for the individual TAG identification [169].

Like acylglycerols, carotenoids are non-polar compounds based on long-chain unsaturated hydrocarbons. In the frame of the COM-CHROM project, we developed a novel approach for carotenoid analysis. Orange essential oil and juice carotenoids were separated by means of comprehensive dual-gradient elution HPLC, when using normal phase with a microbore silica column in the first dimension and reversed phase with a monolithic C18 column in the second dimension incorporating a 10-port switching valve as an interface. For the identification of the resolved carotenoids, we combined the retention data with the UV-visible spectra [170].

Non-linear chromatography in the preparative separations

In the analytical HPLC, the separation phase systems controlled by linear isotherms provide narrow and symmetrical peaks with good resolution of low amounts of sample compounds. However, liquid chromatography is a useful tool also for preparative separations, where large sample volumes enable a high production rate and low production costs. High concentrations of separated samples usually overload the capacity of the LC separation column; a non-linear distribution isotherm controls the retention, which rules out the method development approaches used in analytical HPLC. The main issue is the asymmetric profile of the separated sample bands. The prediction of the retention behavior employs a numerical calculation of the band profiles, based on the actual non-linear isotherm parameters of the separated compounds. The objective of the preparation LC is usually the purification of the product of interest, or the separation of a low number of compounds, frequently the enantiomers of an optically active pharmaceutically active compound.

The author started working on this topic during his visit of the group of Prof. G. Guiochon at the University of Tennessee in Knoxville (USA) in 1990–1991. In the years 1994–1997, our group continued in the co-operation with Knoxville on the development of the overloaded LC separations in the bi-lateral *Non-linear Chromatography* project in the frame of the U.S. – Czechoslovak Science-and-Technology Program, No. 94 036. In the years 1998–2000, the research continued in the frame of the Joint research project KONTAKT-ME 150 of the commission for the cooperation between Czech Republic and NSF (USA). In this period, we cooperated with the universities of Tennessee (USA), Ferrara

(Italy), Magdeburg (Germany), Patras (Greece) and Veszprém (Hungary) on the NATO Science Programme 7 Linkage grant LG.971480 *Fundamentals of non-linear chromatography as the basis of preparative separations by liquid chromatography.* I and my several younger colleagues and PhD students (J. Fischer, D. Komers, M. Škavrada) visited several times Knoxville to use their central computer facilities for numerical calculations of the overloaded band profiles. Prof. Guiochon and his students, K. Mihlbachler and S. Broyles, visited Pardubice, where Prof. Guiochon obtained his *honoris causa doctor* degree.

The adsorption isotherms of the components of a mixture is necessary for a better understanding of the separation process and for the optimization of the production rate and costs in preparative high-performance liquid chromato-graphy (HPLC). A two-component quadratic isotherm describes the effects of the solvents on the overloaded separations in normal-phase solvent systems containing 2-propanol in *n*-hexane and of dichloromethane in *n*-heptane. The numerically calculated band profiles agreed with the experiment [171]. A new type of the competitive isotherm taking into account possible association of solute on its own, the already adsorbed molecules and on the adsorbed molecules of strong solvent improves the fit to the experimental distribution data and enables description of the distribution data in various overloaded LC systems [172–174].

We investigated the effects of various parameters on the production rate and on the recovery yield in overloaded reversed-phase gradient-elution chromatography on the band profiles of binary mixtures of phenol and o-cresol, using the experimental parameters of the distribution isotherms determined by binary frontal analysis. In the numerical calculations, we used a proprietary program based on the semi-empirical distribution model, employing the Rouchon algorithm [175]. The calculated band profiles of the sample components in overloaded reversed-phase chromatography allowed us the determination of the fraction range, recovery yield and production rate necessary to obtain the sample components in the required purity with various loading factors and concentration ratios of sample components in dependence on the concentration of methanol in the mobile phase. There is a composition of the mobile phase, at which the optimum production rate can be achieved [176].

Gradient elution may be helpful in controlling the fraction range, recovery yield and production rate necessary to obtain the required purity of the sample components. If the sample feed in a weak solvent is to be used, combined effects of on-column enrichment, frontal chromatography and sharpening of the later eluted bands may enhance the production rate and the recovery by narrowing the overloaded band profiles. Steep continuous gradients provide higher recovery yields than those obtainable by the isocratic elution, as they permit to inject large volumes of dilute feed [177].

A solvent with a higher elution strength than that of the mobile phase used to dissolve poorly soluble samples in preparative chromatography may cause significant deformation of the band profiles on an overloaded column. Even band

splitting may take place, due to a limited solubility of the sample components in the sample solvent [178]. To explain non-expected band profiles of cholesterol, cholesteryl acetate, cholesteryl formate and cholestanon, we measured their adsorption isotherms on different chemically bonded C18 silica columns in acetonitrile-dichloromethane or in acetonitrile-*n*-hexane non-aqueous mobile phases. Based on the experimental results, we worked out a model explaining the physical meaning of the isotherm parameters on the basis of the limited solubility of the compounds studied in the bulk liquid phase, of the two-layer adsorption implementing an association between the adsorbed molecules [179].

We also adjusted the conditions for preparative separation of the enantiomers of the amino acids phenyl glycine and tryptophan and of mandelic acid as the test compounds on a Chirobiotic T column packed with the glycopeptide Teicoplanin covalently bonded to the surface of the silica gel. The concentration of ethanol or methanol in aqueous-organic mobile phases and the respective pH control the isotherm profile and consequently the retention, selectivity, and the saturation capacity. Also, the solubility of the acids affects significantly the production rate of the preparative separations [180].

The band profiles of the less retained L-phenylglycine are symmetrical, but the band profiles of a more strongly retained D-phenylglycine are tailing and the band broadening does not diminish even at very low concentrations of phenylglycine, so that it cannot be attributed to a possible column overload. The analysis of the band profile using the stochastic theory of chromatography suggests us that the broadening is probably due to at least two additional chiral centers of adsorption on the complex structure of the teicoplanin chiral stationary phase [181].

The measurement of adsorption isotherms by frontal analysis on conventional analytical columns requires relatively large quantities of pure compounds, and hence is expensive, especially in the case of pure enantiomers. We found a good agreement between the isotherm coefficients of the model compounds determined on micro-columns and on conventional analytical columns packed with the same material. The consumption of pure compounds needed to determine the experimental profile of the isotherms decreases proportionally to the second power of the column inner diameter, namely 10 times for a micro-bore column (1 mm I.D.) and 100 times for capillary columns (0.32 mm I.D.) with respect to a 3.3 mm I.D. conventional column. The perturbation technique of the isotherm determination also significantly economizes the consumption of the expensive pure enantiomers [182].

In the last years, the continuous simulated moving bed (SMB) preparative chromatography process emerged as a powerful alternative to the batch elution, providing higher productivities and reduced solvent consumptions. However, the selection of suitable chromatographic systems is more critical in comparison with the single-column preparative chromatography. Finally, we determined the adsorption isotherms of the enantiomers of mandelic acid in five chromatographic systems and compared the achievable productivity rates using SMB chromatography which served as the basis for a numerical analysis of the SMB process [183].

Conclusions

During the past 50 years, the HPLC has evidenced amazing progress from the first attempts at constructing the instrumentation allowing the classical low-pressure liquid chromatography to accelerate the performance working with efficient and selective separation columns at a reasonably high flow rate and pressure until the modern standards are accomplished. Today, we are routinely using Ultra-High-Performance technique (UHPLC) coupled with mass spectrometry, new core-shell, silica-gel or organic-polymer monolithic, capillary and chip column formats, the increased variety of stationary phase chemistry, and two-dimensional HPLC is becoming routine practice, so that HPLC as such represents one of the most useful and most frequently used techniques in modern instrumental analysis. The above-stated advances would not be possible without intense research at the manufacturers, research institutes and universities. The Department of Analytical Chemistry, originally at the Institute of Chemical Technology, now at the Faculty of Chemical Technology of the University of Pardubice, tried to contribute to the efforts in various fields of HPLC. These activities document the number of the PhD (CSc.) dissertations defended at the department in the past half a century, in a period 1969–2018, listed in Table 2. I have supervised many of these young bright scientists and I am very happy that some of them (J. Fischer, P. Česla, L. Češlová, B. Švecová, T. Hájek) still continue in working on the HPLC field at our department on new projects. Especially, Michal Holčapek's HPLC-MS lipidomic analysis team is very successful. I am sure that my younger colleagues will significantly contribute to further development of the HPLC technique. I know that the colleagues at other institutions (Department of Biological Sciences and elsewhere) also very successfully employ HPLC in their research, like some of my colleagues at our department and I really appreciate their work; here, I apologize for not being familiar with the details of their work to discuss it in the present article.

Table 2 PhD theses in the HPLC field defended at the Department of Analytical Chemistry until 2018

Name	Year	Topic (supervisor)
Pavel Jandera	1977	Gradient elution, instrumentation (prof. Churáček)
Marie Janderová	1977	Mobile phase (prof. Churáček)
Ladislav Svoboda	1980	Gradient elution, SPE (prof. Churáček)
Jaromír Kubát	1985	SPE-HPLC (prof. Churáček)
Josef Urbánek	1988	HPLC of surfactants (prof. Churáček)
Jan Fischer	1993	Phase systems, micellar HPLC (prof. Jandera)
Bořivoj Prokeš	1993	Gradient elution, environmental analysis (prof. Jandera)
Michal Holčapek	1998	HPLC/MS of surfactants and oils (prof. Jandera)

Table 2 PhD theses in the HPLC field defended at the Department of Analytical Chemistry until 2018 (continued)

Name	Year	Topic (supervisor)
Simona Bunčeková	2001	Microcolumn HPLC (prof. Jandera)
Václav Staněk	2001	HPLC and CZE of dyes (prof. Jandera)
Michal Škavrada	2003	Overloaded preparative LC (prof. Jandera)
David Komers	2003	Overloaded preparative LC (prof. Jandera)
Lenka Kolářová (Česlová)	2004	HPLC of oligomers and surfactants (prof. Jandera)
Lucie Grynová (Řehová)	2005	HPLC with coulometric detection (prof. Jandera)
Kateřina Novotná (Vyňuchalová)	2006	Column characterization, optimization (prof. Jandera)
Petr Česla	2007	Optimization of CE and HPLC, 2D HPLC (prof. Jandera)
Michal Halama	2007	Overloaded preparative LC (prof. Jandera)
Dana Moravcová	2007	Monolithic capillary columns (prof. Jandera)
Jiří Urban	2007	Monolithic capillary columns (prof. Jandera)
Dana Ansorgová (Vaněrková)	2007	HPLC/MS of dyes (prof. Jandera)
Soňa Řezková	2007	Chromatography in food analysis (assoc. prof. Královský)
Martina Lasáková	2008	Imprinted monolithic columns (prof. Jandera)
Miroslav Lísa	2008	HPLC-MS (prof. Holčapek)
Martina Molíková	2010	Column characterization, liquid crystal phases (prof. Jandera)
Veronika Škeříková	2010	Monolithic columns, 2D HPLC (prof. Jandera)
Tomáš Hájek	2011	2D HPLC, optimization of separation (prof. Jandera)
Zdeňka Kučerová	2011	Gradient HPLC of peptides (prof. Jandera)
Jan Soukup	2012	HILIC chromatography (prof. Jandera)
Blanka Beňová (Švecová)	2013	Chromatography in food analysis (assoc. prof. Královský)
Eva Cífková	2013	Lipidomic HPLC/MS analysis (prof. Holčapek)
Hana Dvořáková	2014	HPLC/MS oil analysis (prof. Holčapek)
Zdeňka Šťastná	2014	Separations of peptides and proteins (prof. Mikšík)
Magda Staňková	2015	Capillary monolithic columns (prof. Jandera)
Petra Drábková	2016	HPLC in assisted reproduction (prof. Kanďár)
Magdaléna Ovčačíková	2016	HPLC/MS of lipids (prof. Holčapek)
Nikola Vaňková	2016	Efficiency, peak capacity (assoc. prof. Fischer, assoc. prof.. Česla)
Petr Janás	2017	HILIC and RPLC of polar compounds (prof. Jandera)
Petra Šilarová (Kalendová)	2018	LC/MS analysis of natural antioxidants (assoc. prof. Česlová)
Soňa Sechovcová (Bencová)	2018	HPLC/ED of sulphur compounds (prof. Ventura)

Acknowledgments

First, I cannot express enough my gratitude to my mentors and friends, who have led me through the early times of my research in chromatography, Prof. Jaroslav Churáček and Prof. Georges Guiochon. Unfortunately, both already passed away. I thank to my PhD and undergraduate students, who did the experimental work overviewed in this article. Further, I appreciate the international co-operation with the partners in the COM-CHROM project, professors Luigi Mondello from Messina, Tyge Greibrokk from Oslo, Peter Schoenmakers from Amsterdam, Lars Blomberg from Karstadt and Didier Thiebaut from Paris. Also, I must thank Prof. Boguslaw Buszewski (Torun), Prof. Attila Felinger (Pecs) and Prof. Georgios Theodoridis (Thessaloniki), as well as the Polish, Italian, Greek, Slovak, and Romanian students coming to work for a shorter or longer time to participate at the HPLC research in Pardubice. Finally, I have to acknowledge the research grants supporting our HPLC activities:

- *U.S.-Czechoslovak Science and Technology Program, No. 94 036 (1994–1997);*
- *KONTAKT-ME 150. (NSF,USA-MSMT,CZ, 1998–2000);*
- *NATO Science Programme 7 Linkage grant LG.971480 (1998–2000);*
- *NATO Collaborative Linkage Grant PST.CLG.979081 (2002–2004);*
- *European Commission COM-CHROM (IHP-RTN-00-2) 2002–2006;*
- *Czech-Greek bilateral project KONTAKT RC_3_16. (2004);*
- *Czech-Greek bilateral project KONTAKT 7-2006-28 (2006–2007);*
- *CEEPUS II project CII-PL-0004-01-0506-PL-130-05/06 (2005–2011);*
- *Czech Science foundation No. GA203/94/1397 (1994–1997), GA203/98/0598 (1998–2000), GA203/01/0238 (2001–2003), GA203/04/0917 (2004–2006), GA203/07/0641 (2007–2011) GA206/12/0398 (2012–2014).*

Last but not least, I thank my younger colleagues, Petr Česla and Tomáš Hájek, for their assistance with arranging the artwork of this article.

References

[1] Tsvet M.S.: On a new category of adsorption phenomena and their application to biochemical analysis. *Trudy Varshavskago obshchestva estestvoispytatelei, Otdelenie Biologii* **14** (1903) 20–39.

[2] Jandera P.: Separation science in the Czech Republic. *Journal of Separation Science* **29** (2006) 465–471.

[3] Horváth C.G., Preiss B.A., Lipsky S.R.: Fast liquid chromatography – an investigation of operating parameters and separation of nucleotides on pellicular ion exchangers. *Analytical Chemistry.* **39** (1967) 1422–1428.

[4] Jandera P., Churáček J.: The flow-through cell for liquid chromatography photometric detector. PV 4098-71, AO 160 771 (1975).

[5] Jandera P., Churáček J: The sample injector head for analytical procedures. PV 4363-71, AO 174 952 (1978).
[6] Jandera P.: The chromatographic column entrance port for increased pressures operations with porous packings. PV 6043-76, AO 186 021 (1980).
[7] Jandera P., Říha V., Churáček J.: The fluorescent adaptor for detection in liquid chromatography. PV 2788-78, AO 201 220 (1983).
[8] Jandera P., Churáček J: The device for exact and reproducible generation of mobile phase gradients in liquid chromatography. PV 8743-79, AO 206 427 (1983).
[9] Jandera P., Churáček J.: Ion exchange chromatography of carboxylic acids. *Journal of Chromatography* **86** (1973) 351–421.
[10] Jandera P., Churáček J.: Ion exchange chromatography of sulphur compounds, phenols, phosphorus compounds and esters of carboxylic acids. *Journal of Chromatography* **86** (1973) 423–449.
[11] Jandera P., Churáček J.: Ion exchange chromatography of nitrogen compounds. *Journal of Chromatography* **98** (1974) 1–54.
[12] Jandera P., Churáček J.: Ion exchange chromatography of aldehydes, ketones, ethers, alcohols, polyols and saccharides. *Journal of Chromatography* **98** (1974) 55–104.
[13] Churáček J., Jandera P.: Separation of the colored derivatives of some organic compounds using liquid chromatography in small-bore columns packed with ion-exchange resins. *Journal of Chromatography* **53** (1970) 69–75.
[14] Mikeš O., Štrop P., Zbrožek J., Čoupek J.: Chromatography of biopolymers and their fragments on ion-exchange derivatives of hydrophobic macroporous synthetic gel Spheron, *Journal of Chromatography* **119** (1976) 339–354.
[15] Jandera P., Churáček J., Čáslavský J., Vojáčková M.: Liquid chromatography on unmodified and modified spheron gels. I. Nucleosides, bases and related compounds. *Chromatographia* **13** (1980) 734–740.
[16] Jandera P., Churáček J., Szabó D.: Liquid chromatography on unmodified and modified spheron gels. II. Barbiturates and sulphonamides. *Chromatographia* **14** (1981) 7–12.
[17] Jandera P., Churáček J., Čáslavský J., Szabó D.: Liquid chromatography on unmodified and modified spheron gels. III. Phenolic compounds. *Chromatographia* **14** (1981) 100–106.
[18] Jandera P.: Comparison of various modes and phase systems for analytical HPLC, in: K. Valko, (Ed) *Separation Methods in Drug Synthesis and Purification*, Elsevier, Amsterdam, 2000, pp. 1–71.
[19] Poole C.F., Poole S.K.: *Chromatography Today.* Elsevier , Amsterdam 1991.
[20] Riedo, F., Kovats E.S.: Adsorption from liquid-mixtures and liquid-chromatography. *Journal of Chromatography* **239** (1982) 1–28.
[21] Snyder L.R.: *Principles of Adsorption Chromatography.* Dekker, New York, (1968).
[22] Snyder L.R.: Column efficiencies in liquid adsorption chromatography – past, present and future. *Journal of Chromatographic Science* **7** (1969) 352–360.

[23] Jandera P., Kučerová M., Holíková J.: Description and prediction of retention in normal-phase high-performance liquid chromatography with binary and ternary mobile phases. *Journal of Chromatography A* **762** (1997) 15–26.
[24] Jandera P.: Normal-phase liquid chromatography, in: Worsfold P., Townshend A., Poole C. (Eds.): *Encyclopedia of Analytical Science*, 2nd Edition, Elsevier, Oxford, 2005, Vol. 5, pp. 142–151.
[25] Halász I., Sebestian I.: New stationary phase for chromatography. *Angewandte Chemie — International Edition* **8** (1969) 453–454.
[26] Kirkland J.J.: Controlled surface porosity supports for high-speed gas and liquid chromatography. *Analytical Chemistry* **41** (1969) 218–220.
[27] Horvath C., Melander W.: Liquid-chromatography with hydrocarbonaceous bonded phases – theory and practice of reversed phase chromatography. *Journal of Chromatographic Science* **15** (1977) 393–404.
[28] Karger B.L., Gant J.R., Hartkopf A., Weiner P.H.: Hydrophobic effects in reversed-phase liquid chromatography. *Journal of Chromatography A* **128** (1976) 65–78.
[29] Vyňuchalová K., Jandera P.: Comparison of a C30 bonded silica column and columns with shorter bonded ligands in reversed-phase LC, *Chromatographia* **79** (2015) 861–871.
[30] Bocian S., Soukup J., Jandera P., Buszewski B.: Thermodynamic study of solvent adsorption on octadecyl modified silica, *Chromatographia* **78** (2015) 21–30.
[31] Krupczyńska K., Buszewski B., Jandera P.: Characterizing HPLC stationary phases. *Analytical Chemistry* **76** (2004) 227A–234A.
[32] Engelhardt H., Löw H., Götzinger W.: Chromatographic characterization of silica-based reversed phases. *Journal of Chromatography* **544** (1991) 371–379.
[33] Jandera P., Bunčeková S., Halama M., Novotná K., Nepraš M.: Naphthalene sulphonic acids – new test compounds for characterization of the columns for reversed-phase chromatography. *Journal of Chromatography A* **1059** (2004) 61–72.
[34] Krupczyńska K., Jandera P., Buszewski B.: Comparison of column properties in reversed-phase chromatography: monolithic, cholesterolic and mixed bonded stationary phases. *Analytica Chimica Acta* **540** (2005) 127–137.
[35] Jandera P., Fischer J.: Chromatographic behaviour in reversed-phase HPLC with micellar and submicellar mobile phases. *Journal of Chromatography A* **728** (1996) 279–298.
[36] Fischer J., Jandera P.: Chromatographic behavior in reversed-phase HPLC with micellar and submicellar mobile phases - Effects of the organic modifier. *Journal of Chromatography B* **681** (1996) 3–19.
[37] Jandera P., Fischer J., Jebavá J., Effenberger H.: Characterization of retention in micellar HPLC, in micellar electrokinetic chromatography and in micellar electrokinetic chromatography with reduced flow. *Journal of Chromato-graphy A* **914** (2001) 233–244.
[38] Jandera P., Fischer J., Jebavá J., Řezáč P.: Comparison of selectivity between HPLC and in micellar electrokinetic chromatography. *Chromatographia* **56** (2002) S-95–S-102.
[39] Jandera P., engelhardt h.: liquid Chromatography Separation of Organic Acid Compounds. *Chromatographia* **13** (1980) 18–23.

[40] Jandera P., Churáček J., Taraba B.: Comparison of retention behavior of aromatic sulfonic acids in reversed-phase systems with mobile phases containing ion-pairing ions and in the systems with solutions of inorganic salts as the mobile phases. *Journal of Chromatography* **262** (1983) 121–140.

[41] Jandera P., Churáček J.: Reversed-phase liquid chromatography of aromatic sulfonic acids and other strongly polar compounds without addition of an ion-pairing counter ion. *Journal of Chromatography* **197** (1980) 181–187.

[42] Alpert A.J.: Hydrophilic-interaction chromatography for the separation of peptides, nucleic-acid and other polar compounds. *Journal of Chromatography* **499** *(*1990) 177–196.

[43] Jandera P.: Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Analytica Chimica Acta* **692 (**2011) 1–25.

[44] Alpert A.J.: Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. *Analytical Chemistry* **80** (2008) 62–76.

[45] Soukup J., Jandera P.: Adsorption of water from aqueous acetonitrile on silica-based stationary phases in aqueous normal-phase liquid chromatography. *Journal of Chromatography A* **2014** (1374) 102–111.

[46] Pesek J.J., Matyska M.T., Boysen R.I., Yang Y.Z., Hearn M.T.W.: Aqueous normal-phase chromatography using silica-hydride-based stationary phases. *Trac-Trends in Analytical Chemistry* **42** (2013) 64–73.

[47] Jandera P., Janás P.: Recent advances in stationary phases and understanding of retention in hydrophilic interaction chromatography. A review. *Analytica Chimica Acta* **967** (2017) 12–32.

[48] McCalley D.V., Neue U.D.: Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography. *Journal of Chromatography A* **1192** (2008) 225–229.

[49] Jandera P., Urbánek J., Prokeš B., Blažková-Brúnová H.: Chromatographic behavior of the oligoethyleneglycol nonylphenyl ether anionic surfactants in normal-phase HPLC. *Journal of Chromatography A* **736** (1996) 131–140.

[50] Jandera P., Prokeš B.: Chromatographic behavior of non-sulfated and sulfated oligoethyleneglycol nonylphenyl ether surfactants in normal phase (HILIC) liquid chromatography systems containing water. *Chromatographia* **42** (1996) 539–546.

[51] Soukup J., Jandera P.: Comparison of nonaqueous hydrophilic interaction chromatography with aqueous normal-phase chromatography on hydrosilated silica-based stationary phases. *Journal of Separation Science* **36** (2013) 2753–2759.

[52] Jandera P.: Optimization of NP and RP systems for analysis of pesticides. Choice of the mode of elution – isocratic and gradient elution. in: Tuzimski T., Sherma J. (Eds.): *HPLC in Pesticide Residue Analysis*, CRC Press, Boca Raton, 2015, pp. 215–242.

[53] Soczewinski E.: Mechanistic molecular model of liquid-solid chromatography – Retention-eluent composition relationships. *Journal of Chromatography* A **965** (2002) 109–116.

[54] Jandera P., Churáček J.: Gradient elution in liquid chromatography I. The influence of the composition of the mobile phase on the capacity ratio (retention volume, band width, resolution) in isocratic elution – theoretical considerations. *Journal of Chromatography* **91** (1974) 207–221.

[55] Jandera P., Colin H., Guiochon G.: Interaction indexes for prediction of retention in reversed-phase liquid-chromatography. *Analytical Chemistry* **54** (1982) 435–441.
[56] Snyder L.R., Glajch J.L.: Solvent strength of multicomponent mobile phases in liquid-solid chromatography – binary-solvent mixtures and solvent localization. *Journal of Chromatography* **214** (1981) 1–19.
[57] Schoenmakers P.J., Billiet H.A.H., Tijssen R., De Galan L.: Gradient selection in reversed-phase liquid chromatography. *Journal of Chromatography* **149** (1978) 519–537.
[58] Jandera P.: Theory of liquid column chromatography in reversed-phase systems, in: J. Churáček (Ed.): *New Trends in Theory and Instrumentation of Selected Analytical Methods*, E. Horwood, Chichester, 1993, pp. 205–236.
[59] Neue, U.D., Kuss, H.-J: Improved reversed-phase gradient retention modeling. *Journal of Chromatography A* **1217** (2010) 3794–3803.
[60] Jandera P., Janderová M., Churáček J.: Gradient elution in liquid chromatography VIII. Selection of the optimum composition of the mobile phase in liquid chromatography under isocratic conditions. *Journal of Chromatography* **148** (1978) 79–97.
[61] Jandera P., Hájek T., Růžičková M.: Retention models on core–shell columns. *Journal of AOAC International* **100** (2017) 1636–1646.
[62] Jandera P., Hájek T., Vyňuchalová K.: Retention and bandwidths prediction in fast gradient liquid chromatography. Part 2 – Core-shell columns. *Journal of Chromatography A* **1337** (2014) 57–66.
[63] Leo A., Hansch C., Elkins D.: Partition coefficients and their uses. *Chemical Reviews* **71** (1971) 525–616.
[64] Tijssen R., Billiet H.A.H., Schoenmakers P.J.: Use of solubility parameter for predicting selectivity and retention in chromatography. *Journal of Chromatography* **122** (1976) 185–203.
[65] Karger B.L. Gant J.R., Hartkopf A., Weiner P.H.: Hydrophobic effects in reversed-phase liquid chromatography. *Journal of Chromatography A* **128** (1976) 65–78.
[66] Horváth C., Melander W., Molnár I.: Solvophobic interactions in liquid chromatography with nonpolar stationary phases. *Journal of Chromatography A* **125** (1976) 129–156.
[67] Martire D.E., Boehm R.E.: Unified theory of retention and selectivity in liquid-chromatography .2. Reversed-phase liquid-chromatography with chemically bonded phases. *The Journal of Physical Chemistry* **87** (1983) 1045–1062.
[68] Jandera P.: Reversed-phase liquid-chromatography of homologous series – a general-method for prediction of retention. *Journal of Chromatography* **314** (1984) 13–36.
[69] Jandera P.: Methods for characterization of selectivity in reversed-phase liquid-chromatography 4. Retention behavior of oligomeric series. *Journal of Chromatography* **449** (1988) 361–389.
[70] Jandera P.: Method for characterization of selectivity in reversed-phase liquid chromatography: I. Derivation of the method and verification of the assumptions. *Journal of Chromatography A* **352** (1986) 91–110.

[71] Jandera P.: Lipophilic and polar indices, in R.M. Smith (Ed):: *Retention and Selectivity in Liquid Chromatography*. Elsevier, Amsterdam, 1995, pp. 269–298.
[72] Colin H., Guiochon G., Jandera P.: Interaction indices and solvent effects in reversed-phase liquid chromatography. *Analytical Chemistry* **55** (1983) 442–446.
[73] Colin H., Guiochon G., Jandera P.: Retention indices in reversed-phase liquid chromatography – calibration of the system. *Chromatographia* **17** (1983) 83–87.
[74] Jandera P.: Characterization of retention and selectivity in reversed-phase liquid chromatography using interaction indices, in R.M. Smith (Ed.): *Retention and Selectivity in Liquid Chromatography*. Elsevier, Amsterdam, 1995, pp. 225–268.
[75] Abraham, M. H., Roses M.: Hydrogen-bonding .38. Effect of solute structure and mobile-phase composition on reversed-phase high-performance liquid-chromatographic capacity factors. *Journal of Physical Organic Chemistry* **7** (1994) 672–684.
[76] Baczek T., Novotná K., Kaliszan R., Jandera P.: Comparative characteristics of HPLC columns based on quantitative structure–retention relationships (QSRR) and hydrophobic-subtraction model. *Journal of Chromatography A* **1075** (2005) 109–115.
[77] Vyňuchalová K., Jandera P.: Selectivity tests of stationary phases for reversed-phase HPLC, *Analytical Letters* **44** (2011) 1640–662.
[78] Janás P., Jandera P., Škeříková V., Urban J.: The effects of mobile phase composition on the retention of phenolic acids, flavonoids and nucleosides on Diol- and Amide-bonded columns. *Journal of Separation Science* **40** (2017) 1434–1448.
[79] Jandera P., Janás P.: Recent advances in stationary phases and understanding of retention in Hydrophilic Interaction Chromatography. A review. *Analytica Chimica Acta* **967** (2017) 12–32.
[80] Ray S., Takafuji M., Ihara H.: Chromatographic evaluation of a newly designed peptide-silica stationary phase in reverse phase liquid chromatography and hydrophilic interaction liquid chromatography: Mixed mode behavior. *Journal of Chromatography A* **1266 (**2012) 3–52.
[81] Zhang K., Liu X.: Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications. *Journal of Pharmaceutical and Biomedical Analysis* **128** (2016) 73–88.
[82] Wu J.Y., Bicker W., Lindner W.: Separation properties of novel and commercial polar stationary phases in hydrophilic interaction and reversed-phase liquid chromatography mode. *Journal of Separation Science* 31 (2008) 1492–1503.
[83] Lammerhofer M., Richter M., Wu J., Nogueira, R., Bicker W., Lindner, W.: Mixed-mode ion-exchangers and their comparative chromatographic characterization in reversed-phase and hydrophilic interaction chromatography elution modes. *Journal of Separation Science* **31** (2008) 2572–2588.
[84] Jandera P., Hájek T.: Utilization of dual retention mechanism on columns with bonded PEG and diol stationary phases for adjusting the separation selectivity of phenolic and flavone natural antioxidants. *Journal of Separation Science* **32** (2009) 3603–3619.
[85] Jandera P., Hájek T., Škeříková V., Soukup J.: Dual hydrophilic interaction-RP retention mechanism on polar columns: Structural correlations and implementation for 2-D separations on a single column. *Journal of Separation Science* **33** (2010) 841–852.

[86] Pesek J.J., Matyska M.T.: Our favorite materials: Silica hydride stationary phases. *Journal of Separation Science* **32** (2009) 3999–4011.
[87] Soukup J., Jandera P.: Comparison of nonaqueous hydrophilic interaction chromatography with aqueous normal-phase chromatography on hydrosilated silica-based stationary phases. *Journal of Separation Science* **36** (2013) 2753–2759.
[88] Soukup J., Jandera P.: Hydrosilated silica-based columns: the effects of mobile phase and temperature on dual HILIC-RP separation mechanism of phenolic acids, *Journal of Chromatography A* **1228** (2012) 125–134.
[89] Jandera P., Staňková M., Škeříková V., Urban J.: Cross-linker effects on the separation efficiency on (poly)methacrylate capillary monolithic columns. Part I. Reversed-phase liquid chromatography *Journal of Chromatography A* **1274** (2013) 97–106.
[90] Staňková M., Jandera P., Urban J., Škeříková V.: Cross-linker effects on the separation efficiency on (poly)methacrylate capillary monolithic columns. Part II. Aqueous normal-phase liquid chromatography. *Journal of Chromatography A* **1289** (2013) 47–57.
[91] Jandera P., Staňková M., Hájek T.: New zwitterionic polymethacrylate monolithic columns for one- and two-dimensional micro-liquid chromatography, *Journal of Separation Science* **36** (2013) 2430–2440.
[92] Staňková M., Jandera P.: Dual retention mechanism in two-dimensional LC separations of barbiturates, sulfonamides, nucleic bases and nucleosides on polymethacrylate zwitterionic monolithic micro-columns, *Chromatographia* **79** (2016) 657–666.
[93] Snyder L.R., Dolan J.W.: *High-performance gradient elution. The practical application of the linear-solvent-strength model*. Wiley, Hoboken 2007.
[94] Jandera P., Blomberg L.G., Lundanes E.: Controlling the retention in capillary LC with solvents, temperature and electric field. *Journal of Separation Science* **27** (2004) 1402–1418.
[95] Jandera P., Churáček J.: *Gradient Elution in Column Liquid Chromatography. Theory and practice*. Elsevier, Amsterdam 1985.
[96] Jandera, P., Churáček, J., Gradient elution in liqud-chromatography. 2. Retention characteristics (Retention volume, band width, resolution, plate number) in solvent-programmed chromatography – theoretical considerations. *Journal of Chromatography* **91** (1974) 223–235.
[97] Jandera P.: Can the theory of gradient liquid chromatography be useful in solving practical problems? *Journal of Chromatography A* **1126** (2006) 195–218.
[98] Jandera P., Churáček J.: Liquid chromatography with programmed composition of the mobile phase. *Advances in Chromatography* **19 (**1981) 125–260.
[99] Jandera P., Churáček J.: Gradient elution in liqud-chromatography. 2. Retention characteristics (Retention volume, band width, resolution, plate number) in solvent-programmed chromatography – theoretical considerations. *Journal of Chromatography* **91** (1974) 223–235.
[100] Jandera P., Churáček J., Svoboda L.: Gradient elution in liquid chromato-graphy: X. Retention characteristics in reversed-phase gradient elution chromatography. *Journal of Chromatography A* **174** (1979) 35–50.

[101] Snyder L.R., Dolan J.W., Gant J.R.: Gradient elution in high-performance liquid-chromatography 1. Theoretical basis for reversed-phase systems. *Journal of Chromatography* **165 (**1979) 3–30.
[102] Schoenmakers P.J., Billiet H.A.H., Tijssen R., Degalan L.: Gradient selection in reverse-phase liquid-chromatography. *Journal of Chromatography* **149 (**1978) 519–537.
[103] Jandera P., Kučerová M.: Prediction of retention in gradient-elution normal-phase high-performance liquid chromatography with binary solvent gradients, *Journal of Chromatography A* **759** (1997) 13–25.
[104] Jandera P., Optimization of gradient elution in normal-phase high-performance liquid chromatography, *Journal of Chromatography A* **797** (1998) 11–22.
[105] Jandera P., Petránek L., Kučerová M.: Characterization and prediction of retention in isocratic and gradient-elution normal-phase high-performance liquid chromatography on polar bonded stationary phases with binary and ternary solvent systems. *Journal of Chromatography A* **791** (1997) 1–19.
[106] Vyňuchalová K., Jandera P.: Possibilities of retention prediction in fast gradient liquid chromatography. Part 1 – Comparison of separation on packed fully porous, nonporous and monolithic columns. *Journal of Chromatography A* **1278** (2013) 37–45.
[107] Jandera P., Hájek T., Vyňuchalová K.: Retention and bandwidths prediction in fast gradient liquid chromatography. Part 2 – Core-shell columns, *Journal of Chromatography A* **1337** (2014) 57–66.
[108] Jandera P., Hájek T.: Possibilities of retention prediction in fast gradient liquid chromatography. Part 3 – Short silica monolithic columns. *Journal of Chromatography A* **1410** (2015) 76–89.
[109] Jandera P., Hájek T., Šromová Z.: Mobile phase effects in reversed-phase and hydrophilic interaction liquid chromatography revisited. *Journal of Chromatography A* **1543** (2018) 48–57.
[110] Molnar I.: Computerized design of separation strategies by reversed-phase liquid chromatography: development of DryLab software. *Journal of Chromatography A* **965** (2002*)* 175–194.
[111] Kormany R., Molnar I., Rieger H.J.: Exploring better column selectivity choices in ultra-high performance liquid chromatography using Quality by Design principles. *Journal of Pharmaceutical and Biomedical Analysis* **80 (**2013) 79–88.
[112] Jandera P., Churáček J., Colin H.: Gradient elution in liquid chromatography. 14. Theory of ternary gradients in reversed-phase liquid chromatography, *Journal of Chromatography* **214** (1981) 35–46.
[113] Jandera P.: Predictive calculation methods for optimization of gradient elution using binary and ternary solvent gradients**.** *Journal of Chromatography* **485** (1989) 113–141.
[114] Soukup J., Janás P., Jandera P.: Gradient elution in aqueous normal-phase liquid chromatography on hydrosilated silica-based stationary phases. *Journal of Chromatography A* **1286** (2013) 111–118.
[115] Jandera P.: Comparison of reversed-phase and normal-phase column liquid chromatographic techniques for the separation of low and high molecular weight compounds. *Journal of Liquid Chromatography & Related Technologies* **25** (2002) 2901–2931.

[116] Fairchild J.N., Horváth K., Guiochon G.: Approaches to comprehensive multidimensional liquid chromatography systems, *Journal of Chromatography A* **1216** (2009) 1363–1371.
[117] Stoll D.R., Li X., Wang X., Carr P.W., Porter S.E.G., Rutan S.C.: Fast, comprehensive two-dimensional liquid chromatography, *Journal of Chromatography A* **1168** (2007) 3–43.
[118] Jandera P.: Comprehensive two-dimensional liquid chromatography-theoretical considerations, a review. *Central European Journal of Chemistry* **10** (2012) 844–875.
[119] Kalili K.M., De Villiers A.: Off-line comprehensive two-dimensional hydrophilic interaction × reversed phase liquid chromatographic analysis of green tea phenolics *Journal of Separation Science* **33** (2010) 853–863.
[120] Guiochon G., Marchetti N., Mriziq K., Shaliker R.A.: Implementation of two-dimensional liquid chromatography. *Journal of Chromatography A* **1189** (2008) 109–168.
[121] Blahová E., Jandera P., Cacciola F., Mondello L.: Two-dimensional and serial column reversed-phase separation of phenolic antioxidants on octadecyl-, polyethyleneglycol- and pentafluorophenyl propyl silica columns, *Journal of Separation Science* **29** (2006) 555–566.
[122] Jandera P., Česla P., Hájek T., Vohralík, G., Vyňuchalová K., Fischer J.: Optimization of separation in two-dimensional HPLC by adjusting phase system selectivity and using programmed elution techniques. *Journal of Chromatography A* **1189** (2008) 207–220.
[123] Jandera P.: Programmed elution in comprehensive two-dimensional liquid chromatography – a review, *Journal of Chromatography A* **125**5 (2012) 112–129.
[124] Jandera, P.: Column selectivity for two-dimensional liquid chromatography. *Journal of Separation Science* **29** (2006) 1763–1783.
[125] Jandera P., Hájek T., Česla P.: Comparison of various second dimension gradient types in comprehensive two-dimensional liquid chromatography. *Journal of Separation Science* **33** (2010) 1382–1397.
[126] Jandera P., Hájek T., Česla P.: Effects of the gradient profile, sample volume and solvent on the separation in very fast gradients, with special attention to the second-dimension gradient in comprehensive two-dimensional liquid chromatography. *Journal of Chromatography A* **1218** (2011) 1995–2006.
[127] Hájek T., Škeříková V., Česla P., Vyňuchalová K., Jandera P.: Multidimensional LC×LC analysis of phenolic and flavone natural antioxidants with UV-electrochemical coulometric and MS detection. *Journal of Separation Science* **31** (2008) 3309–3328.
[128] Stoll D.R., Li, X., Wang X., Carr P.W., Porter S.E.G., Rutan S.C.: Fast, comprehensive two-dimensional liquid chromatography, *Journal of Chromatography A* **1168** (2007) 3–43.
[129] Jandera P.: Stationary phases for hydrophilic interaction chromatography, their characterization and implementation into multidimensional chromatography concepts, *Journal of Separation Science* **31** (2008) 1421–1437.

[130] Jandera P., Hájek T., Staňková M., Vyňuchalová K., Česla P.: Optimization of comprehensive two-dimensional gradient chromatography coupling in-line Hydrophilic Interaction and Reversed Phase Liquid Chromatography, *Journal of Chromatography A* **1268** (2012) 91–101.

[131] Wilson S.R., Jankowski M., Pepaj M., Mihailova A., Boix F., Truyols G.V., Lundanes E., Greibrokk T.: 2D LC separation and determination of bradykinin in rat muscle tissue dialysate with on-line SPE-HILIC-SPE-RP-MS. *Chromatographia* **66** (2007) 469–474.

[132] Kalili K.M., De Villiers A.: Systematic optimization and evaluation of on-line, off-line and stop-flow comprehensive hydrophilic interaction chromatography × reversed phase liquid chromatographic analysis of procyanidins, Part I: Theoretical considerations. *Journal of Chromatography A* **1289** (2013) 58–68.

[133] Cacciola F., Jandera P., Blahová E., Mondello L.: Development of different comprehensive two-dimensional systems for the separation of phenolic antioxidants. *Journal of Separation Science* **29** (2006) 2500–2513.

[134] Cacciola F., Delmonte P., Jaworska K., Dugo P., Mondello L., Rader J.I.: Employing ultra-high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of Stevia rebaudiana extracts, *Journal of Chromatography A* **1218** (2011) 2012–2018.

[135] Jandera P., Hájek T., Staňková M.: Monolithic and core-shell columns in comprehensive two-dimensional liquid chromatography – a review. *Analytical and Bioanalytical Chemistry* **407** (2015) 139–151.

[136] Česla P., Fischer J., Jandera P.: Separation of phenolic acids and flavone natural antioxidants by two-dimensional combined liquid chromatography and micellar electrokinetic capillary chromatography. *Electrophoresis* **31** (2010) 2200–2210.

[137] Hájek T., Jandera P., Staňková M., Česla P.: Automated dual two-dimensional liquid chromatography approach for fast acquisition of three-dimensional data using combinations of zwitterionic polymethacrylate and silica-based monolithic columns, *Journal of Chromatography A* **1446** (2016) 91–102.

[138] Jandera, P.; Hájek, T.; Šromová, Z., Comprehensive two-dimensional monolithic liquid chromatography of polar compounds, *Journal of Separation Science* **42** (2019) 670–677.

[139] Jandera P., Pechová H., Tocksteinová D., Churáček J., Královský J.: Separation and identification of aliphatic amine derivatives with a new fluorescent reagent using high-performance liquid chromatography and thin-layer chromatography. *Chromatographia* **16** (1982) 275–281.

[140] Svoboda L., Jandera P., Churáček J.: Study of the sorption of selected pesticides on nonpolar sorbents. *Collection of Czechoslovak chemical communications* **56** (1991) 317–333.

[141] Jandera P., Ventura K., Hladoniková R., Churáček J.: Comparison of various sorbents for the enrichment of samples of aliphatic amines using solid-phase extraction prior to the determination by HPLC with fluorimetric detection. *Journal of Liquid Chromatography & Related Technologies* **17** (1994) 69–95.

[142] Jandera P.: Theory of liquid column chromatography in reversed phase systems, in: Churáček J. (Ed.): *New trends in theory and instrumentation of selected analytical methods*. E. Horwood, Chichester, 1993, pp. 205–236.

[143] Jandera P., Churáček J., Butzke P., Smrž M.: Liquid chromatography of phenylurea pesticides and related compounds on chemically bonded ion exchange materials. *Journal of Chromatography* **387** (1987) 155–169.
[144] Jandera P., Svoboda L., Kubát J., Schvantner J., Churáček J.: Separation of Phenoxyacid Herbicides Using Liquid Column Chromatography on Chemically Bonded Phases. *Journal of Chromatography* **292** (1984) 71–84.
[145] Svoboda L., Jandera P., Churáček J.: Liquid Chromatography of Carbamate, Urea, Triazine, Phenoxycarboxylic and Organochlorine Pesticides. *Collection of Czechoslovak chemical communications* **54** (1989) 2120–2132.
[146] Jandera P., Fischer J., Prokeš B.: HPLC determination of chlorobenzenes, benzenesulfonyl chlorides and benzenesulfonic acids in industrial waste water. *Chromatographia* **54** (2001) 581–587.
[147] Ansorgová D., Holčapek M., Jandera P.: Ion-pairing high-performance liquid chromatography – mass spectrometry of impurities and reduction products of sulphonated azodyes. *Journal of Separation Science* **26** (2003) 1017–1027.
[148] Sakalis A., Ansorgová D., Holčapek M., Jandera P., Voulgaropoulos A.: Analysis of sulfonated azodyes and their degradation products in aqueous solutions treated with a new electrochemical method. *International Journal of Environmental Analytical Chemistry* **84** (2004) 875–888.
[149] Fischer J., Ventura K., Prokeš B., Jandera P.: Method for determination of plasticizers in industrial exhalates. *Chromatographia* **37** (1993) 47–50.
[150] Adam M., Ventura K., Jandera P., Churáček J.: Combination of solid-phase extraction with supercritical fluid extraction for the determination of contaminants in water. *Journal of Liquid Chromatography & Related Technologies* **23** (2000) 1511–1522.
[151] Manesiotis P., Fitzhenry L., Theodoridis G., Jandera P.: Applications of SPE-MIP in the field of food analysis, in: Pawliszyn J., Mondello L., Dugo P. (Eds.): *Comprehensive sampling and sample preparation*, Volume 4, Elsevier, Academic Press: Oxford 2012, pp 457–471.
[152] Lasáková M., Jandera P.: Molecularly imprinted polymers and their applications in solid phase extraction. *Journal of Separation Science* **32** (2009) 799–812.
[153] Lasáková M., Thiébaut D., Jandera P., Pichon V.: Molecularly imprinted polymer for solid-phase extraction of ephedrine and analogs from human plasma, *Journal of Separation Science* **32** (2009) 1036–1042.
[154] Jandera P., Prokeš B.: Possibilities of enhancing the sensitivity of the determination of UV-absorbing compounds in HPLC. *Journal of Chromatography* **550** (1991) 495–506.
[155] Jandera P., Engelhardt H.: Liquid chromatography separation of organic acid compounds. *Chromatographia* **13** (1980) 18–23.
[156] Jandera P., Churáček J.: Reversed-phase liquid chromatography of aromatic sulfonic acids and of other strongly polar compounds without addition of an ion-pairing counter ion. *Journal of Chromatography* **197** (1980) 181–187.
[157] Jandera P.: Selection of separation conditions for HPLC and HPLC/MS of aromatic sulfonic acids and acid azo dyes. *Journal of Liquid Chromatography & Related Technologies* **30** (2007) 2349–2367.

[158] Vaněrková D., Jandera P., Hrabica J.: Behaviour of sulfonated azodyes in ion-pairing reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **1143** (2007) 112–120.
[159] Holčapek M., Volná K., Jandera P., Kolářová L., Lemr K., Exner M., Církva A.: Effects of ion-pairing agents on the electrospray signal suppression of sulfonated dyes and intermediates. *Journal of Mass Spectrometry* **39** (2004) 43–50.
[160] Jandera P., Fischer J., Staněk V., Kučerová M., Zvoníček P.: Separation of dye intermediates aromatic sulphonic acids by high performance liquid chromatography and by capillary zone electrophoresis. *Journal of Chromatography A* **738** (1996) 201–213.
[161] Fischer J., Jandera P., Staněk V.: Effects of the working electrolyte (cyclo-dextrin type and pH) on the separation of aromatic sulphonic acids by capillary zone electrophoresis. *Journal of Chromatography A* **772** (1997) 385–396.
[162] Staněk V., Jandera P., Claessens H.: Effect of the modified cyclodextrin additives on the separation of anionic dye intermediates by capillary electrophoresis. *Journal of Chromatography A* **948** (2002) 235–247.
[163] Jandera P., Urbánek J., Prokeš B., Blažková-Brúnová H.: Chromatographic behavior of oligoethylene glycol nonylphenyl ether anionic surfactants in normal-phase high performance liquid chromatography. *Journal of Chromatography A* **736** (1996) 131–140.
[164] Česlová L., Jandera P., Česla P.: A study of the thermodynamics of retention of block (co)oligomers using high-performance liquid chromatography/mass spectrometry, *Journal of Chromatography A* **1247** (2012) 89–98.
[165] Jandera P., Fischer J., Lahovská H., Novotná K., Česla P., Kolářová L.: Two-dimensional liquid chromatography normal-phase and reversed-phase separation of (co)oligomers. *Journal of Chromatography A* **1119** (2006) 3–10.
[166] Holčapek M., Jandera P., Zderadička P., Hrubá L.: Characterization of triacylglycerol and diacylglycerol composition of plant oils using High-performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography A* **1010** (2003) 195–215.
[167] Holčapek M., Lísa M., Jandera P., Kabátová N.: Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. *Journal of Separation Science* **28** (2005) 1315–1333.
[168] Holčapek M., Jandera P., Fischer J., Prokeš B.: Analytical monitoring of the production of biodiesel by high-performance liquid chromatography with various detection methods. *Journal of Chromatography A* **858** (1999) 13–31.
[169] Mondello L., Tranchida P., Staněk V., Jandera P., Dugo G., Dugo P.: Silver-ion reversed-phase comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection in lipidic food analysis. *Journal of Chromatography A* **1086** (2005) 91–98.
[170] Dugo P., Škeříková V., Kumm T., Trozzi A., Jandera P., Mondello L.: Elucidation of carotenoid patterns in citrus products by means of comprehensive normal-phase × reversed-phase liquid chromatography. *Analytical Chemistry* **78** (2006) 7743–7750.

[171] Jandera P., Komers D., Anděl L., Prokeš L.: Fitting competitive adsorption isotherms to the distribution in normal-phase systems with binary mobile phases. *Journal of Chromatography A* **831** (1999) 131–148.
[172] Jandera P., Škavrada M., Anděl L., Komers D., Guiochon G.: Description of adsorption equilibria in liquid-chromatography systems with binary mobile phases. *Journal of Chromatography A* **908** (2001) 3–17.
[173] Jandera P., Pošvec Z., Vraspír P.: Mobile phase effects on single-component and competitive adsorption isotherms in reversed-phase systems. *Journal of Chromatography A* **734** (1995) 125–136.
[174] Jandera P., Komers D.: Fitting competitive adsorption isotherms to the experimental distribution data in reversed-phase systems. *Journal of Chromatography A* **762** (1997) 3–13.
[175] Rouchon P., Schonauer M., Valentin P., Guiochon G.: Numerical simulation of band propagation in nonlinear chromatography. *Separation Science and Technology* **22** (1987) 1793–1833.
[176] Jandera P., Komers D., Guiochon G.: Effects of the composition of the mobile phase on the production rate in reversed phase overloaded chromatography. *Journal of Chromatography A* **787** (1997) 13–25.
[177] Jandera P., Komers D., Guiochon G.: Effects of the gradient profile on the production rate in reversed-phase gradient elution overloaded chromatography. *Journal of Chromatography A* **760** (1997) 25–39.
[178] Jandera P., Guiochon G.: Effect of the sample solvent on band profiles in preparative liquid chromatography using nonaqueous reversed-phase HPLC. *Journal of Chromatography* **588** (1991) 1–14.
[179] Jandera P., Guiochon G.: Adsorption isotherms of cholesterol and related compounds in nonaqueous reversed-phase chromatographic systems. *Journal of Chromatography* **605** (1992) 1–17.
[180] Jandera P., Škavrada M., Klemmová K., Bačkovská V., Guiochon G.: Effect of the mobile phase on the retention behaviour of optical isomers of carboxylic acids and amino acids in liquid chromatography on bonded Teicoplanin columns. *Journal of Chromatography A* **917** (2001) 123–133.
[181] Jandera P., Bačkovská V., Felinger A.: Application of stochastic theory of chromatography on the characterization of the retention mechanism of underivatized amino acids on a bonded Teicoplanin column. *Journal of Chromatography A* **919** (2001) 67–77.
[182] Jandera P., Bunčeková S., Mihlbachler K., Guiochon G., Bačkovská V., Planeta J.: Fitting adsorption isotherms to the distribution data determined using packed micro columns for HPLC. *Journal of Chromatography A* **925** (2001) 19–29.
[183] Kaspereit M., Jandera P., Škavrada M. Seidel-Morgenstern A.: Impact of adsorption isotherm parameters on the performance of enantioseparation using simulated moving bed chromatography. *Journal of Chromatography A* **944** (2002) 249–262.