Liquid Chromatography: Theory and Methodology

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Liquid Chromatography: Theory and Methodology

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Review Contents

This review covers fundamental developments in liquid chromatography during the period of approximately October 1995 through approximately October 1997. As with the past four issues of the Fundamental Reviews, there are separate reviews on instrumentation and on size exclusion chromatography; this review is of important developments in the *chemistry* of the separation process. The primary searching method for this work has been *CAS Online*, and each author has supplemented these with search methods of their own. There has been one small change in the organization of this review; due to greatly increased interest, we have added a section on *electrochromatography*, where the mobile

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phase is driven through a packed column by electroosmotic flow, rather than by a pressure drop. This method has the possibility of greatly increasing peak capacity in LC separations, but there is much work left before it becomes a routine tool.

This is not meant to be a comprehensive review of all published papers during this time period; rather, we have tried to select those papers which we feel are significant developments. We have largely restricted the covered material to the English language literature. Comments and suggestions concerning this review are welcomed and encouraged and should be sent to the first author (J.G.D., e-mail, Dorsey@chem.fsu.edu). As readers are all aware, it becomes more and more difficult for any one person to stay in touch with the literature. Even with electronic search methods, some key papers are likely to be missed. To those authors whose work is omitted, we apologize.

As a personal note, I have now led this review for five issues, which spans the entire decade of the 1990s. This will be my last review; it is time for new ideas and new energy. I thank you all for your comments, suggestions, and support during this tenure.

BOOKS, REVIEWS, AND SYMPOSIA PROCEEDINGS

First, the previous Fundamental Review covered work occurring approximately from October 1993 through October 1995 and was published during this review period (*A1*). Many other specialized reviews were published, and they will be cited during the respective sections of this review.

Majors (*A2*) continued his useful surveys of column usage and noted that reversed-phase columns account for 50% of new purchases. Bidlingmeyer also discussed trends in column usage and development (*A3*). It is perhaps only a coincidence that both authors work for major column manufacturers!

Several general, useful books were published during this review period. Snyder, Kirkland, and Glajch (*A4*) published the second edition of their highly popular book, *Practical HPLC Method Development*, and this is perhaps the most complete source of information on LC practice for the chromatography user. Neue (*A5*) published a useful book on *HPLC Columns: Theory, Technology, and Practice*. This book is more academically oriented, but is still a highly useful resource for the practicing chromatographer. Sadek (*A6*) edited a unique book, *The HPLC Solvent Guide*, which is quite likely the most complete volume of information ever assembled on solvents and should find shelf space in virtually every LC laboratory.

Other books published during this period include *HPLC and CE: Principle and Practice* by Weston and Brown (*A7*) and three edited, assembled texts, *High Performance Liquid Chromatography: Fundamental Principles and Practice* (*A8*), *High Performance Liquid Chromatography: Principles and Methods in Biotechnology* (*A9*), and *HPLC: Practical and Industrial Applications* (*A10*). Several other more specialized texts were also published, and where appropriate, they are referenced in the individual sections later in this review.

Many symposia proceedings were published also, as is customary for many of the yearly chromatography meetings, and again, individual papers from these are referenced in the individual sections. It has become much more common for these proceedings to be published as part of the open literature in journals such as *Journal of Chromatography*, and it is not necessary to duplicate their references here.

THEORY AND OPTIMIZATION

Reviews. With the exception of the reviews cited below, we have excluded nearly all theoretical contributions that can logically be placed in another category elsewhere in this review (e.g., reversed phase, optical and positional isomers, preparative, etc.), especially those contributions pertaining to chromatographic properties (efficiency, retention, selectivity).

Lochmuller et al. reviewed the advances in HPLC for the separation of polymers and oligomers. Retention mechanisms, the dynamics of the partition chromatography process, evidence for significant delays in polymer equilibration to mobile-phase conditions, and effect of the temperature dependence of polymer solubility were discussed (*B1*). Conventional chromatographic behavior was verified for the separation of polystyrene, poly(methyl methacrylate), and poly(ethylene oxide) samples. Fabre defined and described the role of robustness testing in liquid chromatography and capillary electrophoresis. Although screening designs may be sufficient to set method limits, response surface designs are more important in method transfer because they give a more comprehensive picture of the method (*B2*). Ooms discussed the ever-increasing role of temperature control in liquid chromatography (*B3*). Many new specialty columns and chemistries require accurate control at sub- or superambient temperatures, and conventional RPLC columns can sometimes show favorable changes in retention or selectivity at temperatures beyond traditional limits.

Davydov reviewed the determination of adsorption isotherms from solution and their use in chromatographic optimization; as well as the effects of intermolecular interactions in mixture on individual component adsorption isotherms (*B4*). Muldoon and Stanker described the recent development and application of molecular imprinting polymer (MIP) technology for residue analysis, reviewing the process of MIP synthesis, the basis for analyte-MIP recognition, and current applications of this technology for residue analysis (*B5*). Pirogov et al. reviewed the simplex algorithm as a tool for finding the optimal analytical conditions and systematically discussed the advantages and disadvantages. The mathematical algorithm was described, its known modifications were compared, and examples were given of the practical optimization of detergent conditions in ion chromatography (*B6*).

Theory. Kanazawa et al. proposed a useful new chromatography concept in which the surface properties and resulting function of the HPLC stationary phase are controlled by external temperature. This method should be ideal for separations of biological and biomedical peptides and proteins using only aqueous mobile phases (*B1*). The influence of column temperature on the retention of steroids on the copolymer-modified stationary phases was more significant than in the case of homopolymer-modified columns (*B8*).

Two research groups examined the effects of pressure on retention and selectivity. Ringo and Evans examined the role of pressure in separations where the primary mechanism for solute retention is inclusion complexation (*B9*). Using the positional isomers of nitrophenol as model solutes and β-CD as the stationary phase, pressure-induced decreases in solute retention factors

ranging from −2 to −35% were observed experimentally for pressures from 40 to 340 bar. For this phase it appears that the pressure dependence of solute retention is due primarily to the pressure-induced dissociation of the cyclodextrin−solute complex (*B9*). Using a warfarin/β-CD system, they were able to measure pressure-induced perturbations in complexation equilibria to determine the change in partial molar volume upon complexation (*B10*). McGuffin and Chen also measured a pressure dependence on retention with a homologous series of derivatized fatty acids. They compared their results to a thermodynamic model derived from regular solution theory, which suggested that state effects alone are not sufficient to describe the measured change in solute retention and that variations in interaction energy with density must also be considered. By using an extended van der Waals relation that better describes polar fluids, good agreement was observed (*B11*).

Foley and Ahuja published without derivation a unifying theory of separation that is valid for both neutral and charge analytes (*B12*). A "master resolution equation (MRE)" that is universally applicable to HPLC, capillary zone electrophoresis, capillary electrochromatography, and micellar electrokinetic chromatography was described; the MRE reduces to equations previously derived by others for each of the specific separation modes when the appropriate constraints are employed. Thede et al. derived analytical peak shape equations for first-order reversible reactions occurring in a chromatography reactor by treating the reversible reactions as consecutive reactions with alternating products. For small to medium conversions, the correspondence between these equations and a numerical solution of the partial differential equation was sufficiently close that the latter could be substituted in fitting procedures for the determination of rate constants (*B13*).

Li and Carr reported the accuracy of various approximate empirical methods for estimating the diffusion coefficients of alkylbenzenes and alkylphenones in acetonitrile (ACN)/water and methanol (MeOH)/water solvent systems and then developed a novel empirical modification of the Wilke−Chang equation in order to more accurately estimate these solute diffusion coefficients (*B14*). The errors of the proposed correlations were no greater than 10% for both ACN/water and MeOH/water systems, which represented a 2−3-fold better acuracy than those of the other two correlations. The authors recommended the use of this modified Wilke−Chang equation with the above homologous series of solutes for the evaluation of column performance in RPLC (*B14*).

Cantwell et al. published three papers concerned with intraparticle sorption rate and liquid chromatographic band broadening in porous polymer packings. In the first paper, they proposed a method by which an experimentally measured sorption−rate curve for a solute on a chromatography sorbent may be used to accurately predict the plate height and peak shape contributions of intraparticle sorption (*B15*). In the second paper, they focused exclusively on macroporous poly(styrene−divinylbenzene) sorbents (e.g., PRP-1) and found that slow intraparticle processes are the major cause of band broadening of naphthalene on PRP-1 (*B16*). In their third paper, they reported that the cause of slow intraparticle mass-transfer rates is the slow rate of diffusion into the polymer matrix (*B17*). Farnan et al. investigated both theoretically and experimentally the effect of intraparticle mass-transfer resistances on the peak shape at flow velocities currently used in high-speed protein chromatography. The peak asymmetry of the protein bands was quantified by the difference between the first statistical moment and the retention volume of the peak apex rather than the peak skewness because the latter was much more difficult to determine. A general method for the evaluation of the mass-transfer characteristics of a given chromatography sorbent from the variation in peak asymmetry with reduced velocity was then introduced (*B18*).

Metting and Coenegracht found that the predictive results of a well-designed neural network were better or comparable to those obtained with linear and nonlinear regression models (*B19*). To predict and understand the profiles of large-size system peaks and vacancy bands in liquid chromatography, Guiochon et al. derived an analytical solution for the ideal model of chromatography. This solution provided a more profound explanation of the formation of the large system peaks than previous studies (*B20*). Later they used an equilibrium-dispersive model to calculate band profiles from the experimentally measured column efficiency and isotherm data (*B21*). Guan-Sajonz and Guiochon showed that the pressure used to pack a column can have an dramatic effect on the performance characteristics of the column. As the packing pressure was increased, the mass of packing material inside each column increased. The column external porosity, determined by an inverse size exclusion chromatography method, decreased as the packing pressure increased, while the internal porosity remained constant. Interestingly, the retention factor and the column efficiency of each compound increased linearly with increasing packing (*B22*).

Trathnigg et al. studied the chromatographic behavior of low-molecular-weight poly(ethylene glycol) (PEG) and its mono- and dimethyl ethers in the isocratic mode of reversed-phase liquid absorption chromatography on a C18-modified silica column (*B23*). Experimental data were interpreted according to simple molecular statistical theory based on the model of a rigid-rod molecule, which is capable of being adsorbed inside the homogeneous layers near the pore walls. This approach allowed the determination of the free energies of transfer of the repeating unit (oxyethylene group) and the two types of end groups (methoxy and hydroxy groups) from a mixed eluents into a bonded C18 phase. The effective thickness of the bonded layer was estimated as well as the critical point of adsorption for poly(oxyethylene), which is of importance for separation of macromolecules by using the crititical-condition mode of liquid chromatography (*B23*).

Chen and Horvath compared both theoretically and experimentally two modes of on-column UV absorption detection with packed capillaries in liquid chromatography. The efficiency of the chromatography system was the same using either detection mode, and the two modes of on-column detection gave equally reproducible results in both qualitative and quantitative analyses. The sensitivity of detection in the second mode, however, was $\epsilon 2T(1 + k')$ times that observed in the first mode for a peak having a retention factor of k' in isocratic elution under otherwise identical conditions (*B24*). Vailaya and Horvath used a simplified version of the solvophobic theory to reexamine a large set of retention data with nonpolar and weakly polar eluites in reversed-phase chromatography (RPC) to test certain predictions by the theory and to clarify further the roles of the mobile and the stationary

phases in the retention process. The results confirmed the dominant role of the mobile phase in governing selectivity changes in RPC of nonpolar eluites (*B25*).

Kantoci developed an empirical equation to evaluate chromatography separation efficiency in terms of the product of a "separation" term, a "capacity" term, and an "alignment between peaks" term. This equation is insensitive to other column or separation parameters; its sensitivity depends only upon the resolution between peaks, which allows the linking of the equation with any column or separation parameter during the optimization process as a response function (*B26*). Miyabe and Takeuchi proposed a nondimensional plate height equation derived from the theory of statistical moments for the kinetic analysis of peak spreading in liquid chromatography (*B27*).

Optimization. Zhu et al. published a series of papers on the combined use of temperature and solvent strength in reversed-phase gradient elution. The first paper showed that four initial experiments (two different gradient times, two different temperatures) yielded sufficient data to allow predictions of separation as a function of temperature as well as gradient and column conditions (*B28*). In the second paper, they discussed selectivity changes as a function of T and b (gradient steepness) and a simple treatment that allows changes in selectivity to be compared quantitatively for different samples and HPLC conditions. Data for a wide range of compound types and conditions are provided in support of the assumption that gradient steepness selectivity (measured by the parameter S) does not change significantly with temperature (*B29*). In the third study, the authors studied the ability to change band spacing via temperature and gradient steepness for several ionizable compounds that included 8 substituted benzoic acids, 9 substituted anilines, 22 basic drugs, 9 structurally related herbicide impurities, 7 chlorophylls, and 72 peptides and proteins (*B30*). In the final study, Zhu et al. examined the selectivity for numerous neutral (nonionized) samples as a function of sample type and other separation conditions. Whereas nonpolar compounds showed only modest changes in band spacing with an increase in temperature and gradient steepness, the polar neutral compounds exhibited relatively large changes in α when temperature and/or gradient steepness was varied; these changes, however, are nevertheless small compared to those observed for ionogenic solutes (*B31*).

de Aguiar et al. discussed and evaluated various criteria for the selection of a rugged optimum in HPLC optimization. The optimal conditions selected by the criteria are Pareto-optimal and in agreement with the ones selected when applying Derringer's desirability function (*B32*). Dzido and Sory used DrylabG software to optimize the RPLC separation of some nucleosides; a good agreement between simulated and experimental chromatograms was observed (*B33*). Vanbel et al. discussed criteria for optimizing the separation of only specified target analytes in a complex sample via the adaptation of two of the most widely used optimization criteria, the minimum resolution and the calibrated normalized resolution product. The case of nonideal separation (featuring asymmetric peaks and/or peaks of vastly different areas) was also studied (*B34*). Gau et al. employed a full factorial design and computer simulation to investigate the effects of the fractions of MeCN and MeOH and the concentration of phosphate buffer in the mobile phase on the retention and separation of nine cardiovascular drugs in an isocratic HPLC system (*B25*).

Heinisch and Rocca made two points with respect to optimization. First, when samples contain solutes at very different concentrations, the use of the classical resolution function to describe the quality of the separation does not provide a true measure of peak overlap and is therefore unsuitable. Second, the validity of an optimum is highly dependent on the accuracy of the predicted retention surfaces; improvements in accuracy may be obtained by restricting the parameter space in which optimum performance is sought (*B36*). Bylund et al. introduced a new chromatographic response function (CRF) that accounts for both quality of separation and retention time for the optimization of the chiral separation of (*R*,*S*)-oxybutynin chloride. The capabilities of three different multivariate techniques (multilayer feed-forward neural networks, multiple linear regression, partial least-squares regression) to model the new CRF and predict its value for new experiments were compared, with the most accuracy achieved by the neural nets (*B37*).

Heinisch et al. utilized Osiris optimization software to optimize separations with nonaqueous or partially aqueous mobile phases, consisting of two, three, or four solvents. Similar to Drylab software, two gradient runs are carried out for each binary mobile phase studied. These gradient runs allow selection of the best composition space for the optimum composition search without requiring additional runs (*B38*). Cela and Lores published a series of papers about the off-line optimization of binary gradient separations using the simulation program PREOPT-W. Part one introduced most of the program's capabilities and characteristics (*B39*), part two was devoted to data management (*B40*), and parts III and IV explored the optimization of isocratic (*B41*) and binary gradient (*B42*) separations. Outinen et al. compared three computer-assisted optimization methods—DryLab, EluEx, and PRISMA—for the optimized separation of 13 biogenic amines that were difficult to separate (*B43*). Galushko reported yet another computer-assisted optimization software program, ChromSword. ChromSword predicted starting conditions for RPLC from structural formulas, without any initial experments, and it supports the optimization of the concentration of the organic fluid media, their pH, temperature, or gradient profile.

Luo and Hsu utilized a two-stage strategy for the optimization of gradient profiles in ion-exchange chromatography for protein purification. In the first stage, rate parameters and gradient correlations were determined according to experimental data from isocratic elution runs. In the second part, they developed the optimization strategy of gradient elution chromatography based on the resolution optimization factor (*B45*). Lukulay and McGuffin compared the optimized separations of corticosteroids obtained by solvent modulation and with premixed mobile phases. The solvent modulation approach compared favorably to premixed solvents in terms of accuracy, total analysis time, critical resolution, and overall quality of the separation; it thus appears to be a very promising optimization strategy (*B46*). Later they presented the concept and theory of parametric modulation, as well as a strategy for the (multivariate) optimization of mobile-phase composition and temperature (*B47*).

Parrilla et al. applied three multivariate calibration methods—partial least squares (PLS-1 and PLS-2), principal component

regression (PCR), and full spectrum calibration—to the simultaneous determination of the three pesticides folpet, procymidone, and triazophos in mixtures by HPLC with photodiode array detection. The effects of several preprocessing techniques are discussed in order to optimize the calibration matrixes by the PLS and PCR methods (*B48*). Osborne and Miyakawa useda fractional factorial design involving 11 separate experiments to generate the required data for the optimization of the pH, percentage of organic solvent and concentration of cyclodextrin in the mobile phase and stationary phase (*B49*). Guillaume et al. employed chemometric methodology to study column efficiency and the separation of 10 benzodiazepines in RPLC. A new response function, which takes into account the separation quality and the analysis time, was proposed for the separation optimization (*B50*). Harrington et al. utilized a two-level factorial design to optimize an ion-pairing-based RPLC method for the separation and of mono-, di-, and tricarboxylic acids (*B51*). Lukulay and McGuffin summarized the rational evolution of optimization methods, from univariate to multivariate, together with selected examples of their application. A novel approach, parametric modulation, is then proposed which overcomes many limitations of the present optimization methods (*B52*). Using the "window diagram" technique of Laub and Purnell, Zivanovic et al. optimized the separation and determination of amiloride and methyclothiazide in tablets via the pH and methanol-to-water ratio in the mobile phase (*B53*).

Zarzycki et al. studied the influence of temperature on the HPLC separation of steroids (*B54*) and estrogenic steroids (*B55*). The plots of capacity factors vs reciprocal of absolute temperature are nonlinear in every case when mobile phase modified with β-cyclodextrin was used. Particularly strong nonlinearity was observed at lower temperature and at high β-cyclodextrin concentration. The complex chromatograms were evaluated using optimization parameters such as capacity factor of the last-eluted peak (k'_{max}), the smallest resolution between adjacent peaks (R_s; min), and relative resolution product (r). The results presented describe precisely the role of temperature in HPLC systems in which mobile phases modified with cyclodextrin were used.

Synder et al. examined the relative value of different variables for controlling selectivity and recommended changes in temperature and gradient steepness (*B56*). Snyder and Dolan showed that a single gradient elution run with acetonitrile (B)/water (A) as mobile phase can be used to estimate preferred conditions for subsequent method development experiments in RPLC. For a broad range of sample types that included both very hydrophilic and very hydrophobic compounds, it was found that isocratic retention is given by $\log k \approx \log k_w - 4.2\phi_v$, where $\phi_v = 0.01\%$ B. The initial gradient run allows $\log k_w$ to be estimated for each compound (*B57*). Lewis et al. then discussed the preferred approach and conditions for isocratic separations in RPLC, in which initial method development experiments for both neutral and ionic samples are carried out with acetonitrile/methanol/buffer mobile phases (*B58*).

Torres-Cartas et al. used a five-parameter retention model and systematic experimental design to optimize the separation of several steroids using a mobile phase containing sodium dodecyl sulfate (SDS) and acetonitrile (*B59*). Rapado-Martinez et al. utilized an interpretive optimization procedure to optimize the micellar mobile phase for the separation and analysis of pharmaceuticals containing β-blockers and other antihypertensive drugs. Whereas one MLC mobile phase (0.15 M SDS, 7% propanol in 0.01 M phosphate buffer at pH 3) was needed for all samples of interest, two different aqueous/organic mobile phases were needed to make the same analyses (*B60*).

DATA ANALYSIS

Reviews. Cserhati and Forgacs provided a practical and thorough tutorial review (118 references) of the use of multivariate mathematical methods for the evaluation of retention data matrixes (*C1*). Sharaf provided a thorough assessment of chromatographic peak purity via single- and multichannel data, including operational and statistical considerations of peak purity detection strategies (*C2*). Swartz and Brown reviewed their use of mathematically enhanced UV/visible absorbance spectral analysis and spectral contrast software techniques in HPLC and MEKC as an aid for the determination of peak homogeneity, identification, and tracking during method development. Cis/trans isomers of doxepin and the diastereomers ephedrine and pseudoephedrine could be distinguished in HPLC (*C3*).

Boque and Rius critically reviewed the state-of-the-art theoretical approaches for the detection limit in multichannel detector systems, discussing the hypotheses and theoretical backgrounds on which they are based and the advantages and limitations of the different techniques and derived estimators (*C4*). Jungbauer described recent insights into the chromatography of proteins provided by mathematical modeling, focusing on intraparticle transport and interactions with the sorbent surface; new theories for intraparticle convection and diffusion were developed (*C5*).

General Papers. Slonecker et al. utilized a qualitative informational similarity technique to describe the orthogonality of projected two-dimensional (2-D) chromatographic separations of complex mixtures from results obtained via 1-D separations. The identification of orthogonal separation modes is useful for the optimization of 2-D separations as well as for choosing a second separation technique for confirmation of separation or peak purity (*C6*). Grainger and co-workers described the identification of chlorinated dibenzo-*p*-dioxins isomers by up to three orthogonal spectroscopic and three orthogonal chromatographic techniques; this integrated approach minimized the probability of isomer misidentification (*C7*). Messick et al. developed practical mathematical tools that can be used to create several figures of merit for *n*th-order instrumentation, i.e., selectivity, net analyte signal, and sensitivity. The performance of a local selectivity measure for second-order instrumentation was tested using simulated second-order data and real second-order data obtained by GC/FT-IR and HPLC/DAD; practical uses of *n*th-order figures of merit were briefly discussed (*C8*). Hayashi and co-workers employed a recently promulgated uncertainty theory of instrumental analyses to minimize the relative standard deviation of chromatographic measurements. Naphthalene, acenaphthene, pyrene, and perylene in HPLC were used as illustrations (*C9*). Ford and Ko compared various methods for extracting linear solvent strength (LSS) gradient parameters from gradient chromatographic data. When realistic experimental uncertainties were incorporated into synthetic retention data sets, the LSS parameters were not recovered accurately unless special precautions were taken. For large molecules, the error in determining the solvent strength parameter

was less than 13%, which is comparable to or better than those for the other methods evaluated (*C10*). Finally, Torres-Lapasio et al. investigated the program MICHROM for the general global treatment of chromatography data, including the determination of dead time, smoothing of chromatograms, measurement of peak parameters, fitting of skewed peaks, deconvolution of overlapped peaks, experimental design optimization of the mobile-phase composition, simulation of chromatograms in several experimental conditions, and graphical representation of chromatograms, resolution surfaces, and contour maps (*C11*).

Peak Purity/Deconvolution. Several studies used chemometric approaches in the qualitative and quantitative analysis of overlapping peaks. Garrido Frenich and co-workers applied three multivariate calibration methods, partial least-squares (PLS-1 and PLS-2) and principal component regression to the simultaneous determination of cypermethrin, fenvalerate, and *cis*- and *trans*-permethrin by HPLC, and discussed several preprocessing algorithms for data pretreatment; mean centering was found to be advantageous (*C12*). Gargallo et al. described the validation of alternating least-squares multivariate curve resolution for the resolution and quantitation of mixtures of the coeluting compounds hydrocortisone and prednisone (impurity) in HPLC with diode array detection. Using initial estimates provided by evolving factor analysis and SIMPLISMA methods, quantitation errors were less than 1.5% for hydrocortisone for resolution values of 0.4 or above, and prednisone concentrations in the range 0.5−20% (*C13*). Chen and Rutan used an adaptive Kalman filter to identify and quantify both major and minor overlapped peaks (chrysene and benz[*a*]anthracene, benzo[*k*]fluoranthene, and benzo[*b*]fluoranthene) in HPLC with diode array detection (*C14*). Prediction errors were within 2% for the major components and within 10% for the minor components. If the reference spectrum of the minor component was unavailable, the prediction error for the major components increased to 7%. The method is valid for highly overlapped peaks even when the chromatographic resolution is zero, but an overlap-free region is required in the spectra (*C14*). Ritter and co-workers demonstrated the improved performance in both simulated and actual data sets of window evolving factor analysis for checking peak purity in HPLC with diode array detection when corrections for heteroscedasticity are made. Garrido Frenich et al. showed that multiwavelength detectors offer improved detection capabilities for HPLC methods, but require improvements in data analysis methodology to fully utilize the available information (*C15*). Tauler illustrated the application of multivariate curve resolution to three types of two-dimensional data (synchronized, nonsynchronized, complex) obtained via HPLC with diode array detection (*C17*). Martinez Galera and co-workers compared two kinds of partial least-squares models and a principal component regression model to the simultaneous determination of iprodione, procymidone, chlorothalonil, folpet, and triazophos by HPLC with diode array detection.

de Juan and co-workers applied a "needle algorithm", based on a uniqueness test in target factor analysis, to several simulated HPLC-diode array detection data sets in both the chromatography and spectral directions to obtain information regarding the number of analytes, the presence of minor compounds, the location of peak maximums, and eventually some characteristics of the spectral shapes (*C19*). Sanchez et al. proposed a stepwise, orthogonal projection approach (OPA) applied to peak purity assessment. The performance of OPA for the assessment of peak purity in HPLC-DAD is described and compared with that of SIMPLISMA (*C20*). They then extended the stepwise OPA to determine the number of compounds present in a multicomponent system with an alternating least-squares procedure that facilitated the determination of the chromatographically and spectroscopically pure compound profiles. The performance of the modified OPA algorithm is compared with that of two window-based self-modeling curve resolution approaches: evolving factor analysis and window factor analysis (*C21*). Finally, Malinowski developed an algorithm called automatic window factor analysis (AUTOWFA) to determine, efficiently and automatically, the concentration profiles of the spectroscopically active components present in such evolutionary processes as chemical titrations, chromatography, and kinetics (*C22*).

Solute Retention/Stationary-Phase Characterization. De Beer and co-workers compared the use of half-fraction and full factorial designs, which required fewer experiments, with central composite design for the retention modeling of four compounds, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, phenylephrine, and chlorpheniramine maleate, in ion-pairing RPLC (*C23*). Marengo et al. employed fractional and star experimental designs to investigate the effect of the following five experimental factors on the retention of simazine and atrazine in ion-interaction chromatography: (1) the chain length and (2) concentration of the ion-interaction reagent; (3) the mobile-phase pH, (4) the flow rate, and (5) the organic modifier concentration (*C24*). Forgacs and Cserhati utilized principal component analysis (PCA) to find the physicochemical parameters that influenced the retention behavior of 13 steroidal drugs. PCA results indicated that five background components contained most (90.67%) of the information present in the 11 original variables (*C25*). Cserhati used PCA followed by nonlinear mapping, varimax rotation, and cluster analysis to demonstrate that the hydrophobic and steric parameters exert the greatest influence on the retention of propargylamine derivatives on a β-cyclodextrin polymer-coated silica column in a mobile phase of 60% THF and 40% 0.05 M K_2HPO_4. Nsengiyumva and co-workers utilized a Draper-Lin small-composite design to study the impact of four different mobile-phase variables on the retention and peak widths of vitamins B_1, B_2 phosphate, B_3, B_6, and C: the percentage of MeOH, the concentration of hexanesulfonate as ion-pairing reagent, the concentration of triethanolamine as competitive base, and pH (*C27*). Finally, Casal et al. compared PLS and multiple linear regression (MLR) for the prediction of the retention of 25 small peptides on four different RPLC columns and found that PLS provided better prediction ability than MLR (*C28*).

Nasal and co-workers compared chemometrically the retention properties of two new HPLC stationary phases, polyethylene-coated silica and polyethylene-coated zirconia. Linear free energy relationship (LFER)-based solute parameters and solute structural descriptors determined by physicochemical methods were regressed as two sets of independent variables vs the logarithm of the retention factors of 25 structurally diverse solutes. The results were very similar (and accurate) and indicated that dipolarity−polarizability intermolecular interactions were stronger on the silica-based phase than on the zirconia-based one, probably as a

consequence of the active free silanols on the silica support (*C29*). Abraham et al. measured the retention of 25 solutes in a variety of mobile phases on six different C18 stationary phases and then used an LFER equation to characterize these phases and 11 others for which similar LFER retention data were available. Although a superficial analysis suggested significant differences among the stationary phases for a given mobile-phase composition, a more detailed analysis led to the conclusion that all the C18 phases examined have roughly the same "intrinsic" hydrophobicity (*C30*). Vervoort and co-workers employed principal component analysis to compare 14 commercially available reversed-phase stationary phases for the determination of basic pharmaceutical compounds. The effect of silanol blocking compounds on retention and peak shape was investigated by using phosphate buffers at pH values of 3 and 7; adding *N*,*N*-dimethyloctylamine to the pH 3 mobile phase at pH 3 resulted in a significant improvement in the peak shapes. As expected, the commercially available stationary phases showed distinct differences in their suitability for the analysis of basic compounds (*C31*). Finally, Turowski et al. synthesized and physicochemically characterized 11 new chemically bonded silica stationary-phase materials in addition to four reference columns for the separation and analysis of 11 fundamental nucleosides and cyclic nucleotides; 8 of the new 11 stationary-phase materials had separation properties similar to a standard octyl silica phase for the target compounds (*C32*).

Miscellaneous. Hartmann and co-workers described the application of a robust orthogonal regression procedure (based on the least median of the squared orthogonal) for the detection of outliers during the evaluation of a method comparison study and, in general, when comparing two series of measurement results (*C33*). Hayashi et al. reported that the statistical reliability of experimentally determined confidence intervals in some linear calibration problems in instrumental analyses can be greatly enhanced if the standard deviation of measurements, which is a theoretical prediction of the instrumental response error, is incorporated into the usual statistical equation instead of the residual of the least-squares fitting (*C34*). Bagur and co-workers described a statistical procedure to validate a chromatographic method. First, the linear dynamic range in both peak height and peak area is established, to choose the best instrumental signal for the quantitative analysis. Second, the performance characteristics of the method (sensitivity, precision, detection limit) are evaluated following published criteria. The evaluation of accuracy, if a suitable standard reference material is not available, is performed using the method of standard addition (*C35*). Bryant et al. outlined the theory of inductive logic programming (ILP) and described the ILP tool Golem and previous applications of ILP. They explained the advantages of ILP over classical machine induction techniques and then presented a case study of the separation of 3-substituted phthalide enantiomer pairs on (*R*)-*N*−(3,5-dinitrobenzoyl)phenylglycine, in which Golem is used to generate rules that predict, with a high accuracy, whether an attempted separation will succeed or fail (*C36*). Nishikawa and co-workers analyzed the peak profiles of sugars undergoing on-column and obtained kinetic rate constants of the isomerization which were then compared with the literature data (*C37*). Yoon et al. employed PCA to distinguish among fruit juices based on HPLC-refractive index detector or GC-FID data (*C38*).

NORMAL PHASE

In keeping with past reviews, "normal phase" will refer to liquid chromatography systems in which the stationary phase is more polar than the mobile phase. This includes stationary phases such as solid adsorbents, bonded and immobilized liquids, and chiral columns operated with nonaqueous, nonpolar, or moderately polar mobile phases. We have also restricted the review to analytical liquid chromatography, generally excluding reports describing preparative, isolation, and concentration applications.

The term normal phase usually implies that adsorption−displacement processes control retention, and this convention is followed here. The few fundamental studies of retention in normal phase LC (NP-LC), particularly those that address adsorption−displacement processes, are discussed first. Descriptions of new normal-mode stationary phases and novel mobile phases follow. Finally, some unique applications of normal-phase HPLC are noted.

Most of the studies describing fundamental retention processes in liquid−solid chromatography were concerned with solute molecular properties rather than characteristics of the stationary and/or mobile phase. However, there did appear a number of reports in which the prediction of retention based on fundamental molecular parameters was investigated. Cheong and Choi used linear solvation energy relationships (LSERs) to characterize retention on bare silica gel in 2-propanol/hexane mobile phases (*D1*). These authors based their LSER analysis on Kamlet−Taft solvatochromic polarity scales for 58 solutes. LSER regressions had lower significance than those observed in reversed-phase HPLC using similar scales. The authors concluded that LSER predictions were not useful in predicting absolute solute retention with bare silica stationary phases but were in agreement with "chemical senses".

Jandera and co-workers published a series of studies of retention in gradient-elution normal-phase HPLC with silica columns. The retention of phenylurea herbicides was characterized in binary (*D2*) and ternary (*D3*, *D4*) gradients of *n*-heptane, 2-propanol, and dioxane. In the case of a binary gradient, they observed that, above an initial polar modifier concentration of 1−3%, preferential adsorption of the polar modifier was not important and the concentration of the modifier in the stationary phase was independent of its concentration in the stationary phase. Predictive calculations based on two- and three-parameter equations were thus in good agreement (∼2%) with observed behavior. These studies were extended to include ternary gradients (*D3*) with a constant ratio of polar solvents (elution strength gradients) and constant sum of polar solvents (selectivity gradients). The retention behavior of the phenylurea herbicides and nitrophenols in these same gradients were compared to theoretical predictions based on the previous studies (*D4*). The authors concluded that, to fit three-component data at any combination of concentrations of the three solvents, a nine-parameter equation was necessary. Meyer also investigated gradient elution in normal-phase HPLC and showed that, contrary to conventional assumptions, bare silica does not exhibit long equilibration times when low and moderately polar solvents are used, and reproducible retention with short reequilibration times are possible (*D5*).

The specific retention characteristics of aromatic hydrocarbons in normal-phase systems continue to attract interest. Wan et al.

compared the retention of aliphatic and aromatic hydrocarbons of similar molecular polarizability and concluded that electrostatic interactions that involve a solute's quadrupole moment dominate the retention of aromatics (*D6*). Lanin et al. modeled the retention of benzene derivatives on aminopropoyl silica, a column known to have a high affinity for aromatic compounds, focusing on solute−mobile-phase modifier interactions (*D7*). This same group also looked at polycyclic aromatic hydrocarbons in a hydroxylated silica/*n*-hexane system and observed that retention depended not only on the number of aromatic rings and methyl groups but also on the nature of the bond between the aromatic rings (*D8*). This observation is in agreement with numerous previous studies that have demonstrated a "planarity effect" in adsorption-based LC separations.

Several reports describing novel stationary phases and their retention characteristics appeared since the last review. Silver-modified silica was shown to have superior separating capabilities for model unsaturated fatty acids (*D9*) and saturated fatty acid methyl esters and triacylglycerols (*D10*). Silver- and palladium-modified silicas were compared as chelating phases for the ligand-exchange separation of polycyclic aromatic sulfur heterocycles (*D11*). Metal loading was found to be crucial in determining selectivity, with Pd-loaded silica providing group isolation of the sulfur heterocycles from the rest of the aromatic fraction in petroleum mixtures.

One of the most interesting new stationary phases to appear was fullerene C60 immobilized on silica (*D12*). The C60 fullerenes increased the surface area of the silicas and were used to study the adsorption and separation of fullerenes, calixarenes, and proteins with both normal- and reversed-phase solvents. Zirconia continues to attract attention and was compared to silica, alumina, and titania in one study (*D13*), and to mesoporous aluminosilicate, silica, alumina, and titania in another (*D14*). The first study demonstrated the basic character of alumina, titania, and zirconia relative to silica, as well as the possibility of base separations, and the high hydrolytic stability in strongly acidic or alkaline mobile phases of lipophilic packings synthesized from these materials. The second study described the pore structure and surface properties of a novel mesoporous aluminosilicate, MCM-41, and showed that it was a suitable stationary phase for separating acids, bases, and neutrals.

Other novel stationary phases that appeared useful in the normal-phase mode include polyacrylate-based film phases for open-tubular LC (*D15*). The moderate polarity of these films actually makes them suitable for either normal- or reversed-phase LC. Silica-immobilized bovine serum albumin has been shown to be an effective material for a number of applications, and Gao and Gilpin reported on the influence of HCl on retention properties of these phases (*D16*). The HCl treatment appears to enhance hydrogen-bonding interactions between solutes and the immobilized protein. Finally, Danielson et al. used hexamethyldisiloxane in the mobile phase to generate what appears to be a partitioning retention mechanism in a C1-silica column (*D17*). This partitioning is due to adsorption of hexamethyldisiloxane on the silica surface.

Examples of the advantages of chiral separations in the normal-phase mode continue to appear in the literature. Chankvetadze et al. synthesized a series of dichloro-, dimethyl-, and chloromethylphenyl carbamate derivatives of cylodextrins and characterized their enantiomeric recognition (*D18*). High enantioselectivity was observed for a few model compounds, although these phases showed generally lower enantioselectivity than their polysaccharide analogues. Alebic-Kolbah and Zavitsanos (*D19*) demonstrated the usefulness of small-bore (2.1 mm) normal-phase chiral columns for LC−MS analysis of several chiral drugs. The convenience of the normal-phase mode outweighed the need for postcolumn addition of an aqueous mobile phase in order to interface directly to an atmospheric pressure ionization MS source.

As always, the number of interesting applications of normal-phase HPLC exceeds the space available in this review to discuss them. Thus, the following is only a representative sampling of some of the more novel uses of NP-LC. Brykina et al. used a silica column and hexane/butanol mobile phase to separate Cu, Zn, Pb, Hg, and Ce complexes of pheophytins (*D20*). Pheophytins a and b can be separated on the basis of structural differences between the a and b series, as well as the nature of the central metal atom. Yoshida demonstrated that certain hydrophilic peptides that could not be separated in reversed-phase LC could be resolved with carbamoyl-derivatized silica and aceonitrile/water mobile phases (*D21*). The retention mechanism was "normal phase", since retention times increased with increasing acetonitrile content. Various forms of vitamin E (tocopherols) that could not be separated in the reversed-phase mode were separated on a diol normal-phase column (*D22*). Phenyl-silica columns were used with hexane/toluene and hexane/dichloromethane to separate triphenylphosphine (PPh_3)-substituted homo- and heterodinuclear metal carbonyl complexes of Mn and Re (*D23*). Amino stationary phases gave superior separations of oligoethylene glycol nonylphenyl ether surfactants compared to nitrile phases in NP-LC (*D24*).

A number of reports described applications of normal-phase separations coupled with mass spectrometry detection. Andersen and Hansen used a normal-phase LC separation with particle beam mass spectrometry detection to analyze metabolites of the dopamine receptor antagonist Odapipam and the muscarine receptor agonist Xanomeline (*D25*). They point out the advantage of the normal-phase mode is the fact that solvent changes with low-polarity solvents have much less of an impact on the particle beam interface parameters than is the case with aqueous-based solvents. Class separations of phospholipids by normal-phase HPLC were followed by electrospray ionization mass spectrometry for specific species determinations (*D26*). Similarly, different classes of polycyclic aromatic hydrocarbons and nitro-PAH were separated with a silica column and heptane/tetrahydrofuran mobile phase and followed by particle beam mass spectrometry (*D27*).

REVERSED PHASE

Reversed-phase liquid chromatography is still the workhorse of the chromatographic methods, and many of the early problems of reproducibility of columns from lot to lot were solved 10 or more years ago. However, there are still many unanswered questions about this simple to use but complex to understand separation technique. Remaining critical issues include the question of column equivalency, which is of great interest in the European community with the debate over the creation of a "standard" column, the issue of "phase transitions", the quanti-

tation and blocking of residual silanols, an unambiguous definition and process for measuring the volume phase ratio, and many remaining questions about the thermodynamics of the phase-transfer process.

There were three papers of great general interest. Nakanishi published a thorough review of the sol−gel process and pore structure control of silica gels (*E1*). Snyder published an excellent tutorial on developing selectivity in reversed-phase LC and showed a quantitative evaluation of different variables for their effect on selectivity (*E2*). Klatte and Beck (*E3*) performed molecular dynamics simulations of the transfer of a simple nonpolar molecule from a water/methanol mobile phase into a C18 stationary phase at room temperature. They note that the overall free energy change is consistent with that expected for hydrophobic transfer, but specific interfacial effects make bulk partitioning models questionable.

Three other papers also should be of widespread interest. Guiochon et al. (*E4*, *E5*) published two studies of the packing of chromatographic columns. There have been surprisingly few thorough studies of this important process, and these papers should prove highly useful. They showed that the packing of chromatographic columns is heterogeneous, being denser and less well organized close to the column wall (*E4*). They further studied the effect of packing pressure on the performance of reversed-phase columns and showed that retention factor and column efficiency increased linearly with increasing packing pressure (*E5*). Still discussing pressure, MacNair et al. (*E6*) showed that the use of extremely high pressure can be useful for long columns with small-diameter stationary phases, generating as many as 300 000 plates!

The use of retention indexes still generates interest, although there is mounting evidence that this approach will never be as viable as it is in GC. Reese et al. (*E7*) used principal component analysis and target testing to develop a universal solute and solvent retention index system, which they applied across different reversed-phase columns. Sanagi et al. (*E8*) used a homologous series of alkylbenzene compounds as a retention index system and showed that while it did not work well for less retained compounds, it was fairly useful for relatively nonpolar compounds. Bogusz et al. (*E9*) compared three databases that have been established using the 1-nitroalkane scale and noted that the standardization of chromatographic conditions is a minimum prerequisite for interlaboratory reproducibility of retention indexes.

Temperature optimization is a very underutilized variable for controlling retention and selectivity in chromatography. There are an increasing number of reports showing that both low and high temperatures can be useful. Ooms published a review of temperature control, discussing both instrumental aspects and chemical interactions (*E10*). Sheng et al. (*E11*) studied temperatures from 24 to 80 °C and showed both improved efficiencies and an increased optimum velocity with a packed capillary column. Trones et al. (*E12*) also studied elevated temperatures with packed capillary columns, using nonaqueous mobile phases. They showed that with temperatures up to 150 °C compounds completely retained at room temperature would elute with good peak shape. Alternatively, Hirano and Takahashi (*E13*) showed that low temperatures, down to −20 °C, can also be useful for improved resolution of triglyceride species. Kamiyama et al. (*E14*) studied the temperature dependence of retention using a porous acrylic support and showed that with this stationary phase retention increased with increasing temperature.

There were also a number of general papers describing retention processes. Sajonz et al. (*E15*) studied and compared profiles of large-size system peaks and vacancies and showed that the isotherm data could be fitted to a simple Langmuir equation. Tan and Carr (*E16*) published a continuing discussion of driving forces in reversed-phase LC. Using measured free energies of transfer of a methylene group from organic/aqueous mixtures to bulk hexadecane, they concluded that retention on monomeric bonded phases with octyl chains or longer is dominated by a partitioning-like mechanism. Lochmuller et al. (*E17*) used factor analytical modeling to predict retention of some neutral, basic, and acidic organic compounds in water/methanol/acetonitrile solvent systems at pH 7, 5, and 3. Guillaume and Guinchard (*E18*) compared retention mechanisms of weak polar solutes in acetonitrile/water mobile phases and methanol/water mobile phases, comparing entropy and enthalpy of transfer and enthalpy−entropy compensation data. Lukulay and McGuffin (*E19*) performed experimental and computer simulation studies of solute−solute interactions, including self-association and mixed association for several steroids. Haegglund and Staahlberg (*E20*) applied an ideal model of chromatography to charged solutes and studied adsorption isotherms and resulting peak shapes. Collantes et al. (*E21*) used the moment of inertia in comparative molecular field analysis to model chromatographic retention of nonpolar solutes. Sun et al. (*E22*) studied the relationship between retention behavior and molecular structure parameters of substituted benzenes. While these type studies work well for congeneric species, it is difficult to extend them to more dissimilar solutes.

Mobile-Phase Studies. Once again, for convenience, we have arbitrarily divided the reports into mobile-phase and stationary-phase sections. This has been done only for organizational purposes, as it allows more easy categorization of the voluminous original reports each reporting period. It should be stressed once again that retention is a result of the chemical potential difference between the two phases and that most chromatographic studies designed to show only effects from only one or the other are not totally deconvoluted.

Nyiredy published a thorough study of solvent classification for liquid chromatography (*E23*), discussing both solvent strength and solvent selectivity factors, based on Snyder's solvent groups. Vailaya and Horvath (*E24*) published the first original work on the solvophobic theory in many, many years and reexamined a large set of retention data with nonpolar and weakly polar eluites. They showed that this theory can be useful for the evaluation of physicochemical parameters associated with retention of hydrocarbon solutes and that the mobile phase is dominant in governing selectivity changes of nonpolar solutes. While their theory does fit the data well, it still must be reiterated that the solvophobic theory does not account for any interactions in the stationary phase and cannot completely accurately model the retention process.

Lochmuller and Hangac (*E25*) reviewed and studied the use of mobile-phase additives for selective interactions with solutes and selectivity adjustment. They compared coordinatively unsat-

urated β-diketonates as additives with ionic, metal dopants and showed the neutral complexes generally perform better. In a more specific study, Cole and Dorsey showed that cyclohexylamine additives were beneficial in peptide separations and were easier to use than the more common triethylamine (*E26*).

Accurate values of diffusion coefficients are necessary for theoretical modeling of chromatographic performance. Li and Carr performed a very careful study measuring diffusion coefficients of alkylbenzenes and alkylphenones in water/methanol and water/acetonitrile mixtures (*E27*). They compared these measured values with those predicted by the Wilke−Chang equation and the Scheibel equation. They proposed a modification of the Wilke−Chang equation that gave errors no greater than 10% for either system, and their values were generally 2−3 times better than either of the other equations.

McGuffin and Chen (*E28*) measured the molar enthalpy and molar volume of methylene and benzene homologues for a typical monomeric column and typical polymeric column and found significant differences between the two. They further studied the effects of pressure on solute retention using a homologous series of derivatized fatty acids and reported changes in capacity factors of up to 24% for inlet pressures up to 5000 psi (*E29*).

McCalley (*E30*) examined eight silica-based reversed-phase stationary phases with nine bases of different structure and found that the nature of the probe significantly affected the results of column performance. This remains a significant problem; it would be very useful to have a standard probe of silanol activity that could be compared from laboratory to laboratory and from column to column.

Two studies were published comparing the performance of methanol/water systems to acetonitrile/water systems. Miyabe and Takeuchi (*E31*) studied surface diffusion and heats of adsorption of small molecules, and Guillaume et al. (*E32*) used chemometric methodology to study the separation of 10 benzodiazepines.

Two more anecdotal studies of retention behavior were published, using the assumed linearity of log k' vs volume fraction organic modifier. Sun et al. (*E33*) used substituted benzene derivatives and discussed the relationship of the intercept (log k_w) and the slope (S) and discussed these in relation to the total solubility parameter and the octanol/water partition coefficient. Nasuto et al. (*E34*) used PAH compounds to study effects of retention vs mobile-phase composition.

Gradient elution separations are still a very underutilized method by most industries. There is still an unfounded belief by many people that transfer of these methods is more difficult, that reproducibility is not as good, and that reequilibration times are painfully long. Snyder continued his efforts to show the utility of these methods for routine analysis and published a series of four papers showing that the combined use of temperature and solvent strength in gradient elution can be a very powerful set of variables for selectivity control in method development and that optimization software can reliably predict the effect of changes of both variables (*E35*−*E38*). They also published a tutorial article summarizing these results (*E39*). In a further paper, they described the use of a short precolumn between the pump and injector for removing trace organic impurities from the water component of the mobile phase and showed improvements in baseline stability by 5−100 times (*E40*). Warner and Dorsey (*E41*) discussed the use of a constant composition of 3% 1-propanol in both the weak and strong solvents as a means of reducing reequilibration time in reversed-phase gradient separations. The rule of thumb that 20 column volumes of the original mobile phase is necessary for reequilibration is virtually always a gross overestimation, and using the recommended system, reequilibration can be reduced to approximately one column volume.

Lee et al. (*E42*) showed several applications of the use of enhanced fluidity mobile phases for complex mixture separations and showed that the lower pressure drops of these systems allows the connection of serial columns for improvements in peak capacity.

There was significant interest in the use of water as a mobile phase during this reporting period, with two different approaches to solving the problem of extraordinarily long elution times. Synovec et al. first showed the utility of water as a mobile phase, and they synthesized a unique stationary phase giving a volume phase ratio reduced by about 2 orders of magnitude compared to traditional reversed-phase columns (*E43*). This allows elution of small molecules in reasonable times. They further showed the compatibility of this system with a flame ionization detector FID), a detector long envied by practitioners of LC (*E44*). Smith and Burgess took a different approach to using water and showed that, by pressurizing the column to prevent boiling, superheated water was a medium-polarity mobile phase, with UV transparency as low as 190 nm (*E45*, *E46*). Miller and Hawthorne combined the previous two approaches and showed the compatibility of heated water with an FID detector (*E47*). Hu (*E48*, *E49*) described the separation of nucleosides and their bases using water as the mobile phase and used both zwitterionic surfactants (*E48*) and temperature (*E49*) to reduce retention on conventional columns.

Stationary-Phase Studies. There were more reports that were classified as "stationary-phase studies" than "mobile-phase studies". This is indicative of the complexity of the problem of understanding the retention process as related to especially alkyl chain structure and also is indicative of the many, many variations of new stationary phases that are possible. This section is also subdivided arbitrarily into studies of traditional alkyl chain-based stationary phases, of other more polar silica-based phases, and of stationary phases on other than silica.

Four reviews relevant to this section appeared during this review period. Nawrocki published a thorough review of the silanol group and its role in LC (*E50*). Van Der Voort and Vansant (*E51*) published an excellent review of silylation of the silica surface, Wirth et al. (*E52*) reviewed the use of self-assembled monolayers in chemical separations, and Pesek et al. (*E53*) reviewed the synthesis, characterization, and applications of hydride-based surface materials.

Kirkland et al. (*E54*, *E55*) published two *highly* practical papers investigating the effect of various buffer salts on stability of commercially available reversed-phase stationary phases and showed that the ubiquitous phosphate buffer leads to fastest degradation! Most organic buffers lead to much slower degradation, and these articles should be thoroughly read by all practitioners of reversed-phase LC!

There were a number of "mechanistic" papers, that is, papers that further our understanding of the chemical interactions that

lead to selective retention. The Kamlet−Taft formalism of solvatochromic effects continues to be useful to examine these processes, and there were four publications describing this approach. Lu and Rutan described the synthesis of a new solvatochromic dye and showed its utility for characterizing chromatographic stationary phases (*E56*) and then compared three dyes for the study of a C18 phase from 5 to 100% methanol, acetonitrile, and THF in water (*E57*). Tan et al. (*E58*) studied five stationary phases, varying in silanol activity, using 87 aliphatic and aromatic solutes. Abraham et al. (*E59*) studied 25 solutes on six different C18 columns and showed that, after normalizing for differences in phase ratio, all columns studied had about the same "hydrophobicity". This is in agreement with *many* other studies which have shown that the volume phase ratio is the most important parameter controlling absolute retention.

As well as solvatochromic studies, a number of other papers examined processes related to retention. Park et al. (*E60*) examined the adsorption vs partitioning question on a number of different polymeric phases and concluded that partitioning is the dominant process at most mobile-phase compositions. Burns et al. (*E61*) used fluorescent solutes and quenching techniques to study the interfacial region of typical stationary phases. Buszewski et al. (*E62*) compared the retention properties and physical characterization of a C18 phase with an aminopropyl phase, and Jaroniec (*E63*) used sorption and retention data to further characterize the interfacial region of the stationary phase. Akapo and Simpson (*E64*) studied the influence of temperature and mobile-phase composition on retention properties of oligomeric bonded phases, Elizalde-Gonzalez et al. (*E65*) studied adsorption isotherms on two types of phases and reported a method for the measurement of these isotherms that is a combination of a frontal experiment and peak shape analysis; Miyabe and Takeuchi (*E66*) measured surface diffusion phenomena with both methanol/water and acetonitrile/water.

There were four reports studying physical characterization of reversed-phase materials. Guan-Sajonz et al. (*E67*) studied the pore structures of four conventional stationary phases and showed that the agreement among nitrogen sorptometry, inverse size exclusion chromatography, and mercury intrusion porosimetry was excellent. Morel et al. (*E68*) used differential scanning calorimetry and specially prepared phases to study the "phase transition" which some workers believe occurs. This is an area of stationary phases that is still not well understood. Doyle et al. (*E69*) reported Raman spectra of various ligands used in reversed-phase stationary phases. The novelty of their approach is that their spectra were taken with a fiber-optic fitting into an LC column, so that they were measured under flow and under pressure, rather than on a flat plate at ambient pressure! Haegglund and Staahlberg (*E70*) proposed a method for measuring the chromatographically accessible surface area using measured adsorption isotherms of charged amphiphiles.

There were also five reports of practical characterization techniques for comparing different stationary phases. This is a very practical problem, as the question of column equivalency is heating with the debate in Europe over the development of a "standard column". Cruz et al. (*E71*) used principal component analysis and other chemometric techniques to classify 30 commonly used phases, Barrett et al. (*E72*) used neutral, basic, and acidic test solutes to probe the effect of chain length and showed that short chains that achieve high densities best mask residual silanol effects. Bolck et al. (*E73*) proposed a statistical approach using a few test solutes for the study of "aging" of reversed-phase columns, with the goal being to predict column failure before loss of analytical data occurs. Sandi et al. (*E74*) used a gradient elution technique and principal component analysis to compare commercial columns, and Eymann (*E75*) described tests for determination of hydrophobic and silanophilic interactions and for trace metal impurities. They also measured phase stability and showed column performance at two different pH conditions.

There were an additional five reports describing the study of the population and interaction of residual silanols. Scholten et al. (*E76*) performed a fundamental study by deuterium exchange and CP-MAS NMR of two commercial phases and the native silica. They further chromatographically confirmed the NMR results using four column tests proposed in the literature (*E77*). Sykora et al. (*E78*) studied several different phases as a function of mobile-phase composition and pH. McCalley (*E79*) studied the performance of columns as a function of pH and proposed (quite logically!) that columns be evaluated at the pH of intended use. Vervoort et al. (*E80*) compared 14 commercial columns with basic pharmaceutical compounds and used principal component analysis to classify them.

There was also significant interest in the use of nonporous silica for fast analysis. The trade here is for sample capacity, and some older instruments are not able to handle the sharp, fast peaks resulting from these columns. Hanson and Unger reviewed the synthesis and characterization of nonporous particles (*E81*) and then discussed their application, mostly for the separation of biomolecules (*E82*). Barder et al. (*E83*) also wrote a tutorial review of the application of these materials. Jenke (*E84*) performed a thorough, practical comparison of these materials with a traditional column and concluded that faster separations are possible with a considerable reduction in solvent usage. Yu and El Rassi (*E85*) prepared 0.8- and 1.1-μm nonporous materials with various functionalities and evaluated their performance with small and large molecules.

There were four reports of the preparation of monolithic column materials. That is, rather than discrete stationary-phase particles, the stationary phase is now a continuous bed and may offer advantages in terms of efficiency and column stability. Liao et al. (*E86*) prepared a polymeric bed which they surface modified with C18 groups. Minakuchi et al. (*E87*, *E88*) prepared continuous, porous silica rods and characterized them with small and large molecules. Fields (*E89*) prepared a silica xerogel column and showed its use for both pressure-driven and electrochromatography.

Guo and Colon (*E90*) prepared an open-tubular capillary column with a sol−gel coating and showed very high separation efficiencies. Akapo et al. (*E91*) described retention characteristics and applications of cyclic siloxane-based C18 phases. There were two reports of characterization of the C30 phase originally developed by Sander and Wise. This material is especially useful for the separation of carotenoids (*E92*) and tocopherol isomers (*E93*).

While the C8 and C18 materials are the workhorses of reversed-phase LC, there are always new phases being prepared

which may offer unique separations for particular classes of compounds. In the interest of space, these phases will simply be listed, and the reader is referred to the original paper for further details. These new phases include an amide functionalized column (*E94*), a new base-deactivated phase with basic ligands incorporated into the silica matrix (*E95*), propiophenyl and multifunctional (cyanopropyl−octadecyl) materials (*E96*), alkylamide phases (*E97*), silica-bound fullerene derivatives (*E98*), polysiloxane-encapsulated materials (*E99*), a 3-(1,8-naphthalimido)-propyl phase (*E100*, *E102*), mono-ol, butylphenyl, and perfluorinated columns (*E101*), a spacer-bonded propanediol (*E103*), bonded cholesteryl 10-undecenoate (*E104*), phenylsilicone (*E105*), immobilized porphyrins (*E106*), and a comb-shaped polymer as a lipid membrane analogue (*E107*).

Restricted access media continue to be of interest, and a tutorial review appeared (*E108*), as well as an original report describing the use of various buffers to clean the plasma matrix from the columns after use (*E109*).

There is much interest in the use of alternative materials to silica for derivatization and preparation of reversed-phase materials. One of the more promising substrates is zirconia, and Carr's group continued their extensive study of this material, with the publication of six original works (*E110*−*E115*). Two other groups also reported use of zirconia materials (*E116*, *E117*).

Carbon-based materials continue to be of interest, and Knox and Ross published two reviews of these materials, one on structure, performance, and retention mechanisms (*E118*) and the second on applications (*E119*). In addition, there were four original publications describing further developments of these materials (*E120*−*E123*).

There were three reports of the use of titania as a support material for reversed-phase columns (*E124*−*E126*). Porous polymers continue to generate interest, and Svec and Frechet (*E127*) published a thorough review of the use of macroporous disks and rods as stationary-phase materials. In addition, there were reports of perfluorooctyl- and perfluorobutyl-bonded alumina materials (*E128*), the influence of solvent on PS-DVB materials (*E129*), and the swelling behavior and kinetic performance of polyacrylate stationary phases (*E130*).

CAPILLARY ELECTROCHROMATOGRAPHY

Simply put, electrochromatography is a liquid chromatographic experiment run in a CE instrument. That is, electroosmotic flow drives the mobile phase through the column, rather than a pressure drop. Since the capillary is now packed with chromatographic stationary phase, the surface-to-volume ratio is increased by orders of magnitude. This will ultimately allow much larger injection volumes, lowering the concentration LOD, and should also give *much* improved EOF reproducibility. The ultimate driving force for the interest in CEC, however, is not these two minor advantages, but rather the solution to the limited peak capacity of traditional LC. In traditional LC, mobile-phase velocity at a given pressure drop is proportional to the square of the stationary-phase particle diameter. As the stationary-phase particles are made smaller, giving better chromatographic efficiency, the pressure limit of the system is quickly reached. In electroosmotic flow, however, there is no particle size dependence on flow velocity, and in theory, long columns of very small diameter can be packed to generate open-tubular GC-like efficiencies in an LC experiment.

Electrochromatography is not a new technique. Pretorius published a seminal paper almost 25 years ago, "Electroosmosis: A New Concept for High-Speed Liquid Chromatography" (*F1*), showing that a potential gradient could be used to pump the mobile phase through a packed column, rather than a pressure gradient. However, they were using rather large glass tubes, and Joule heating was a serious problem. The enabling technology for electrochromatography, as for capillary electrophoresis, open-tubular GC, and virtually every other capillary separation technique was the development of fused-silica capillary columns. Knox then published two papers that were key in reigniting the interest in electrodriven chromatographic flow (*F2*, *F3*) and showed that dramatically lower plate heights could be generated with electroflow as compared to traditional pressure-driven flow.

This is the first time that this subject has been covered in this review, but there has been intense research interest in the past few years. Again, this will not be a comprehensive review of all published papers, rather we will highlight some of the key works and cite more thorough review articles. Dittmann and Rozing (*F4*) published a thorough tutorial, discussing the origins of electroosmotic flow in packed capillaries and showing the possibilities of high-efficiency separations by this method. Colon's group published two reviews, the first a tutorial article (*F5*), and the second a more comprehensive review (*F6*). Rathore and Horvath (*F7*) published an excellent review of theories of electroosmotic flow in porous media, with particular emphasis on packed capillaries.

One of the key issues for electrochromatography is the development of extremely small particle stationary phases which will generate electroosmotic flow. One of the ways this is being approached by several groups is through the development of monolithic stationary phases. That is, the stationary phase is not composed of discrete particles, but is a continuous porous bed, providing extraordinarily short diffusional distances between phase boundaries. Ericson et al. (*F8*) described the synthesis and characterization of continuous polymer beds which were derviatized with C18 ligands. A similar approach was taken by Peters et al. (*F9*). Maruska and Pyell (*F10*) developed microparticulate cellulose-based packing materials and showed their use for both reversed- and normal-phase separations. Robson et al. (*F11*) approached the problem from a different perspective and studied packing traditional materials into capillary columns using supercritical carbon dioxide as the carrier solvent; they showed that extremely efficient columns could be prepared.

Other papers of great general interest include work by Dittmann and Rozing (*F12*) investigating the influence of mobile-phase and stationary-phase properties on electroosmotic velocity, retention, and selectivity. Behnke et al. (*F13*) discuss the practical parameters affecting electrochromatography, including eluent, frits, packed columns, and optional supplemental pressure. Euerby et al. (*F14*) discussed the operational parameters and factors controlling performance of electrochromatography with commercially available instrumentation.

While it is conventional wisdom that some background electrolyte is necessary to generate current flow and electroosmotic flow, Wright et al. (*F15*) showed that significant electroos-

motic flow could be generated in pure organic and aqueous solvents with no background electrolyte. This has the potential of dramatically limiting Joule heating and allowing both higher potential drops and larger capillaries. Lister et al. (*F16*) further showed that the currents generated by these solvents are in the very low nanoampere (!) range.

A very practical problem that must be solved for this to be a viable technique is the ability to generate gradient elution separations. Two methods for doing this have been proposed. Yan et al. (*F17*) developed a two-power-supply approach analogous to the two-pump system in pressure-driven flow. They used computer control to ramp from the weak solvent to the strong solvent and used a "tee" connector to introduce the two solvents into the separation capillary. Huber et al. (*F18*) used a traditional LC system to generate a solvent gradient which was flowing past the inlet to the separation capillary. This allows the solvent gradient to be introduced to the capillary by purely electroosmotic flow.

New developments in this area are coming extremely rapidly, and those interested in seeing the latest developments are strongly encouraged to attend either the general LC meetings, where there is significant discussion of electrochromatography, or the new International Symposium on Capillary Electrochromatography, which will be held in San Francisco at the end of August.

BIOPOLYMER SEPARATIONS

Reviews. Eksteen presented a guide for column selection, organized according to the mode of chromatographic separation, in the analysis of biomolecules (*G1*). Wirth reviewed the advantages of mixed self-assembled monolayers, especially for the separation of biopolymers and pharmaceuticals (*G2*). These stationary phases demonstrate reduced electrostatic interactions with solutes; such interactions can limit chromatographic performance in these bioseparations. Stulik et al. critically reviewed the use of stationary phases in size-exclusion, ion-exchange, reversed-phase, and hydrophobic interaction modes of chromatography for the separation of peptides (*G3*). Svec and Frechet reviewed pore-size-specific stationary-phase modification in liquid chromatography for use in two-dimensional separations of proteins (*G4*). Two modes of chromatography such as reversed-phase, ion-exchange, hydrophobic interaction and others may be combined in a single column. More specific reviews addressed the separation of antimicrobial peptides (*G5*), amylases (*G6*), oligosaccharides (*G7*, *G8*), DNA topoisomerases (*G9*), nucleic acids (*G10*), and synthetic DNA (*G11*). The use of carbon-based packing materials for the separation of carbohydrates (*G12*), and additionally sugars and glucuronides (*G13*) was reviewed as well.

In an extensive review article, Benedek addressed protein composition, structure, and adsorption isotherms in relation to protein separations in LC (*G14*). Hunkeler et al. critically compared experimental conditions for chromatographic adsorption and solubility of macromolecules, as both phenomena involve thermodynamic considerations (*G15*). Mantle and Noon provided an overview of the technique of chromatofocusing of proteins (*G16*). Nice summarized the micropreparative purification of peptides and proteins in LC to yield nanogram and microgram quantities (*G17*). Practical aspects of the separation of peptides and proteins (*G18*) and protein-based biopharmaceutical products (*G19*) were reviewed as well. Rapid protein separation methodologies in LC were reviewed by Regnier and co-workers (*G20*), as well as by Godt and Kamp (*G21*). The latter article focused on the use of nonporous silica particles. In contrast, Rodrigues reviewed the applications of large-pore supports for enhanced intraparticle convective solute mass transfer (*G22*).

The separation of carbohydrates and their derivatives using hydrophilic interaction chromatography with both silica- and polymer-based supports was reviewed by Churms (*G23*). Large-scale process development in hydrophobic interaction chromatography (HIC) was reviewed by Gagnon and Grund (*G24*). Wu and Karger provided a general overview of chromatographic conditions for the separation of proteins in HIC (*G25*). Fischer discussed the effectiveness of HIC in revealing the heterogeneity of macroamphiphiles such as lipoteichoic acids, lipoglycans, and lipopolysaccharides, differing in number of fatty acid residues, fatty acid composition, and length of the hydrophilic chain (*G26*). O′Farrell described the principles of HIC and provided a method for the purification of aspartate aminotransferase from *Thermus aquaticus* (*G27*). El Rassi summarized the fundamental principles and chromatographic systems involved in the separation of oligosaccharides, glycopeptides, and glycoproteins using RPLC and HIC (*G28*). Neville discussed the separation of proteins using RPLC, including various columns, mobile-phase compositions, and mobile-phase additives (*G29*).

Batey reviewed the ion-exchange chromatographic separation of legume and cereal proteins (*G30*). Sheehan and Fitzgerald discussed the use of microgranular DEAE-cellulose for the separation of proteins in ion-exchange chromatography (IEC) (*G31*). Lee assessed the use of anion-exchange chromatography for the separation of carbohydrates (*G32*). Crimmins reviewed the application of strong cation-exchange chromatography for the separation of synthetic or proteolytically derived peptides (*G33*). No pre- or postcolumn derivatization was required, and carbohydrates and their positional isomers were separated according to the number of hydroxyl groups and degree of polymerization. Fast protein liquid chromatography (FPLC) employing both ion-exchange and size-exclusion chromatography (SEC) was reviewed by Sheehan (*G34*). Churms reviewed the use of SEC for the separation and molecular weight distribution analysis of carbohydrates (*G35*). Irvine summarized the theoretical basis of SEC, especially for the purification and molecular weight estimation of peptides (*G36*).

Column Packing Materials. Fundamental investigations were conducted involving mass-transfer properties and flow rate dependencies of liquid chromatographic stationary phases in the separation of biopolymers. Horvath and co-workers investigated from both theoretical and experimental standpoints the effects of intraparticle resistance to mass transfer on chromatographic peak shape for gigaporous, mesoporous, and gel-filled gigaporous stationary-phase supports (*G37*). An important finding from these studies was that, at high flow rates, intraparticle convection contributed greatly to intraparticle mass transfer. Rodrigues et al. determined that mass transport in Poros Q/M large-pore particles was dominated by intraparticle convection, which in turn enhanced intraparticle diffusivity and narrowed chromatographic bands (*G38*). Ellenberger et al. used Poros perfusion chromatographic media for the separation of synthetic oligonucleotides

(*G39*). High flow rates and extremes in pH could be employed in conjunction with this medium for increased sample throughput. Moriyama et al. performed rapid separations of peptides and proteins in the reversed-phase mode using 2-mm porous silica gel (TSKgel Super-ODS) (*G40*). Short gradient times and high flow rates were applied, yielding sharp chromatographic peaks and high analyte recoveries (>80%).

Several investigators revealed the advantages of nonporous silica chromatographic supports. Nimura and Itoh compared the chromatographic efficiency of nonporous vs macroporous alkylsilyl-bonded silica gels for the separation of proteins in RPLC (*G41*). In the fast separation mode, the nonporous matrixes yielded higher recoveries, especially for relatively large and hydrophobic proteins. Yu and El Rassi evaluated nonporous, monodispersed, microspherical silica particles (0.8 and 1.1 mm in diameter) modified with lectin, octadecyl, or triphenyl ligands for the separation of a variety of analytes, including proteins (*G42*). Overall, the nonporous stationary-phase supports demonstrated rapid separations due to negligible resistance to mass transfer. Using short (33 mm) columns packed with nonporous silicon dioxide microspheres (Kovasil MS-C14 and Kovasil MS-H), Jelinek et al. observed fast analysis times, reduced mobile-phase consumption, and enhanced stationary-phase stability to harsh chromatographic conditions in the analysis of proteins and peptides (*G43*). Ohmacht revealed the advantages of Kovasil-H, another nonporous silica-based chromatographic support, in peptide mapping studies (*G44*). Short column lengths (33−66 mm) and high column temperatures were employed in conjunction with the support.

Unger and co-workers characterized the performance of silica, alumina, titania, and zirconia supports that had been modified with lipophilic organosilanes and polymers in the analysis of proteins (*G45*). These stationary phases demonstrated high hydrolytic stability to strongly basic or strongly acidic mobile-phase compositions. Dunlap and Carr investigated the effects of mobile-phase composition on the separation of proteins using a carboxymethylated zirconia stationary phase (*G46*). A minimal concentration of a Lewis base in the mobile phase was necessary in order to elute the proteins from this ion-exchange stationary phase. The ionic strength and pH of the mobile phase also influenced the elution and recovery of the proteins; however, optimal experimental conditions were highly dependent on the particular proteins being analyzed. Klint and Eriksson investigated ultrastable zeolite Y with a Si/Al ratio of greater than 240 as a chromatographic support for the purification of proteins (*G47*). Strong protein adsorption to the support surface and to other protein molecules in layers above the support surface could be controlled as a function of mobile-phase pH.

Benmoussa et al. qualitatively analyzed two new stationary-phase supports, spherical aggregates of calcium phosphate hydroxyapatite and lead phosphate hydroxyapatite, for the separation of globular protein mixtures (*G48*). Barroug et al. analyzed the adsorption of succinylated lysozyme on chromatographic grade hydroxyapatite at 20 °C from pH 5.9 to 7.4 and determined that this phenomenon is driven by hydrophobic interactions (*G49*). Akazawa et al. compared the chromatographic performance of hydroxyapatite stationary phases derived from cattle bones and hydroxyapatite derived synthetically from reagents for the separation of bovine serum albumin and egg white lysozyme (*G50*). Ogawa and Hiraide analyzed the desorption of various proteins from ceramic hydroxyapatite types I and II as a function of molar concentration and pH of the phosphate buffer eluent, as well as the isoelectric points of the proteins (*G51*). In general, proteins with high isoelectric points eluted earlier than proteins with low isoelectric points; regardless of pH, proteins eluted later at pH 6 vs 9, and later on type I vs type II.

A variety of polymer-based stationary phases were employed recently for the separation of biopolymers. Svec and Frechet demonstrated the utility of macroporous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) and poly(styrene-*co*-divinylbenzene) rods as monolithic separation media for the separation of proteins and peptides (*G52*). These stationary phases prepared in situ have relatively large pore diameters (1 mm) and demonstrate low column backpressure and flow rate-independent efficiency. In a separate report, the same research group prepared macroporous 2,3-epoxypropyl vinylbenzyl ether−divinylbenzene copolymer beads and noted their unexpected chromatographic performance in the separation of proteins in the RPLC mode (*G53*). The concentration of divinylbenzene was found to control the pore size distribution and stationary-phase surface area of these new matrixes. Finally, this research group evaluated the use of porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolithic columns that had been derivatized with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) for the ion-exchange separation of proteins (*G54*). The characteristically large pores of the chromatographic media allowed efficient and rapid separations (within 1.5 min) of a three-protein mixture at high flow rates. Green et al. introduced a new polymeric resin-based stationary phase, PolyFlo, for both large-scale and small-scale separation of oligodeoxyribonucleotides (18−41 bases in length) (*G55*). The chromatographic performance of this resin was excellent for nucleic acids, with high yield (>90%), high purity (>95%), and no observable loss of biological activity. Nilsson et al. showed the utility of molecular imprinting methacrylic polymers for the selective separation of acetylated carbohydrate derivatives; α- and *β*-configurations of the carbohydrate derivatives could be discriminated chromatographically (*G56*). Polystyrene−divinylbenzene columns were applied in the separation of acid-soluble nuclear proteins in RPLC (*G57*). Electrophoretically pure protein fractions were achieved with minimal sample preparation. Additionally, Huber et al. applied short columns, packed with 2.3-mm particles of highly cross-linked polystyrene−divinylbenzene, for the separation of proteins and peptides at relatively high column temperatures (*G58*). These columns demonstrated high efficiencies, short analysis times, and fast regeneration due to the high diffusivity of the analytes and short diffusion path lengths.

Porous graphitized carbon (PGC) was used as a stationary-phase support for the separation of sulfated oligonucleotides (*G59*). As opposed to separation methodologies employing anion-exchange or reversed-phase ion-pairing chromatographic stationary phases, salts were not necessary to achieve satisfactory chromatographic performance. In addition, Lipniunas et al. applied two-dimensional crystallized carbon stationary phases for the separation of oligomannosidic branch isomers that differ only in the position of a single residue (*G60*).

Temperature-Responsive Packing Materials. Kanazawa et al. designed a temperature-responsive stationary phase and suggested its utility in the separation of peptides and proteins in LC (*G61*). Thermally responsive polymer chains composed of carboxyl semitelechelic oligomers of *N*-isopropylacrylamide (NiPAM) were covalently attached to aminopropylsilica. The hydrated form of the polymer exhibits hydrophilic properties above 32 °C (the lower critical solution temperature, LCST), and the dehydrated form exhibits hydrophobic properties below 32 °C. Interactions between the stationary phase and analytes were controlled via external column temperature. The same investigators covalently attached homogeneous poly(*N*-isopropylacrylamide) (PNiPAM) as well as a copolymer of NiPAM and butyl methacrylate to aminopropyl silica for the separation of proteins and peptides (*G62*, *G63*). Three peptides, insulin chains A and B and β-endorphin fragment 1−27, were separated as a function of column temperature and NaCl concentration in the aqueous mobile phase. Ivanov et al. applied the temperature-responsive 70:30 copolymer of NiPAM and *N*-hydroxyethylacrylamide as a chromatographic ligand for the separation of proteins (*G64*). The ligand was subsequently attached to wide-pore glass, resulting in a weakly hydrophobic stationary phase that demonstrated a subtle temperature dependence for lysozyme near the LCST of the copolymer. Peters et al. derivatized the internal pores of ethylene dimethacrylate−glycidyl methacrylate monolithic supports with NiPAM and methylenebisacrylamide for use as stationary phases in HIC separations of proteins (*G65*). Characteristics of the stationary phases, such as pore size, were temperature dependent.

Hydrophobic Interaction. Theoretical and experimental investigations involving HIC were performed. Perkins et al. applied a simple thermodynamic model to describe protein retention in HIC as a function of stationary-phase ligand hydrophobicity and mobile-phase composition (*G66*). The adsorption of a protein molecule was associated with the release of water molecules. Analogous to entropically driven hydrophobic interactions, the number of water molecules released was related to the surface area of the protein. Horvath and co-workers studied the retention of hydrophobic dansylated amino acids in HIC as a function of temperature (5−50 °C) and compared the results to previous calorimetric studies (*G67*, *G68*). Changes in heat capacity, enthalpy, and entropy associated with retention in HIC were analogous to those observed in other processes involving the hydrophobic effect. Justification for enthalpy−entropy compensation was presented, and correlations with molecular area were established. In a related research paper, these researchers focused primarily on enthalpy−entropy compensation in HIC (*G69*). De Frutos et al. related chromatographic peak shape in HIC to protein structure for β-lactoglobulins A and B (*G70*). A variety of experimental parameters were investigated including sample concentration, amount of sample injected, column temperature, mobile-phase composition and pH, and time of interaction between the protein and the stationary phase. Bai et al. demonstrated that HIC was superior to weak anion-exchange chromatography and SEC in monitoring the refolding of α-amylase and discriminating between α-amylase conformers of differing hydrophobicities (*G71*).

New monolithic supports were introduced during the review period for the separation of proteins by HIC. Xie et al. prepared columns of macroporous poly(acrylamide-*co*-butyl methacrylate-*co*-*N*,*N*′-methylenebisacrylamide) for the rapid separation of protein mixtures (*G72*). The hydrodynamic and chromatographic properties of the new columns were analyzed. Additionally, Zeng et al. introduced a new continuous-bed support for the separation of model proteins; acrylamide monomers, including a monomer containing isopropyl functionalities, were polymerized in situ (*G73*). Characteristic features of the new supports used in conjunction with an ammonium sulfate mobile-phase gradient included high rigidity, as well as low rate-independent resolution and high separation efficiency of proteins.

Reversed Phase. Two mathematical treatments of peptide and protein separations in RPLC were presented in the review period. The retention behavior of octreotide, a somatostatin analogue, and its glycosylated derivatives was modeled by Kuhn et al. (*G74*). The model included a variety of eluent conditions and was extended to alkylated and acetylated derivatives of the peptide. Linear functions between the logarithm of the retention factor and hydrophobicity of the solute were obtained in all instances; the slope of the linear function indicated the selectivity of the system. Hortacsu and McCoy treated a protein capable of undergoing dynamic conformational transitions as a multicomponent mixture undergoing first-order reversible reactions (*G75*). Moment analysis using Laplace Transforms was utilized to predict distinct or overlapping peaks.

Fundamental advances in the separation of nucleic acids, oligosaccharides, and glycopeptides in RPLC have involved the addition or exclusion of mobile-phase additives, as well as control of the column temperature. Hu and co-workers applied pure water as a mobile phase and a strongly negative or positive charged zwitterionic surfactant-modified octadecyl stationary phase in the separation of nucleosides and their bases (*G76*). Hydrophobic interactions between the stationary phase and the analytes were tempered by the electrostatic interactions present on the silica surface. Additionally, Hu and co-workers controlled the elution of nucleosides and their bases by employing elevated column temperatures, unmodified octadecyl stationary phases, and pure water as the mobile phase (*G77*). Two factors appeared to be contributing to the observed chromatographic behavior: reduced hydrophobic interaction between the stationary phase and mobile phase and enhanced transfer of the solutes through the mobile phase due to higher solubilities in the mobile phase. An elevated column temperature of 45 °C was employed by Kwon and Kim in the separation of mono- and oligosaccharides derivatized with *p*-aminobenzoic ethyl ester (*G78*). Oligosaccharides containing (1→4) linkages were retained longer than those containing (1→6) linkages. Robles et al. separated a variety of agar-type polysaccharides according to their degree of polymerization at an optimal column temperature of 30 °C (*G79*). Arghavani and Romano demonstrated enhanced resolution of synthetic oligonucleotides containing a high guanine content using a PRP-1 column in RPLC, relative to the more traditionally used IEC (*G80*). The addition of 20% formamide to the sample loading buffer in these separations inhibited intra- and intermolecular complexations that are characteristic of these oligonucleotides and did not interfere chromatographically. Wang and Barofsky studied the effect of column temperature on the separation of glycopeptides in RPLC and found no general trends (*G81*). The authors suggested that the

experimenter investigate a variety of temperatures for each glycopeptide mixture in order to optimize the corresponding separation.

Comparison studies were performed on various stationary phases for the separation of peptides and proteins in RPLC. Jedrzejewski and Taylor compared silica-, alumina-, and polymer-based stationary phases (Unisphere-PBD, gRP-1, PRP-1, Deltabond C8) for the separation of a variety of analytes, including peptides (*G82*). Many chromatographic parameters were examined including peak asymmetry, selectivity, reduced plate height, column flow resistance, total porosity, the Knox-Parcher ratio, and linear velocity of mobile phase; each stationary phase demonstrated characteristic chromatographic properties. Ricker et al. compared the selectivity of peptides and proteins on CN- and C3-bonded silica stationary phases that were protected by sterically bulky side groups vs more conventional longer chain stationary phases (*G83*). The former stationary phases were less susceptible to hydrolysis and high-temperature conditions.

Several investigators optimized peptide and protein separations in RPLC by altering the mobile-phase composition, especially with the addition of ion-pairing agents. For instance, the addition of dimethylformamide to an acetonitrile/water mobile phase resulted in high recovery and purity values for protected peptides, including peptides containing a long segment of relatively high hydrophobicity (*G84*). Barbosa optimized mobile-phase pH and organic modifier content for the separation of low-molecular-weight peptides according to Reichardt's ETN scale of solvent polarity and the Kamlet−Taft multiparameter solvent scale (*G85*). De Frutos et al. applied the linear solvent strength model to study the chromatographic behavior of bovine whey proteins in RPLC (*G86*). Chromatographic retention and band broadening were evaluated as a function of parameters of the applied mobile phase gradient.

Hoffmann et al. compared the effects of the ion-pairing agents trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) on the separation of unphosphorylated, monophosphorylated, and diphosphorylated synthetic peptides in RPLC (*G87*). Separation parameters were dependent on hydrophilic interactions with the use of TFA and dependent on hydrophobic interactions with the use of HFBA. Separation of unphosphorylated and monophosphorylated peptides using these conditions yielded sharp chromatographic peaks, especially with the use of TFA; however, diphosphorylated peptides were characterized by broadened chromatographic peaks. Additionally, Pearson and McCorksey demonstrated the advantages of perfluorinated carboxylic acids (pentafluoropropionic acid, heptafluorobutyric acid, perfluoropentanoic acid, perfluorohexanoic acid, perflouroheptanoic acid) as ion-pairing agents relative to the use of TFA in the separation of peptides and proteins in RPLC (*G88*). Likewise, Siebert et al. applied pentafluoropropanoic acid and heptafluorobutyric acid as ion-pairing agents for the separation of platinum(II) complexes of methionine- and histidine-containing peptides (*G89*). The retention of the peptides was a function of their respective charge, coordination number, and hydrophobicity, whereas selectivity of separation was controlled by varying the concentration and chain length of the ion-pairing agent. Cole and Dorsey observed an enhancement in the separation of peptides using cyclohexylamine as an ion-pairing agent relative to the use of triethylamine or TFA for the separation of peptides in RPLC (*G90*). Gazdag et al. employed either hexanesulfonate or perchlorate as ion-pairing agents for the separation of the synthetic peptides thymotrinan and thymocartin in RPLC (*G91*). Optimization of chromatographic conditions enabled separation of diastereomeric and isopeptide derivatives. Hodges and co-workers studied the effect of sodium perchlorate as a mobile-phase additive at low pH for the RPLC separation of helical and nonhelical peptides containing positively charged amino acids (*G92*). Ion pairing of the peptides with the negatively charged perchlorate ion decreased peptide hydrophilicity, resulting in increased chromatographic retention and enhanced resolution.

Ion Exchange. Predictive models and techniques involving IEC were presented. Staahlberg and Joensson augmented the "slab model" that describes the interaction between a protein and a charged stationary-phase surface to include the effect of charge regulation on the capacity factor (*G93*). New retention data were analyzed, representing a more realistic aspect of such separations, where the net charge of the protein changes as it approaches the oppositely charged electrical field of a charged stationary phase. Yamamoto introduced a method for predicting chromatographic plate height from elution profiles in IEC of proteins in the linear gradient mode (*G94*).

The separation of immunoglobulins (Ig's), nucleic acids, and oligosaccharides in IEC involving either enhanced or alternative selectivity was demonstrated by several investigators. Yang and Harrison investigated the use of IEC for the purification of Ig's as opposed to the more commonly used affinity chromatographic techniques (*G95*). Five IgG antibodies were separated on strong cation-exchange, strong anion-exchange, and weak anion-exchange columns as a function of experimental parameters, to probe the separation mechanism involved.

Yamakawa et al. demonstrated the utility of a nonporous anion-exchange resin (DNA-NPR) for the separation of DNA fragments up to 50 base pairs in length according to both size and sequence (*G96*). Both high resolution and recovery were achieved for the DNA fragment mixtures. Yamazaki et al. optimized the separation of the 1-kbp DNA standard on the same support according to amount of sample loaded, mobile-phase flow rate and gradient time, and temperature (*G97*). The optimized chromatographic system was effective in the separation of tRNAs and oligonucleotides. Tomizawa et al. applied the DNA-NPR resin to the separation of antisense phosphorothioate oligonucleotides and polymerase chain reaction products of cDNA from hepatitic C virus and human immunodeficiency virus (*G98*). Superior resolution of the DNA products was achieved on this anion-exchange resin relative to their separation on a reversed-phase stationary phase. Separation of peptide nucleic acid oligomers, even oligomers with the same base composition but different sequences, was achieved on a RPC-5 anion-exchange column (*G99*).

As an alternative to silica- and organic polymer-based anion-exchange resins, Simms et al. applied a β-cylcodextrin column for the separation of oligogalacturonic acids (*G100*). The separation selectivity of oligosaccharides of varying degrees of polymerization was optimized as a function of the sodium acetate-based mobile-phase composition in either the isocratic or the gradient mode. The separation of neutral N-linked oligosaccharides was achieved using high-pH anion-exchange chromatography (*G101*).

Similar to the separation of sialylated oligosaccharides, the neutral species eluted according to monosaccharide substitution. Corradini et al. synthesized a new polymer-based, strong anion-exchange resin for the separation of carbohydrates at high pH (*G102*). Highly cross-linked styrene−divinylbenzene copolymer beads were functionalized with quaternary amino groups; separations were optimized as a function of the ionic strength of the mobile phase. Cataldi et al. investigated the effect of the divalent cations Ba^{2+}, Sr^{2+}, and Ca^{2+} in alkaline mobile phases for the anion-exchange separation of carbohydrates and alditols (*G103*). Peak symmetry and separation efficiency were enhanced due to the ability of the cations to complex with the polyhydroxy compounds.

Loidl-Stahlhofen et al. introduced the concept of thermodynamically controlled charge-selective peptide and protein separations in LC (*G104*). Temperature gradients are applied in order to alter the phase transition between the fluid and the gel phases of phospholipid bilayers attached to silica supports and, hence, alter chromatographic selectivity. Hearn and co-workers studied the effects of temperature on the chromatographic band broadening of peptides and proteins using "tentacle-type" LiChrospher-1000 SO^{3-} and Polysulfoethyl A cationic-exchange adsorbents in the gradient elution mode of LC (*G105*, *G106*). Distinct chromatographic behavior was observed for each adsorbent. One noteworthy observation was that band broadening, presumably associated with conformational unfolding of the analyte, was more prominent on the "tentacle-type" adsorbent at higher temperatures where hydrophobic interactions dominate. These studies support the hypothesis that "tentacular" ligands provide a greater contact area and multipoint binding with the biomacromolecules.

Lui and Anderson developed the technique of gradient chromatofocusing, employing both internal and external linear pH gradients in conjunction with weak anion-exchange supports (*G107*). Practical advantages were demonstrated vs conventional modes of separation for fibrinogen degradation products, including cost-effectiveness of more common buffer components and ease of adjustment of the slope of the gradient. In a related investigation, these researchers presented the theoretical aspects of gradient chromatofocusing (*G108*). Computer simulation modeling and derived equations revealed the versatility of this technique in terms of experimental optimization. Strong and Frey demonstrated chromatofocusing of protein mixtures on a strong anion-exchange resin (Q Sepharose FF) using simple monovalent buffering components to create an internal pH gradient (*G109*). Mathematical simulations of the experimental results were used in a predictive mode to design preparative chromatofocusing systems. Dubinina et al. studied the effects of gradient times on protein separations in IEC (*G110*). The focus of this investigation was the relationship between the gradient steepness and the working length of the column, where quasi-steady-state conditions are achieved.

In a comprehensive investigation, Levison et al. evaluated the physical properties and chromatographic performance of 70 different ion-exchange stationary phases based on a variety of support materials for the separation of proteins (*G111*). As the chromatographic performance varied greatly from medium to medium, the authors recommended adequate screening of chromatographic materials in order to achieve optimal separation parameters. Weaver and Carta compared protein adsorption on the macroporous adsorbent, Poros 50 HS (a styrene−divinylbenzene copolymer), relative to S-HyperD (high-porosity polystyrene-coated silica particles with polyacrylamide-based hydrogel-filled pores) in cationic-exchange chromatography (*G112*). The macroporous adsorbent demonstrated a higher diffusivity and higher uptake of the model protein lysozyme, but overall, the gel composite adsorbent provided superior chromatographic performance. Adachi et al. demonstrated the utility of sulfopropyl ProtEx-SP and diethylaminoethyl ProtEx-DEAE columns for the separation of cytcochrome *c* variants and deaminated isoforms of human growth hormone, respectively, with no irreversible adsorption (*G113*). The highly favorable chromatographic properties of the columns were attributed to the uniformity of the particles and hydrophilicity of the functional ligands. Levison et al. observed slower desorption kinetics for agarose- vs cellulose-based anion-exchange media (Express-Ion Exchanger Q vs Q-Sepharose Fast Flow) in the separation of hen egg white proteins (*G114*). In addition, chromatographic resolution was monitored as a function of mobile- phase flow rate.

Gustavsson and Larsson synthesized and characterized super-agarose spherical chromatographic particles; the particles were derivatized subsequently with polyethyleneimine for use as an ion-exchange support for the separation of three model proteins (*G115*). Due to the presence of superpores, or flow pores, in addition to normal diffusion pores, improved mass transfer enabled higher efficiencies in these separations relative to the use of homogeneous materials of the same particle size. Yang et al. derived a new strong cation-exchange resin from a hydrophilic polystyrene−divinylbenzene-based resin for protein separations (*G116*). The new stationary phase demonstrated highly efficient and rapid protein separations and high chemical stability upon exposure to strongly basic or strongly acidic elution conditions. McCreath et al. prepared poly(vinyl alcohol)-coated perfluoro polymer particles for subsequent derivatization with anion-exchange (DEAE and Q) and cation-exchange (SP) functional groups; the new supports displayed high resolution of proteins differing slightly in their isoelectric points (*G117*).

The advantages of lower molecular weight displacer molecules have been realized in IEC. Chen and Scouten demonstrated the utility of hydrolyzed l-carrageenan (5.4−48 kDa) as an efficient displacer molecule in anion-exchange chromatography for the separation of β-lactoglobulins A and B (*G118*). In general, the lower molecular weight displacers yielded better separation parameters; the displacer molecules of higher molecular weight were more effective at lower concentrations. Kundu and co-workers introduced protected amino acid esters of arginine and lysine as low-molecular-weight displacers in the cation-exchange chromatographic separation of proteins (*G119*, *G120*). The practical and fundamental advantages of the low-molecular-weight displacers was shown to be a function of the concentration of the displacer and the initial ionic strength of the mobile phase. Additionally, Gallant and Cramer applied a low-molecular-weight displacer, neomycin sulfate, in IEC (*G121*). Both experimental and theoretical investigations were conducted, the latter employing the steric mass action model. The use of the low-molecular-weight displacer molecules provided adequate selectivity and comparable

displacement relative to the more commonly employed high-molecular-weight displacers.

Size Exclusion. Ricker and Sandoval generated a set of practical guidelines for the SEC separation of biomacromolecules, such as antibodies and proteins (*G122*). In general, minimal sample volumes and low flow rates as well as longer columns (or columns in tandem) provided optimal chromatographic resolution, but overall, experimental conditions were highly dependent on the analyte of interest. Fournier et al. coated porous polystyrene−divinylbenzene beads with phenoxy-dextran and subsequently chemically cross-linked the functionalities; these new size-exclusion supports were used in the separation of proteins in aqueous media (*G123*). Ferreira et al. compared two size-exclusion supports, Superose 6 and Sephacryl S1000, for the separation of supercoiled plasmid DNA (*G124*). The latter support was superior in terms of resolving individual plasmid conformers.

Preparative Separations. Theoretical treatments and experimental investigations of preparative chromatography received moderate attention during the review period. Gagnon and Grund summarized the unique effect of high salt concentrations on preparative HIC separations (*G125*). Kaltenbrunner and Jungbauer developed a model to describe step mobile-phase gradient control in preparative HIC and IEC (*G126*). The model was in agreement with experimental results involving the in-line mixing of two salt buffers; effects of buffer pH were excluded from this model. Raje and Pinto developed a model for IEC of proteins under overload conditions that employs both the steric mass action and nonideal surface solution models (*G127*). Salt−salt interactions on the support surface greatly affected protein adsorption. Gallant et al. utilized the steric mass action model and mass balance equations to predict linear gradient separations of proteins in IEC (*G128*, *G129*). Jungbauer and Kaltenbrunner optimized protein separations in preparative IEC using computer-aided process design (*G130*). The distribution coefficient, *K*, a function of both protein and salt concentration, was derived from chromatographic experiments and related to other experimental parameters for both single-component and multicomponent systems. Luo and Hsu optimized gradient elution parameters for protein separations in IEC based on chemical engineering concepts (*G131*). The time-saving and economical optimization methodology involved two aspects; first, experimental data from isocratic runs were utilized, and second, the resolution optimization factor was developed in the gradient elution mode. Frey developed a local-equilibrium theory to investigate protein retention in preparative strong anion-exchange chromatography and aid in the choice of elution gradient conditions (*G132*). Hossain and Do modeled the displacement chromatographic separation of denaturing proteins in the presence of an impurity according to both first-order kinetics and competitive Langmuir isotherms (*G133*). Simulated chromatograms generated as a function of experimental parameters revealed conditions for optimal chromatographic performance.

From a more experimental standpoint, Hellberg et al. characterized three Superdex preparative grade chromatographic supports according to chromatographic selectivity, amount of charged functionalities, hydrophobicity, and functional stability (*G134*). The three dextran-coated agarose supports, primarily employed in the separation of peptides and proteins, demonstrated characteristically different properties. Svec and Frechet prepared porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) rods that were subsequently derivatized in situ with weak ion-exchange 1-*N*,*N*-diethylamino-2-hydroxypropyl groups (*G135*). The chromatographic separation of protein mixtures and yeast enzymes was demonstrated; an especially high dynamic capacity (>420 mg) for bovine serum albumin was observed. Eriksson et al. introduced a rigid phenyl-bonded silica stationary phase for both preparative and semipreparative separations of proteins in HIC (*G136*). Feng et al. cited the effects of sample solvent on the chromatographic peak shape of the proteins cytochrome *c* and lysozyme under sample overload conditions (*G137*). Two peaks were observed for a single protein when the sample solvent contained a lower concentration of salt than the starting mobile phase of the gradient. The retention time of the less retained peak shifted to a greater extent relative to the more retained peak as a function of injection volume. Staby and Mollerup studied the chromatographic retention of egg white lysozyme in semipreparative HIC using perfusion stationary phases (*G138*). A database was constructed of chromatographic retention as a function of ammonium sulfate concentration in the mobile phase, mobile-phase pH, and isocratic vs gradient modes of elution. The capacity factor was correlated to the activity coefficients of the protein, both in the mobile phase and in contact with the stationary phase.

AFFINITY CHROMATOGRAPHY

Reviews and Theoretical Models. Many review articles involving affinity chromatography, ranging in both scope and depth, were retrieved during the review period. The historical aspects, current technology, and future utility of affinity chromatography were addressed by Lowe (*H1*). According to this author, advances in future affinity chromatographic applications point toward the development of new chromatographic supports that can withstand relatively harsh chemical treatment and yet retain appropriate chromatographic properties. Johnson and Arnold provided an overview of multiple interaction adsorption of engineered proteins in affinity chromatography (*H2*), whereas Liapis and Unger reviewed both chemical and engineering aspects of the technique (*H3*). The affinity purification of proteins (*H4*) and therapeutic protein products (*H5*) was reviewed by Burton; the affinity purification of recombinant proteins was reviewed by Hentz et al. (*H6*). The general use of cyclic antibiotics as affinity ligands for the purification of proteases was discussed by Ibrahim-Granet and Bertrand (*H7*). An interesting mode of biotin−streptavidin affinity chromatography that involves elution via ultraviolet irradiation was reviewed by Thiele and Fahrenholz (*H8*) and Fabry and Brandenburg (*H9*). This relatively gentle elution technique may be applied to particularly labile proteins without observable loss of binding affinities.

Van Oss discussed the long-range, noncovalent interactions, as well as the origin of enthalpy−entropy compensation in antigen−antibody systems as they relate to affinity chromatography (*H10*); whereas Cutler reviewed the practical aspects of immunoglobulin separations (*H11*). Price and Beyzavi summarized the use of immunoaffinity chromatography, including general principles of the technique, and the options that exist in the selection of supports, ligands, spacers, and activation chem-

istries (*H12*). Lectin affinity chromatography was reviewed by Endo (*H13*) in the purification and structural elucidation of oligosaccharides, as well as by West and Goldring (*H14*) for the purification of glycoproteins. The use of protein affinity chromatography to identify DNA replication products was addressed by Wittmeyer and Formosa (*H15*).

Several researchers reviewed various aspects of immobilized metal ion affinity chromatography (IMAC) and dye−ligand affinity chromatography. Yip and Hutchens discussed the methods of IMAC in general (*H16*). Andersson focused on the chemical structure, metal binding capacities, and nature of interactions involved in the separation of peptides and proteins (*H17*). Jack and Beer reviewed the principles of IMAC for the purification of antigens using immobilized antibodies (*H18*). Holmes and Schiller highlighted advances in the Fe^{3+}-IMAC purification of biomolecules, especially those containing phosphate, carboxylate, and phenol functionalites (*H19*). Xiao et al. reviewed the application of porphyrin-based silica stationary phases in the IMAC mode for the separation of peptides and proteins (*H20*). The purification of proteins using dye−ligand affinity chromatography was reviewed by Worrall (*H21*), whereas new innovations in dye−ligand affinity chromatography were reviewed by Garg et al. (*H22*). In the latter review, dye molecules that decrease nonspecific protein adsorption were emphasized.

Sridhar employed mathematical models of film mass transfer and intraparticle diffusion as a function analyte concentration, ligand content, and particle diameter in order to compare batch and fixed-bed affinity chromatography performance (*H23*). Orthogonal collocation of these parameters revealed that fixed-bed adsorption processes were more efficient than batch adsorption processes. The exceptional case to this finding was in the low-feed mode where the batch adsorption process was more advantageous. Mingalyov and Fadeev proposed a descriptive model of the biospecific adsorption of proteins on rigid silica supports; biospecific adsorption of a protein decreased as the surface concentration of the attached ligand increased (*H24*). Mathematical modeling was also employed by Lawden et al. to predict specific ligands that would demonstrate high binding affinities for lipid A (*H25*). Hydrophobic interactions with lipid tails and electrostatic interactions with negatively charged phosphate headgroups of the lipid were implicated in the binding of the ligands. Tejeda-Mansir et al. modeled the regeneration of a protein A affinity column (*H26*). Breakthrough curves from protein adsorption on a fixed-bed column were obtained experimentally and analyzed to determine rate constants of protein adsorption. Overall, adsorbent selectivity decreased with increased exposure to sodium hydroxide. The authors suggested that these results be applied to the design and optimization of future protein A affinity chromatographic applications. Similarly, Noriega et al. modeled column regeneration in dye−ligand affinity chromatography via analysis of breakthrough curves of lysozyme and bovine serum albumin (*H27*). Various chemical treatments and elution times directly affected protein adsorption capacity and adsorbent selectivity, but not protein binding affinity.

Theoretical treatments involving IMAC were presented. Goud et al. developed a procedure to identify peptides to be used as more efficient affinity "tails" in IMAC (*H28*). Peptides were chosen from a combinatorial library according to relative affinities with cellular proteins from the expression host of the target protein. Kim developed a theoretical model based on experimental results to predict the effects of mobile-phase-modifier gradient parameters in IMAC separations (*H29*). The results of this investigation facilitate the understanding of complex metal affinity binding, future methods development, and overall optimization of separation. Patwardhan et al. formulated a mathematical model for IMAC of protein−metal binding, film mass transfer, effective protein diffusivity, flow rate of eluent, and chromatographic ligand density (*H30*). By constructing breakthrough curves and determining pH dependencies of elution for various proteins, optimal chromatographic parameters could be predicted. Vunnum and Cramer employed the metal ion affinity interaction model in addition to chromatographic transport equations to investigate the nonlinear chromatographic elution of proteins (*H31*). Langmuir and double-plateau profiles were observed with isocratic elution of low- and high-mobility modulators, respectively. Loading of proteins in the absence of modulators yielded higher throughput and protein concentrations during elution. Johnson et al. constructed a thermodynamic model for the multipoint attachment of engineered proteins to randomly distributed binding sites on a surface; lateral interactions between adsorbed protein molecules were included in the model (*H32*). These results correlated with those predicted by the Temkin and stoichiometric displacement models; a correlation between the Temkin model and the adsorption of cytochrome *c* variants in IMAC was demonstrated.

Affinity Supports. Several reports focused on the use of natural products as support media. Gustavsson et al. applied superagarose as a support for affinity chromatography (*H33*). In addition to nominal pores, the support contains "superpores" ($^1/_{10}$−$^1/_3$ of the particle diameter in size) which allow for enhanced mass transfer of analytes. Nicotinamide adenine dinucleotide (NAD) and protein A were independently attached to both the superagarose beads and homogeneous agarose beads; the former provided 3−5 times reduction in analysis time. Yang et al. demonstrated the utility of a commercially available Sepharose-based IMAC resin, Talon, which contains a tetradentate chelating agent for the subsequent immobilization of Co^{2+} (*H34*). The resin demonstrated enhanced resolution of (histidine)6-tagged proteins as well as high sample loading capacity, relatively mild elution conditions, and more efficient protein elution. Beads of chitosan, a naturally occurring deacylated derivative of chitin, were investigated for their ability to covalently and physically bind the triazine dye Procion Red HE-3B for the purification of proteins (*H35*). These chitosan beads possess a heterogeneous surface but appear to be a viable alternative to the more expensive commercially available agaroses with respect to dye−ligand immobilization. Shi et al. immobilized chicken ovomucoid on chitosan beads via a glutaraldehyde linkage for the affinity chromatographic separation of trypsin (*H36*). Experimentally, the new supports demonstrated high mechanical strength, were stable upon storage, and could be reused. Preparation of the functionalized supports was facilitated relative to the use of a Sepharose matrix. Yoshida and Fujiwara developed chitosan/dextran DEAE composite matrixes for the separation of proteins (*H37*). This new packing material demonstrated a high, pH-dependent adsorption capacity for bovine serum albumin.

Many reports documented the usefulness of acrylamide- and acrylate-based supports in affinity chromatography. Cocker et al. introduced spherical composite beads of polyacrylamide/magnetite for use in magnetically stabilized fluidized-bed chromatography (*H38*). The physical strength of the beads was comparable to that observed for polyacrylamide gels. Bead diameters ranged from less than 60 mm up to 600 mm according to the reaction conditions employed during the suspension polymerization method, and the beads had a high magnetism at a relatively low applied field strength (60 mT). Soybean trypsin inhibitor was immobilized onto the beads via a carbodiimide linkage and the affinity separation of chymotrypsin from trypsin was demonstrated. Hemoglobin, cytochrome *c*, and transferrin were molecularly imprinted into a copolymer of acrylamide and *N*,*N*′-methylenebisacrylamide (*H39*). Each protein was selectively retained and could be eluted using 0.5 M NaCl or 10% acetic acid containing 10% SDS. Molecular imprinting was also employed by Matsui and Takeuchi in the preparation of a nicotine-selective affinity rod-type stationary phase (*H40*). The use of 2-(trifluoromethyl)acrylic acid as a functional monomer provided superior selectivity vs the use of methacrylic acid. In a separate report, rods of porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) molded within a chromatographic column were derivatized with trypsin (*H41*). Comparison studies were performed with poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) beads derivatized with the same enzyme. The "molded" supports provided enhanced resistance to mass transfer at high flow rates. Mislovicova et al. demonstrated the advantages of filling poly(hydroxyethyl methacrylate) gels with dextran for the Cibacron Blue 3G-A affinity ligand chromatographic separation of proteins (*H42*). Overall, higher binding capacities and higher protein recoveries were observed. Denizli et al. copolymerized ethylene glycol dimethacrylate (EGDMA) and 2-hydroxyethyl methacrylate (HEMA) in aqueous media to produce poly(ethylene glycol dimethacrylate) microbeads (150−200 μm in diameter) for use as a support in metal chelate affinity chromatography (*H43*). Congo Red was attached to the microbeads for the specific adsorption of Cd^{2+} ions. The stationary phases were characterized using optical microscopy, spectroscopy, and elemental analysis. Adsorption investigations of bovine serum albumin at various pH and ionic strength conditions revealed negligible nonspecific adsorption to the microbeads.

Staak et al. demonstrated the usefulness of polystyrene as an affinity matrix for the purification of antibodies (*H44*). Bovine immunoglobulins were immobilized on the solid supports for the purification of anti-bovine Ig. The new supports were characterized using radial immunodiffusion and ELISA procedures. Comparable purification levels, but significantly lower yields of the antibodies, were achieved on the polystyrene-based supports, relative to the use of conventional agarose beads. The new supports offered the additional advantages of enhanced stability, immunological inertness, and low cost. Reports of modified poly(styrene−divinylbenzene) supports for use in affinity chromatography have appeared as well. Porous poly(styrene−divinylbenzene) beads were coated with phenoxy-dextran, chemically cross-linked, and subsequently functionalized with an affinity ligand for the purification of trypsin (*H45*). Nash et al. modified a poly(styrene−divinylbenzene) chromatographic matrix (CG1000-sd) with poly(vinyl alcohol) (PVA) and subsequently covalently attached the dye Procion Blue MX-R for protein purification in affinity chromatography (*H46*). According to BET nitrogen adsorption−desorption analysis, the PVA fills the micropores of the matrix, but not the macropores, for enhanced stability during sodium hydroxide solution rinsing. Prior to the dye immobilization, no nonspecific adsorption of proteins to the new modified matrixes was observed. In a separate study, these researchers investigated the effects of the nature of PVA cross-linking and the attachment of different dyes; irreversible binding of the model analyte human serum albumin decreased as the extent of cross-linking of PVA increased, and different dye molecules produced different adsorption characteristics (*H47*). In addition to CG1000-sd matrixes, PLRP4000s matrixes were modified by the adsorption of cross-linked PVA in order to investigate the effects of pore size and particle diameter on mass-transfer rates (*H48*).

Siliceous supports have received considerably less attention than their polymeric counterparts as supports in affinity chromatography. Dawidowicz et al. prepared protected siliceous supports for subsequent attachment of affinity ligands (*H49*). A layer of cross-linked dextran and polyimine, instead of the more conventional DEAE-dextran, was deposited above the solid support. The authors suggested that the polymeric layer could be customized according to the chemical cross-linking or chemical modification desired. The recent trend of applying sol−gel technology to separation science has now been extended to immunoaffinity chromatographic supports. Cichna et al. immobilized IgG from antipyrene antiserum in the pores of a silica gel (sol−gel) matrix (*H50*). The columns were applied to the analysis of pyrene in aqueous samples. Selectivity of the columns was compromised by the adsorption of polyaromatic hydrocarbons. A phosphate-buffered saline eluent containing high-molecular-weight poly(vinyl pyrrolidone) and a nonionic surfactant was employed to achieve optimal selectivity.

Several novel affinity supports were also introduced during the review period. Dong et al. applied graphite fibrils (*0.01 × 1−10 μm, concentric layers of graphite) for the affinity purification of proteins (H51*). Horseradish peroxidase, β-galactosidase, and alkaline phosphatase were each covalently attached to carboxylated fibrils via an amino linkage without loss of catalytic activity. Morgan et al. investigated iminodiacetic acid (IDA) functionalized nonporous perfluorocarbon supports for Zn^{2+} chelate affinity chromatography (*H52*). Three distinct perfluorocarbon matrixes were evaluated according to ligand density and binding capacity of a monoclonal antibody to the Zn^{2+}−IDA functionality. Ji et al. derivatized superparamagnetic beads with a hydrocarbon chain ($n = 6$) and nitriloacetic acid for the metal−chelate affinity purification of relatively hydrophobic or labile peptides and proteins (*H53*). The magnetic beads were also particularly suited for purification of limited quantities of the peptides and proteins and displayed less nonspecific adsorption in comparison with a commercially available nickel chelate affinity support.

Affinity Elution Conditions. Several investigations have focused on the use of alternative elution conditions for enhanced chromatographic performance in affinity separations. Mattiasson et al. employed water-soluble polymers, such as poly(vinylpyrrolidone) or PVA, in dye−ligand affinity chromatography of crude protein extracts to shield nonspecific chromatographic interactions (*H54*). Purcell and Wallace added substrates of pyruvate car-

boxylase to the mobile phase for monomeric avidin affinity chromatography (*H55*). The authors propose that the presence of such compounds alters the accessibility of the biotin label on the protein, resulting in significant improvements in chromatographic yield. In a quest for a universal elution buffer for efficient immunoaffinity chromatography of monoclonal antibodies, Ben-David and Firer demonstrated the general applicability of 4 M $MgCl_2$ in 25% ethylene glycol (*H56*). Hale designed affinity stationary phases where antibodies could be reversibly bound to a cobalt−iminodiacetate resin according to the oxidation state of the cobalt ion (*H57*). Therefore, elution of the antibodies could be conducted under relatively mild conditions (conditions causing reduction of the cobalt ion), in the absence of high salt concentrations, detergents, metal chelating reagents, or chaotropic agents.

The specific effects of various elution conditions in IMAC were addressed by many research groups. Patwardhan and Ataai observed anomalous behavior in the elution of bovine serum albumin during IMAC on a chelating Superose matrix; a significant increase in capacity was observed near the elution pH for this protein (*H58*). Due to an apparent low degree of accessibility of the copper chelating site, the estimated saturation capacity of the column, according to the plateau regions of breakthrough curves, could appear to be much lower than the true saturation capacity. This phenomenon is more likely to be observed in the analysis of relatively large proteins or for highly cross-linked matrixes where copper sites become less and less accessible. The impact of high ionic strength conditions on binding of hen egg white lysozyme on a Cu^{2+}−IDA Sepharose CL-4B support was demonstrated by Jiang and Hearn (*H59*). The Langmuir model was observed at NaCl or KCl concentrations less than or equal to 0.2 M, whereas the Freundlich−Langmuir model was followed at concentrations greater than or equal to 0.5 M. These interactions increased with increasing salt concentration and are indicative of heterogeneous, multipoint attachment of the protein to the support. The authors suggested that these results be extended to preparative-scale applications. Lee and Chuang studied, both experimentally and theoretically, the effect of mobile-phase buffer pH on the elution of proteins from a nonporous silica support (1.4 mm in diameter) functionalized with the affinity ligand, protein A (*H60*). Using this technique, the elution pH altered both the association and desorption constants, as well as chromatographic elution time, peak shape, peak height, and sample loading capacity. Johnson et al. studied independently the effects of an imidazole mobile-phase gradient, in addition to mobile-phase pH, on the elution of proteins from an IMAC support (*H61*). The imidazole mobile-phase gradient allowed the separation of cytochrome *c* variants differing only in histidine content and location. The study of the effect of mobile-phase pH (especially above pH 7.5) on elution revealed that, in addition to surface histidine functionalities, additional surface amines are responsible for the chromatographic retention of the cytochrome *c* variants. In addition to imidazole, Cramer and co-workers investigated N-protected histidines and tryptophan as metal affinity chromatographic displacers of proteins (*H62*). Imidazole is a small molecule with low steric hindrance due to single site coordination; however, when used in the appropriate concentration range, imidazole is highly efficient in the displacement of multicoordinated protein molecules. N-Protected histidines as protein displacer molecules yielded distinct protein elution profiles, whereas tryptophan yielded less distinct elution profiles with significant peak tailing.

Support Activation: Methodology and Characterization. Benes et al. developed a new synthetic procedure for enhanced reactivity of the diazonium functionality with phenol-, imidazole-, thiol-, or amine-containing affinity ligands (*H63*). A diazotizable amine precursor was prepared resulting in a SO_2 group in the para position relative to the diazonium group *after* diazotization. "Reversed" diazonium coupling was employed to investigate the reactivity of the enhanced derivatization chemistry, where the support has been derivatized with reactive functional groups and the "ligand" contained the diazonium groups. In another investigation, the hydroxyl groups of Sepharose CL-4B and Fractogel HW75F were activated by bis(4-nitrophenyl)carbonate (BPNPC) for subsequent attachment of amines (*H64*). The affinity ligands containing amines could be attached to the BPNPC-activated supports at ambient temperature in pH 7−10 buffers within 30 min. Alvarez et al. synthesized new pseudobiospecific stationary phases for affinity chromatography. Poly(butadiene−hydroxyethyl methacrylate) supports were activated with either epichlorohydrin (ECH) or 1,4-butanediol diglycidyl ether (BDGE) (*H65*). Histidine, Cu^{2+}- and Zn^{2+}-chelating IDA, and Cibacron Blue F3GA were subsequently attached as ligands. In a separate report, the hexamer and monomer of urease were independently immobilized on several different supports for affinity chromatography, yielding varied immobilization efficiencies and enzymatic activity; the optimal spacer length was determined to be 10−15 atoms for these ligands (*H66*).

Zumbrink et al. applied solid-state ^{13}C CP/MAS NMR spectroscopy to the analysis of carbonyldiimidazole- and tresyl chloride-activated agarose and silica gels (*H67*). For carbonyldiimidazole-reacted supports, the spectra agreed with previously reported reaction mechanisms. The reaction mechanism between the novel tresyl chloride-activated supports and alkylamines was revealed spectroscopically; however, the reaction mechanism for the attachment of alkanethiols to the tresyl chloride-activated supports could not be confirmed. FT-IR spectroscopy, electron microscopy, and magnetic measurements were used by Mehta et al. to confirm the attachment of bovine serum albumin to magnetite particles previously activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (*H68*). Scoble and Scopes developed a titration assay to determine the number of reactive groups on affinity chromatography gels resulting from ECH and divinyl sulfone activation chemistries (*H69*). The results of the assay were applied to control and optimize activation experimental parameters such as reaction pH and reaction time.

Affinity Ligand Immobilization, Characterization, and Applications. Fundamental investigations involving IMAC were conducted by several research groups. Hearn and co-workers determined potentiometrically the acid−base dissociation constants of three metal ion chelating ligands, IDA, *O*-phosphoserine, and 8-hydroxyquinoline, and the stability of their immobilized metal complex counterparts (*H70*, *H71*). Immobilization of the ligands alters their electron-withdrawing properties and, ultimately, the stability constants of their respective metal−ligand complexes. The new affinity supports developed for the purification of peptides and proteins exhibited alternative metal ion:ligand (M:L) binding stoichiometries as a function of buffer composition, ionic strength,

and pH. More specifically, $M(OH)_mL_n$ binding stoichiometries (m or $n > 1$) were observed as opposed to the simple 1:1 M:L or 1:2 M:L types. These studies should facilitate the understanding and prediction of binding behavior of proteins or peptides in IMAC for a variety of elution conditions. Diez et al. investigated the affinity of the model analyte glycine for a chromatographic resin covalently derivatized with a 8-hydroxyquinoline:Pd^{2+} complex (*H72*). Experimental and theoretical studies were in agreement for the partitioning and the elution of the amino acid from the "soft" metal ion affinity column. The authors suggested that this chromatographic system be extended to the separation of amino acid mixtures. Liesiene et al. showed that desorption of human growth hormone in IMAC could be controlled via ligand density of a cellulose-based support containing Cu^{2+} (*H73*).

Several investigators employed new stationary-phase supports for the separation of amino acids, peptides, and proteins in IMAC. Hearn and co-workers immobilized the "cis" and "trans" isomers of *N*-(carboxymethyl)-4-amino-L-proline as tridentate metal ion chelators on an epoxy-activated Sepharose CL-4B support (*H74*). The effect of ligand chirality on chromatographic selectivity of human serum proteins was evaluated from pH 5 to 9; adsorption capacities and dissociation constants (K_d) were evaluated using horse heart myoglobin. Chromatography employing the "cis" vs the "trans" conformation of the ligand yielded similar protein binding properties but higher adsorption capacities and K_d values. Leibler et al. immobilized various salicylaldehyde−copper−amino acid complexes as affinity ligands for protein separations (*H75*). In some instances, the new chelate ligands demonstrated enhanced selectivity over more conventional IDA supports. Xiao and Meyerhoff covalently attached metalloprotoporphyrins (MProP) to silica for IMAC of amino acids and peptides (*H76*). Peptides containing L-histidine or aromatic amino acids (L-tryptophan and L-phenylalanine) were retained via metal−nitrogen axial ligation or p−p interactions, respectively. The former interaction was highly dependent on the metal ion incorporated in the metalloprotoporphyrin; supports containing Fe^{3+} yielded the strongest interaction. The authors observed an unusually strong retention of the metal ion to the ProP affinity ligand upon excessive washing of the column with 50 mM EDTA. A heterobifunctional affinity chelating ligand with biotin and IDA attached to a single poly(ethylene glycol) molecule was applied in IMAC by Ehteshami and co-workers (*H77*). The bifunctionalized supports were found to have comparable binding constants with copper and avidin, relative to their unconjugated counterparts.

Heparin-immobilized affinity supports were the focus of several investigations. Bjoerklund and Hearn covalently attached heparin to amino-functionalized silica supports of varying pore size and surface area (*H78*). LiChroprep Si60 silica supports (60 Å, 500 m^2/g) demonstrated chromatographic properties similar to nonporous supports due to the exclusion of heparin from the pores during ligand immobilization. Fractosil 1000 silica adsorbents (1000 Å, 20 m^2/g) yielded relatively lower ligand binding densities but greater binding site accessibilities. Using a new 96-well microtiter plate-based quantitative absorption spectroscopic assay, ligand leaching from the supports was found to be minimal at pH 5. These new adsorbents were analyzed further in both packed- and expanded-bed column chromatography according to purification and recovery factors for thrombin from crude extracts (*H79*). Reversible binding of thrombin to the support was optimal at relatively low ligand density values. Zhao et al. probed the affinity interactions between a heparin-derivatized stationary phase and the aromatic functionalities of phenylalanine- or tyrosine-containing oligopeptide tetramers (*H80*). As an alternative to heparin-immobilized stationary phases, Ishimura et al. derivatized a hydrophilic vinyl−polymer gel matrix with β-cyclodextrin sulfate for the chromatographic separation of heparin binding molecules (*H81*). The new affinity support demonstrated several advantages over heparin-immobilized stationary phases including enhanced stability under acidic or basic conditions and ease of analyte elution.

New stationary-phase supports were developed for the affinity purification of specific proteins. Gorner et al. synthesized a new affinity chromatography support demonstrating biospecificity for the allosteric site of hemoglobin (*H82*). A hexamethylamine spacer was attached to EAH Sepharose-4B, and benzenetetracarboxylic acids were subsequently bound to the spacer ligands. Association constants for different forms of hemoglobin (oxyhemoglobin and deoxyhemoglobin) and support loading capacities were reported for the new support. Koyama and Terauchi synthesized a new affinity chromatography support for the separation of glycated proteins in diabetic serum (*H83*). A porous polymeric matrix was derivatized with 1,1′-carbonyldiimidazole (CDI) and tested for reactivity with *m*-aminophenylboronic acid hemisulfate (APBA). In contrast to previous reports, the highest coupling yields of APBA to the CDI-polymeric matrix were achieved under acidic reaction conditions. Regnier and co-workers demonstrated the effectiveness of the tetrapeptide GPRP and a cytochrome *c* peptide analogue (CAQCHTVEK, containing a heme group bound to the two cysteines), as ligands for the purification of native fibrinogen and human serum albumin, respectively, in perfusive affinity chromatography (*H84*). Optimal elution conditions and binding capacities were reported for each protein. Zembower et al. immobilized peptide boronic acids on Sepharose 6B for the purification of bovine α-chymotrypsin and porcine pancreatic elastase (*H85*). The peptide boronic acids were synthesized specifically as transition-state analogue inhibitors for the two corresponding serine proteases. The authors suggested that this technique could be adapted to the purification of other serine proteinases.

Fundamental advances in dye affinity chromatography were cited as well. Gypei-Garbrah et al. developed "tentacle" affinity matrixes from polystyrene/poly(vinyl alcohol) cross-linked with terephthalaldehyde (*H86*). Dextran with an average molecular weight of 500 000 was bound to the polymeric support via an epoxide linkage; the dextran molecules were derivatized subsequently with triazine dye Cibacron blue. The dye ligands project from the support surface and provide significantly faster binding kinetics relative to more conventional supports, presumably due to reduced steric hindrance. Shen and Giese separated BODIPY hydrazide, a fluorescent dye, and BO-IMI (BODIPY derivatized with *N*-acetylhistidine) on three different Sepharose-IDA affinity chromatographic columns containing Cu^{2+}, Ni^{2+}, or Zn^{2+} (*H87*). As potential ligands in dye−ligand affinity chromatography, BODIPY hydrazide demonstrated strong, bidentate binding with columns containing Cu^{2+} and Ni^{2+}, whereas BO-IMI showed preferential interaction with the column containing Zn^{2+}.

Affinity separations of nucleotides were the focus of many investigations during the review period. You et al. attached NAD to a phospholipid-coated aminated macroporous silica support for the separation of nucleotides (*H88*). Imai et al. covalently attached single-stranded DNA to latex particles (0.22 μm in diameter) for the purification of complementary RNA or DNA from cellular matrixes (*H89*). Wils et al. developed a new technique for the purification of supercoiled plasmid DNA, triple-helix affinity chromatography (THAC) (*H90*). The basis of the affinity interaction in THAC is the sequence-specific formation of a triple helix between an oligonucleotide and a DNA plasmid molecule; a sequence complementary to the oligonucleotide is inserted into the plasmid. A 100-fold reduction in chromosomal DNA contamination of plasmid DNA was achieved via a two-step elution with an alkaline phosphate buffer. Min and Verdine attached to DNA an affinity "tail" consisting of six successive 6-histaminylpurine residues for selective retention on a Ni^{2+}−NTA-agarose (*H91*). The purified DNA strands eluted with 200 mM imidazole and could be employed in polymerase chain reaction methodologies and other molecular biological techniques. The applicability of this affinity "tail" in the purification of proteins was described by Buening et al. (*H92*) and Crowe et al. (*H93*). Wheatley et al. immobilized synthetic oligonucleotides on an epoxide-activated hydroxyethyl methacrylate affinity support for the specific purification of proteins from nuclear extracts (*H94*). Immobilization of the oligonucleotides was facilitated at high salt concentrations; elution of the proteins was achieved using a salt gradient.

Imbert et al. applied "DNA-like" phosphorylated cross-linked polystyrene as affinity chromatography matrixes for specific interaction with DNA binding proteins, such as transcription factors (*H95*). The transcription factors were eluted without loss of biological activity and as a function of the extent of substitution of phosphoester functionalities on the polymeric support. These affinity supports were applied also to the separation of anti-DNA antibodies (*H96*). Computer simulations of the active sites of the support and the anti-DNA antibodies were performed in order to give insight into the separation mechanism involved. Ohlson et al. applied weak monoclonal antibodies to the separation of structurally similar carbohydrate antigens in weak affinity chromatography (*H97*). Studies of the effects of experimental factors on chromatographic performance revealed that nonspecific interactions were responsible for retention.

Three biological applications of affinity chromatography appeared during the review period. Rye coated magnetic microspheres with carbohydrates to facilitate the analysis of carbohydrate recognition proteins in cell biology (*H98*). Frontal affinity chromatography was used to probe interactions between metal ions and complex biomaterials, such as plant cell fragments immobilized within a polysilicate matrix (*H99*). Metal binding isotherms revealed the presence of two classes of metal binding sites. Quantitative zonal affinity chromatography was applied for the first time to measure nonspecific DNA−protein equilibrium binding constants (*H100*). Results obtained from the rapid and simple procedure were in agreement with literature values for the binding of bovine pancreatic RNase A and *Escherichia coli* lac repressor to double-stranded DNA immobilized on a cellulosic matrix.

ION CHROMATOGRAPHY

Ion chromatography (IC) continues to generate much interest, both in research directed toward improving the technology and especially into new applications. Except for those reports devoted exclusively to the development of one or the other of the methodologies, we will not distinguish between suppressed and nonsuppressed techniques. During the period of this review, there have been advances made in the design and synthesis of ion-exchange stationary phases, in the understanding of the retention process, in the development of better mobile phases for certain applications, and in new detection techniques. The areas of stationary-phase design and development of theory were especially active.

The second edition of Weiss' popular book appeared (*I1*), and three general reviews were published. Lucy (*I2*) published an excellent perspective on the development of the technique, including the place of IC in relation to capillary electrophoresis and the rapid development of this technique for high-resolution ion analysis. Singh et al. (*I3*) published a thorough review of the application of suppressed IC for the analysis of low concentrations of anions in the presence of a high concentration of matrix ions. Brzychcy et al. (*I4*) reviewed the use of IC for gas analysis, including sampling techniques.

A number of papers also appeared discussing retention mechanisms for ion exchange and ion exclusion. Foti et al. (*I5*) compared theory and experiment for the classical retention mechanism and derived equations for system peaks, labeled eluent ions, and analytes in a system containing only strong electrolytes. Yang and Hu (*I6*) developed a statistical thermodynamic model for retention, especially emphasizing the relation between the retention volume and the ion potential of the solute ion. Haddad's group published two papers on computer optimization, a review (*I7*) discussing eluent optimization and an original work discussing the development of an expert system using a database of over 4000 published methods (*I8*). Glod discussed parameters influencing retention in ion exclusion chromatography (*I9*) and developed equations and computer simulations of column performance. He also discussed the use of strong acids as eluents to suppress dissociation of weak acid analytes (*I10*).

Watanabe et al. published two works discussing the retention volume of the second system peak with monobasic (*I11*) and dibasic acid eluents (*I12*). Retention models for ion interaction chromatography were published for anionic, neutral, and cationic analytes (*I13*) and for singly and doubly charged analytes (*I14*). Other theoretical papers of general interest include a discussion of the effects of mass action equilibria on fixed-bed multicomponent ion-exchange dynamics (*I15*), a study of the effect of pH and concentration on column dynamics of weak electrolyte ion exchange (*I16*), a presentation of retention models for the ion chromatographic separations of metals in the presence of complexing agents (*I17*), a new approach to determining each component of a two-component overlapping peak by single-column IC (*I18*), and a new approach to the simultaneous separation of inorganic cations and anions based on ion-exchange and electrostatic interactions (*I19*).

Mobile Phase and Sample Effects. We have arbitrarily broken the IC review into *mobile-phase* and *stationary-phase* sections. This has been done merely for convenience of organiza-

tion, and it should be kept in mind that retention, for any chromatographic process, is controlled by the difference in the chemical potentials of the solute between the two phases.

Chelation is still an often used technique for eluting ions from IC columns. Liu et al. (*I20*) discussed the use of various complexing agents for the separation of the lanthanide series and showed a limit of detection of 2.5 ng/mL by concentrating 25 mL of seawater. Nesterenko and Jones used a single-column chelation system for the separation of transition metals (*I21*) and studied a variety of eluents. Hajos et al. (*I22*) extended a multiple species retention model to the retention behavior of EDTA complexes of Cu, Zn, and Pb together with inorganic anions Cl^-, NO_2^-, Br^-, and NO_3^-.

The exploitation of chemical equilibria in aqueous mobile phases is a very powerful way of controlling separations. Jansen et al. (*I23*) studied the effect of dissociation equilibria on ion-exchange processes of weak electrolytes by varying pH and running binary displacement experiments. Hu et al. published three works involving the study of aqueous mobile phases. They investigated the problem of confusing distribution of analyte ions in quantitative analysis and showed that by adding specific ions to the sample solution that only a single ion pair was formed, greatly simplifying identification and quantitation (*I24*). They also showed the simultaneous separation of inorganic cations and anions using a dynamic stationary phase of mixed SDS and Zwittergent-3−14, a zwitterionic surfactant and an aqueous mobile phase (*I25*). They further studied the properties of a stationary phase of only a zwitterionic surfactant with water as the mobile phase and showed that this provides the basis for a simple method for the simultaneous separation of cations and anions (*I26*).

A variety of other novel eluents were proposed for specific separations. These include cyanuric acid-based eluents for suppressed anion chromatography (*I27*), pyromellitate eluent for the nonsuppressed chromatography of inorganic anions, magnesium and calcium (*I28*), a study of the alkanesulfonate homologous series as eluent components in anion chromatography (*I29*), poly(vinyl alcohol) as an eluent for aliphatic carboxylic acids on a cation-exchange resin (*I30*), benzenedisulfonic acid for the separation and indirect detection of both cationic and anionic species (*I31*), sulfoisophthalic acid for separation and high sensitivity conductivity detection of common anions by nonsuppressed chromatography (*I32*), and a polyanionic eluent of perchlorate anion and phosphate buffer for the separation of selenite and selenate (*I33*).

Stationary Phases. The development of new stationary phases and the characterization of existing phases generated much interest during this review period. Two papers of general interest appeared; Marton discussed the characterization of ion-exchange stationary phases and recommended that the thermodynamic exchange constant and the free energy interaction parameter be measured (*I34*). Levison and Pathirana (*I35*) quantitatively studied the phenomenon of ligand leakage from ion-exchange media, which is a key issue in the validation of ion-exchange processes in many industrial applications.

While there is increasing interest in fiber columns and monolithic columns in other areas of chromatography, only one report appeared for ion exchange during this time. Liquan et al. (*I36*) studied a suppressed system, with both the separation and suppression columns prepared from ion-exchange fibers, and reported efficiency comparable to small-particle stationary phases, but at one-tenth the pressure drop, allowing much faster separations.

Ohta et al. published three reports of the use of unmodified (bare) silica as an ion-exchange medium. They reported the separation of Mg and Ca in environmental waters using 3 mM nitric acid as eluent (*I37*), a more complete study of the separation of alkali, alkaline earth, and transition metal cations using a number of weak organic and inorganic eluents (*I38*), and the simultaneous separation of common mono- and divalent cations using dilute nitric acid and 2,6-pyridinedicarboxylic acid as eluent (*I39*).

The dynamic modification of other stationary phases continues to be of interest for generating ion-exchange surfaces. Paull and Jones (*I40*) studied a number of chelating dyes as impregnating agents on traditional substrates. Jun et al. (*I41*) modified a polymeric PRP-1 reversed-phase column by coating it with hexadecyltrimethylammonium bromide and used it for the separation of inorganic anions and monocarboxylic acids. Knox and Wan (*I42*) adsorbed polyethyleneimine onto porous graphitic carbon and showed chromatographic performance similar to that of bonded ion-exchange silica gels. Umemura et al. (*I43*) studied the adsorption of three sulfobetaine surfactants onto a C18 column and studied the separation of inorganic anions and cations with water as the eluent.

Macrocyclic receptors are also useful for the separation of ionic species, and three different systems were reported during this review period. Glennon et al. (*I44*) studied the retention behavior of alkali and alkaline earth metal ions on a silica-bonded calix[4]-arene phase, using water as the eluent. Okada (*I45*) showed that complexing a bonded phase having a primary ammonium ion with a crown ether significantly modified the anion-exchange selectivity. Glennon et al. (*I46*) further reported on the use of the silica bonded calix[4]arene phases for the separation of metal ions and amino acid ester hydrochlorides.

The use of novel polymeric resin materials continues to be of interest. Revesz et al. (*I47*) reported the retention behavior of mono- and dicarboxylate and inorganic anions on latex-based pellicular anion exchangers. Rey and Pohl (*I48*) described a new cation exchanger based on a polymeric, highly cross-linked macroporous substrate to which two different monomers, one with carboxylate and the other with phosphonate functional groups, were covalently grafted and showed that these phases were useful for the separation of amines and of six common inorganic cations. Nair et al. (*I49*) described a highly chemically and mechanically stable poly(divinylbenzene)-based resin with dimethylethanolamine functional groups. McCreath et al. (*I50*) described a poly(vinyl alcohol)-coated particulate perfluoro polymer support functionalized with ion-exchange groups for the separation of proteins.

Several original reports of silica-based ion-exchange stationary phases also appeared. Elefterov et al. (*I51*) described the ion-exchange properties of glutamic acid bonded silica for the separation of alkali, alkaline earth, and transition metals. Zein et al. (*I52*) immobilized bovine serum albumin on silica and used it as a stationary phase for the separation of inorganic anions. Nesterenko and Jones (*I53*) compared iminodiacetic acid functional groups immobilized on silica and hydrophilic and hydro-

phobic polymer matrixes for the separation of various alkaline earth and transition metals. Yang et al. (*I54*) reported the preparation and properties of multifunctional ion exchangers, including one cation and two zwitterion types covalently bonded to silica and showed these multifunctional phases were more efficient for the separation of organic weak bases and organic weak acids than simple ion exchangers.

Suppressor Technology, Quantitation, and Detection. New detection schemes for IC are not explicitly covered in this review; the reader is referred to the LC equipment and instrumentation review also in this issue. We will include, however, a few reports dealing with detection aspects of IC.

Novic et al. (*I55*) studied the influence of high concentrations of chloride on the accuracy of the quantitation of nitrite and nitrate by suppressed IC and found that as the chloride concentration increased, retention of the two anions increased and resolution decreased. Watanabe and Sato (*I56*) studied both theoretically and experimentally the response of the analyte and system peaks with bulk property detectors, such as conductivity and UV absorbance in indirect mode. They reported sensitivity enhancement dependent on the retention volume of the second system peak relative to the solute peak.

Caliamanis et al. (*I57*) showed that a commercial micromembrane suppressor can be used to effect ion replacement reactions in suppressed eluent streams. They showed that with proper choice of replacement reagent enhancement of up to 250 times in peak height and 1400 times in peak area are possible! Sjoegren et al. (*I58*) described a simple system for high-performance suppressed anion chromatography on a capillary scale, which provides on-line high-pressure electrodialytic NaOH eluent production and gradient generation.

There was significant effort in improving concentration limits of detection in IC during this review period. Kaiser et al. (*I59*) described a system allowing injection of up to 1300 μL with no significant loss in peak efficiency and detection limits for most ions from 10 to 400 ng/L. Toofan et al. (*I60*) studied the preconcentration behavior of inorganic anions and organic acids on a commercial concentrator column. Umile and Huber (*I61*) showed the use of a column switching technique for relative analyte enrichment and showed that the detection limit could be lowered (improved) by up to 5000 times! Hissner et al. (*I62*) replaced the loop of an IC injection valve with an electrochemical flow-through cell with a gold electrode and preconcentrated metal ions on the electrode. They found improvement in detection limits of between 7 and 14 times.

Fung and Tam (*I63*) studied the factors affecting the use of a ruthenium(II) complex as an ion interaction reagent for indirect fluorometric detection of simple analyte ions separated by nonsuppressed IC, with detection limits in the low-nanogram per milliliter range.

SECONDARY CHEMICAL EQUILIBRIA

The investigation and utilization of secondary chemical equilibria (SCE) such as acid−base, complexation, ion-pairing, and solute−micelle associations for modern LC continued in this review period, with many reports concerned with the simultaneous use of two or more phenomena.

Reviews. Dolezal and Sommer reviewed the complex solution equilibria of metal chelates of N-heterocyclic azo dyes and decribed their application to the separation of metal ions by RPLC and ion-pairing RPLC (*J1*, *J2*). Lochmuller and Hangac provided first a generalized discussion of the strategy for mobile-phase additives that promote selective secondary equilibria and then a specific review of coordinatively unsaturated metal β-diketonates as mobile-phase additives. Unlike previous ionic, metal dopants, these neutral complexes combined reasonable elution times, high chromatographic efficiency, and enhanced chromatographic selectivity for polar compounds in normal and reversed-phase modes (*J3*). Ward and Ward summarized the structure and properties of micelles, (nonhomogeneous) solubilization theory, and applications in micellar liquid chromatography (MLC) and micellar electrokinetic chromatography (MEKC) (*J4*). Szczepaniak and Szymanski reviewed the theory and applications of MLC for the analysis and physicochemical characterization of biologically active compounds (*J5*).

Several reviews on MLC were published in a special issue of the *Journal of Chromatography* devoted to the applications of micelles in analytical chemistry. Marina and Garcia described the use of MLC for the evaluation of solute-micelle association constants (binding model), distribution coefficients of solutes between stationary-aqueous, stationary-micellar and aqueous-micellar phases (partition model), and the application of these constants to the study of retention mechanisms and prediction of separation (*J6*). Garcia-Alvarez-Coque et al. reviewed models for predicting retention in MLC; these models permit the evaluation of the strength of micelle−solute and stationary phase−solute interactions in addition to the optimization of resolution (*J7*). Jimenez and Marina also reviewed retention models in MLC, first using physicochemical models to explain the variation of retention with one or more experimental variables (such as micellar concentration, organic modifier concentration, and pH) and then illustrating new trends for empirical models of solute retention (*J8*). Berthod reviewed the causes of reduced efficiency in MLC, which are slow solute mass-transfer kinetics between micelles, the aqueous phase, and the surfactant-covered stationary phase. Surfactant adsorption results in an increase in both the flow anisotropy (*A*) and mass-transfer (*C*) terms of a Knox plot. These losses in efficiency may be recovered by increasing the operating temperature, adding alcohol in proportion to the surfactant concentration, or by reducing the flow rate (*J9*). Khaledi provided an extensive perspective (263 references) on micelles as separation media in MLC and MEKC. The roles of micelles and organic modifiers in controlling retention and selectivity in MLC are described, along with the differences between MLC and RPLC in terms of chromatographic behavior and the scope of applications. Despite the efficiency limitations of MLC, it is shown to be superior to ion-pair LC and ion-exchange LC for the separation of charged molecules and mixtures of charged and uncharged solutes (*J10*). Takeuchi reviewed the advantages and limitations of micelles as fluorescent signal enhancers and selectivity modifiers in MLC, using dansyl (Dns) amino acids as probe molecules and ion-exchange-induced stationary phases (*J11*). Finally, Nishi provided an extensive review (230 references) of the pharmaceutical applications of MLC and MEKC (*J12*).

Micellar Liquid Chromatography. Research continued in the area of solute retention. Xie and co-workers explored neural networks for the modeling and prediction of retention in RPLC and MLC. Because more retention data are needed to train neural networks than to fit most mathematical models, their use is recommended only for those cases where adequate theoretical or empirical models do not exist (*J13*). Garcia-Alvarez-Coque et al. provided a physicochemical interpretation of a previously reported empirical retention model, in which the partition coefficient between stationary phase and water and the solute−micelle association constant are functions of modifier concentration. An extension of the elution model was proposed for very hydrophobic solutes, and several curve-fitting procedures of the experimental data to the model were compared. The effect of errors in the cmc on the accuracy of physicochemical parameters and in the prediction of retention factors was reported (*J14*). They also proposed and compared four different criteria for the determination of (dead time) in MLC under a variety of mobile-phase conditions (*J15*). Takeuchi and Miwa examined the retention, selectivity, and enhanced fluorescence of dansyl amino acids on an ion-exchange-induced stationary phase. Several mobile-phase parameters affected the retention of the analytes, including the type and concentration of micellar agent and modifier ion and the concentration of acetonitrile (*J16*).

Garcia and Marina examined the effects of alcohol organic modifiers on the association constants and retention mechanism for aromatic compounds in MLC with SDS and hexadecyltrimethylammonium bromide (CTAB). As the surfactant concentration in the mobile phase was increased, a shift from the three-phase partition equilibrium mechanism to a direct transfer of solutes from micellar to stationary phase was observed, particularly for highly hydrophobic compounds; this facilitated the prediction of separation selectivity from the ratio of two stationary−micellar partition coefficients (*J17*). Escuder-Gilabert and co-workers described a novel retention model in MLC that includes both hydrophobic and electrostatic solute−stationary phase interactions and compared it with other previously reported models. Especially suited for charged compounds, this model provided high correlations between log k and structural parameters for partially ionized local anesthetics using a nonionic surfactant solution as mobile phase. A predictive model for estimating the hydrophobicity of local anesthetics was also proposed (*J18*). Jandera and Fischer derived and experimentally verified equations describing the dependence of solute retention on the concentration of surfactant in both aqueous micellar and submicellar mobile phases. With submicellar mobile phases, they observed superior selectivity relative to hydroorganic mobile phases and better efficiency and RI detector sensitivity compared to micellar mobile phases (*J19*). Interestingly, Li and Fritz also observed better RPLC separations (shorter retention times, sharper peaks) of alkylbenzenes, polycyclic aromatic hydrocarbons, alkylphenols, and other aromatic compounds with relatively high concentrations of (submicellar) surfactant *monomers*; i.e., the surfactant concentration exceeded the aqueous cmc but not the hydroorganic cmc (*J20*).

Jimenez and co-workers reported a theoretical model for the prediction and modeling of retention factors of 27 dihydropyridines in MLC with CTAB or SDS as surfactants and 1-propanol and 1-butanol as organic modifier (*J21*). They later proposed a new physicochemical retention model that successfully described the effect on solute retention of adding 1-propanol and 1-butanol to micellar mobile phases containing CTAB or SDS (*J22*). Finally, they presented an overview of the capabilities of neural networks for modeling retention data in MLC with hybrid eluents. Several neutral net parameters were evaluated, including the type of activation function, number of neurons in the hidden layer, and use of some input and/or output data transformations. The best results were obtained with a linear activation function, a recurrent network, and a logarithmic transformation of the retention factor (*J23*).

Torres-Lapasio and co-workers proposed, for ionizable solutes, a "global" retention model that takes into account, simultaneously, the pH and concentrations of surfactant and 1-propanol over relatively narrow ranges of values; mean relative errors in the retention factors of seven compounds were usually lower than 6% (*J24*). Medina-Hernandez and Sagrado studied the chromatographic quantification of hydrophobicity using MLC using an empirical model and found that nonlinear relationships between k or log k and log P are to be expected and only in particular circumstances can linear relationships be obtained. In contrast, linear relationships were found between log P and the logarithm of the retention factor at zero micellar concentration, k_m (*J25*).

Argiles et al. proposed two new regression models for the estimation of k_m and solute−micelle association constants; their reliability was evaluated with the aid of simulated data and compared with the conventional model (*J26*).

By using a 1-propanol-modified SDS mobile phase on a short-chain bonded-phase column, Kayali and co-workers were able to separate with a reasonable degree of retention five PAHs that would have been too highly retained on a long chain column (*J27*). Rapado-Martinez et al. utlized an interpretive optimization procedure to select a suitable micellar mobile phase for the simultaneous determination of the β-blockers atenolol, metoprolol, and oxprenolol, the diuretics amiloride, bendroflumethiazide, chlorthalidone, and hydrochlorothiazide, and the vasodilator hydralazine in pharmaceutical products. Whereas two different hydroorganic mobile phases were needed in order to analyze all of the above drugs, one micellar mobile phase was found to suffice (0.15 M sodium dodecyl sulfate, 7% propanol in 0.01 M, pH 3 phosphate buffer).

Somewhat fewer studies were concerned with efficiency and selectivity. Lavine and Hendayana used the Knox equation to identify the most important sources of band broadening in MLC; the slow rate of surfactant desorption from the stationary phase was shown to play a critical role (*J29*). Rodriguez Delgado et al. examined the influence of surfactant concentration, iso-PrOH content, temperature, and flow rate on efficiency and peak shape in MLC using polynuclear aromatic hydrocarbons as test compounds; the efficiency increased as the surfactant concentration and iso-PrOH were increased (*J30*).

Diaz and co-workers utilized the unique selectivity of reversed cholic acid micelles dissolved in THF to achieve a normal-phase MLC separation of seven plant hormones (*J31*); chromatographic results conformed closely to those predicted by the pseudophase MLC theory. Marina et al. used multiple linear regression to interpret the influence of the surfactant nature and concentration

on the separation selectivity of 15 benzene and naphthalene derivatives in MLC with an octadecylsilica column; SDS, CTAB, and Brij-35 were employed at concentrations ranging from 0.02 to 0.1 M (*J32*). Delgado and co-workers measured the enthalpy and entropy of transfer of polynuclear aromatic hydrocarbons from the bulk aqueous mobile phase to the stationary phase and from the bulk aqueous phase to the micellar phase. They observed better separation selectivities at higher temperatures and better resolution and shorter analysis times when alcohol modifiers were added to the mobile phase (*J33*).

The solution- and stationary-phase interactions of ionic or nonionic surfactants were the focus of several studies. Lavine et al. used MLC and solid-state ^{13}C NMR spectroscopy to study the interactions of three ionic surfactants (SDS, DTAB, CTAB) with cyanopropyl (*J34*, *J35*), C8 (*J35*), and C18 (*J36*) bonded-phase columns. Differences observed in the amount of surfactant adsorbed on each phase as well as differences in the nature and degree of surfactant monomer−bonded-phase interactions (*J34*) are postulated as reasons for the different selectivities observed. Models based on the spectroscopic data are in good agreement with retention data. Whereas SDS with its sulfate headgroup appeared to form an anionic hydrophilic surface layer on C18 with the headgroup outside of the C18, with DTAB and CTAB, the quaternary ammonium headgroups appeared to incorporate themselves into the bonded phase (*J36*).

Portet et al. studied the behavior of pure alkylethoxylated nonionic surfactants (e.g., C10E6 and C14E6, both as single components and as binary mixtures of various composition) in solution and at silica/water interfaces. In both environments, the data obtained suggested a partial demixing of the micelles into segregated aggregates in the C10E6-rich composition range and by an increased stability of the aggregates in the C14E6-rich domain (*J37*). Desbene and co-workers used HPLC to measure the adsorption of pure nonionic alkylethoxylated surfactants (C12En series, $n = 2-9$) down to low concentrations at a silica/water interface. At very low concentration, the amount adsorbed was higher as the number of ethoxylated groups increased, but at higher surfactant concentration, the reverse trend was found (*J38*).

Finally, given the usually deleterious effects of micelle polydispersity, it is appropriate to mention reports of this period that are concerned with surfactant analysis. Kiewiet and de Voogt described the developments over the last few decades in the routine determination of two major types of nonionic surfactants in environmental samples, i.e., alcohol ethoxylates (AE) and alkylphenol ethoxylates (APE). Because of differences in (bio)-degradation patterns observed in the aqueous environment between AE and APE, RPLC is recommended for AE and normal-phase LC for APE, and LC−MS for more complete information on isomer, oligomer, and homologue distribution of both AE and APE (*J39*). Desbene et al. used RPLC with a C8 column to separate and determine trace levels of complex nonionic poly(ethylene oxide)-type (PEO) surfactant mixtures (*J40*). Cuccovia and co-workers described methods for determining the concentration of halide co-ions or counterions, respectively, in the presence of added salt and SDS micelles or cetyltrimethylammonium halide micelles (*J41*, *J42*).

Acid−Base Equilibria. Wan and co-workers compared, over a pH range from 2.0 to 7.0, the retention behavior of 36 positional isomers of ionizable, substituted benzenes on porous graphitic carbon and octadecyl bonded silica (ODS) using 35% aqueous acetonitrile as the mobile phase. With PGC, the theoretical equations based on solute ionization fit the observed retention data for each class of solute, indicating that the retention mechanism was uniform over the whole pH range. However, with ODS, the amine-containing compounds exhibited serious deviations from the theory, suggesting that strongly acidic silanols gave added retention at low mobile-phase pH (*J43*). Lord and Stringham studied the effects of ionic strength and pH on the retention and elution order of four dicarboxylic acids with a silica-based amine HPLC column. Hydrophobic effects, observed when ionization of either the probe acids or the amine column was minimized, were moderated by incorporation of organic solvents into the mobile phase (*J44*). Roses et al. derived and tested a new model that relates the retention of a weak acid in HPLC columns with pH, ionic strength, and mobile-phase composition in methanol−water mobile phases. The proposed model uses the pH value in the mobile phase instead of the pH value in water, takes into account the effect of the activity coefficients, and considers different holdup times for the ionic and neutral species, the latter of which is independent of the mobile-phase properties (pH, solvent composition, ionic strength). The proposed equations can be combined with previously derived equations that relate solute retention with mobile-phase composition to yield a general model that simultaneously relates solute retention to all three mobile-phase variables (*J45*).

Pappa-Louisi and Zougrou studied the role of mobile-phase pH in the determination of different types of catecholamine-related compounds (acidic, basic, amphoteric, neutral) by RPLC with amperometric detection. The effect of pH on peak width and peak shape was investigated in addition to its effect on retention (*J46*). Using a LSER model, Barbosa and co-workers optimized the pH of the hydroorganic mobile phase and the proportion of organic modifier for the separation of several quinolones (*J47*, *J48*) and low-molecular-mass peptides (*J49*). They also measured the ionization constants of several carboxylic acids in acetonitrile−water mixtures up to 70% (w/w) in order to predict the influence of pH on retention and selectivity in RPLC (*J50*). Marengo and co-workers employed fractional and star experimental designs to investigate the effect of the following five experimental factors on the retention of simazine and atrazine in ion-interaction chromatography: (1) the chain length and (2) concentration of the ion-interaction reagent; (3) the mobile-phase pH, (4) the flow rate, and (5) the organic modifier concentration (*J51*).

Sykora et al. studied the influence of pH, sorbent character, and mobile-phase composition on the retention of 19 basic compounds on 5 silica-based sorbents, a polybutadiene-coated alumina, and 2-hydroxyethyl methacrylate-based stationary phase. Deviations in the theoretically predicted sigmoidal dependence of retention on pH observed on some phases were apparently due to a complex retention mechanism in which not only hydrophobic interactions but other interactions such as ion exchange occurred. The effect of methanol on retention, however, was generally in good agreement with theory (*J52*). Hanai and co-workers compared pK_a values of 64 phenolic and 50 nitrogen-containing

compounds obtained via RPLC measurements and computational chemistry calculations. Good agreement was observed for the phenols, but the pK_a's for the nitrogen-containing compounds were lower than the reference values (*J53*).

Jano and co-workers derived a general equation relating the observed retention factor of polyprotic acids and bases to the pH of the mobile phase, the dissociation constants, and the retention factors of the different ionic species (*J54*). They then utilized a two-stage least-squares fitting procedure to calculate the dissociation constants of polyprotic leukotrienes from retention data obtained at each of several percentages of organic modifier over a pH range from 2 to 12. The dissociation constants thus obtained for leukotriene B4, leukotriene E4, and *N*-acetylleukotriene E4 were extrapolated to give their pK_a values in 100% aqueous solutions (*J55*).

Vervoort et al. employed principal component analysis to compare 14 commercially available reversed-phase stationary phases for the determination of basic pharmaceutical compounds. The effect of silanol blocking compounds on retention and peak shape was investigated by using phosphate buffers at pH values of 3 and 7; addition of *N*,*N*-dimethyloctylamine to the pH 3 mobile phase resulted in a significant improvement in the peak shapes. As expected, the commercially available stationary phases showed distinct differences in their suitability for the analysis of basic compounds (*J56*). Claessens and co-workers studied the effect of buffers on silica-based column stability in RPLC over a wide pH range and found that while pH is an important factor, the kinetics of column degradation vary considerably with the chemical composition of the buffer, i.e., the identity and concentration of the buffer anions and cations, particularly at neutral and alkaline pH. Much more rapid degradation was observed at 1:1 MeOH−aqueous pH 10 carbonate and phosphate buffers than with borate and glycine buffers. Differences were also observed as the buffer cation was varied (*J57*). McCalley evaluated at pH 3.0 the performance of eight silica-based RPLC columns evaluated previously at pH 7.0, using the same relatively high-pK_a bases as test compounds and the same isoeluotropic mixtures of methanol, acetonitrile, or THF in combination with phosphate buffer. Differences in the dependence of column performance on organic modifier identity were shown to be considerably reduced at acid pH. The ranking of columns according to average asymmetry of the basic compounds was found to vary somewhat with pH; however, all solutes except pyridine gave superior results at acid pH (*J58*). Finally, Chen and Zhang exploited the differential ionization of *o*-, *m*-, and *p*-nitrobenzoic acid at pH 2.99 for their separation on a C18-bonded column and a mobile phase consisting of 2-propanol−water−acetic acid (20:80:0.4) (*J59*).

Ion Pairing. Pearson and McCroskey examined higher perfluorinated "homologues " of TFA as alternative ion-pairing agents to TFA, which has been employed almost universally in RPLC for separations of peptides and proteins. They compared the retention and selectivity provided by TFA, pentafluoropropionic acid, heptafluorobutyric acid, perfluoropentanoic acid, perfluorohexanoic acid, and perfluoroheptanoic acid and found that the largest increases in selectivity were usually obtained for compounds that were insufficiently retained with TFA in the mobile phase (*J60*). In contrast, Sereda et al. examined the use of sodium perchlorate as an alternative ion-pairing agent to TFA for peptide separations by RPLC and showed practical examples of increased retention, selectivity, and resolution of mixtures of nonhelical and amphipathic α-helical peptides when perchlorate was employed at pH 2. Markedly different selectivities for peptides are demonstrated with aqueous perchlorate/acetonitrile mobile phases compared to the more traditional aqueous TFA/acetonitrile system (*J61*). Angelino and Gennaro reported, tested, and validated (through intercalibration with a GC/MS method) an ion-interaction RPLC method for the determination of the 11 EPA priority pollutant phenols, based on their ability to form ion pairs with alkylammonium ions. The optimized mobile phase was a water−acetonitrile solution of octylammonium orthophosphate at pH 8. With UV detection at 285 nm, detection limits lower than 30 μg/L were achieved without preconcentration (*J62*). Zhao and Fleet separated 20 isomers of ribonucleotides and deoxyribonucleotides by RPLC with ion pairing; mobile and stationary phases consisted of a C18 column and a gradient elution system consisting of phosphate buffer (0.05 M, pH 5.45) and methanol (*J63*). Using a C18 stationary phase equilibrated with 60 mM Bu_4NOH, 150 mM phosphate buffer, and a small but variable concentration of perchlorate ion (0.32−5.62 mM), Huang and co-workers developed a stable ion-interaction system for the separation of metallocyanide complexes. The linear relationship between the logarithms of the retention factor and perchlorate ion concentration facilitated the optimization of selectivity. Three differing elution orders of metallocyanide complexes were achieved by varying the concentration of from 0.94 to 5.62 mM (*J64*, *J65*). Jezorek and Wagner simultaneously separated and detected in one run divalent metal ions and neutral organics by isocratic RPLC using standard ion-interaction reagents and water−MeOH−tartrate mobile phases on a C18 reversed-phase column (*J66*).

Miscellaneous Complexation Equilibria. With the hope of determining the mechanism by which thorium(IV) and uranyl are retained, Hao et al. studied their retention behavior on a reversed-phase column with glycolate and mandelate as eluents. The observed elution behavior could not be explained from the predicted forms of the complexes present. However, the susceptibility of these metals to form mixed-ligand complexes with hydroxide ions occupying one or more positions in the metal's coordination sphere suggested that mixed anionic complexes were present, and thus Th(IV) and uranyl were eluted as neutral or weakly charged complexes (*J67*). To develop a unified rational approach to the selection of eluents for the analysis of porphyrins, Grykina and co-workers studied the RPLC retention behavior of some porphyrin ligands (pheophytins a and b, protoporphyrin IX, protoporphyrin IX dimethyl ester, 1,4,5,8-tetramethylporphyrin, tetraphenylporphyrin) and their metals (Mg, Zn, Cu, Pd, Pt, Pb), and considered mechanisms of the sorbate−sorbent−eluant interactions (*J68*). Hoshino et al. reported an accurate method for determining traces of Be(II) ion at nanomolar levels with photometric detection coupled with ion-pair RPLC. The chelate, $[BeIIL]^{3-}$, was efficiently separated on an Asahipak ODP-50 column using tetrabutylammonium bromide as an ion-pairing agent in a methanol (35%)−water eluent. The HPLC separation, coupled with an EDTA masking procedure, allows detection of Be(II) ion at 20 nM in the presence of metals at their natural abundance levels, such as Al, Fe, Ca, Mg, Zn, and Pb (*J69*). Li and co-workers reported an isocratic NPLC separation of tri-

phenylphosphine (PPh_3)-substituted homo- and heterodinuclear metal carbonyl complexes $[MM'(CO)_{10-n}(PPh_3)_n$, where M, M′ = Mn, Re; $n = 1, 2]$ using a phenyl-derivatized silica stationary phase and either a hexane−toluene (98:2) or hexane−dichloromethane (90:10) mobile phase (*J70*). Nowack et al. described an HPLC method for determining EDTA species in various environmental samples at low molar concentrations. The EDTA is desorbed from suspended particles or to sediments using phosphate and then detected at 258 nm as the Fe(III) complex (*J71*). Adlof separated the acetate derivatives of fatty acid mono- and diacylglycerol positional isomers by argentation liquid chromatography with an isocratic nonpolar mobile phase (1.2% acetonitrile in hexane) and flame ionization (*J72*).

OPTICAL AND POSITIONAL ISOMERS

The volume of literature emerging on optical and positional isomer separation continues to increase, and our literature search turned up over 650 original papers. Many of these were specific applications and will not be discussed here. However, numerous review articles were published which will alert the reader to many of the articles that we were forced to exclude.

Reviews. By far the most extensive review was provided by Bojarski, whose review with 758 references summarized important developments in the chromatographic separation of enantiomers in the last couple of years, including both direct and indirect separation modes, new derivatizing reagents, chiral stationary phases, and applications to enantioseparations of drugs and other biologically active substances (*K1*). Davankov provided a singular review of the nomenclature of chiral separation methods and chiral products (*K2*). Francotte summarized the present state of the chromatographic chiral separation from the analytical to the preparative scale, discussing the established methods and the new trends (*K3*). Chandwani and Dhaneshwar discussed the different modes and mechanisms of chiral separation of compounds (*K4*) including numerous examples of molecular recognition in nature. Ducharme et al. reviewed critical issues in chiral drug analysis in biological fluids, with particular emphasis on stability, stereoconversion, enantiomeric separation, recovery, and drug concentration determinations. An overview of the requirements for the validation of a chiral method was also presented (*K5*).

Bojarski and Aboul-Enein reviewed the principles and applications of the chromatographic separation of enantiomers in pharmaceutical analysis, including a comparison of "direct" and "indirect" methods for several racemic drugs (*K6*). Aboul-Enein summarized the advantages of chiral chromatography for the direct resolution of racemates on an industrial scale over the classical methods of optical resolution. Various chiral stationary phases (CSPs) were described, along with examples of the drug racemates separated on a large scale. The potential use of chiral membranes for resolution of racemic drugs was also presented (*K7*). Fried and Wainer addressed the use of coupled LC columns in chiral separations (including two-dimensional modes) and the application of the resulting arrangements to bioanalytical analyses (*K8*).

Toyo′oka reviewed the separation of enantiomers based upon diastereomer formation with a selection of fluorescent chiral derivatization reagents. The tagging reagents for various functional groups, i.e., amine, carboxyl, carbonyl, hydroxyl, and thiol, were evaluated in terms of optical purity, handling, flexibility, stability, sensitivity, and selectivity, and the applicabilities of each reagent to drugs and biologically important substances were included (*K9*). A similar review on the strategy and design of novel reagents for the fluorometric analysis of biomolecules was provided by Meguro and Ohrui (*K10*).

Several reviews of the synthesis and role of chiral polymers were published during this period. Yashima and Okamoto (*K11*) provided an extensive review of the chiral discriminating power of polysaccharide derivatives, particularly those involving tribenzoates and tris(phenyl carbamates) of cellulose and amylose; significant mechanistic aspects were also briefly reviewed. They also reviewed the characteristics of chiral synthetic polymers as packing materials for CSPs (*K12*), including proteins, polysaccharides and derivatives, polyamides, polymethacrylates, polyacrylamides and polymethacrylamides, polyurethanes, and synthetic polymers with chiral cavities (*K13*). One-handed helical polymethacrylates and polysaccharide derivatives in chiral HPLC (*K14*) were emphasized in yet another review of this topic. Finally, Okamoto et al. reviewed the synthesis and chiral recognition of a number of helical polymers, including optically active polymethacrylates, polyacrylates, polyacrylamides, and polyisocyanates; optically active polyisocyanates with a predominantly one-handed helical conformation showed the ability to discriminate enantiomers in solution (*K15*). Hirtopeanu et al. provided an extensive review of polyacrylamides and polyacrylates as CSPs, with an emphasis on the synthesis and applications (*K16*).

Oi reviewed CSPs based on hydrogen-bonding, π-donor, and π-acceptor characteristics, ligand exchange, and host−guest complexation (*K17*). Allenmark and Schurig provided a broad review of gas and liquid chromatographic CSPs for the direct separation of enantiomers, including those based on hydrogen bonding, coordination, and inclusion; temperature effects, current trends in analytical and preparative applications, and enantiomerization and chiral inversion taking place in thermally labile chiral molecular structures were described (*K18*).

Bressolle et al. summarized basic principles and new developments of enantiomeric separations of drugs by liquid chromatography and capillary electrophoresis using native and derivatized cyclodextrins (*K19*). Han described the historical development and applications of native and derivatized cyclodextrins in HPLC as CSPs, chiral mobile-phase additives, and chiral counterions (*K20*). Deng et al. reviewed principles and methods for direct enantioseparation of drugs and metabolites using cyclodextrin CSPs with a coulometric array detection system (*K21*). A similar review was provided by Maruyama et al. for the analysis of endogenous enantiomers of neurotoxins (e.g., isoquinolines) in clinical samples (*K22*). Leloux reviewed the determination of β-blocking drugs, which included many references to chiral separation (*K23*). Meyer reflected on the challenges in the chromatographic quantitation of very low concentrations of an enantiomer in the presence of its antipode, i.e., the much greater resolution required when separating hugely disparate concentrations of enantiomers, geometrically induced integration errors when resolution is incomplete, peak tailing, detector nonlinearity, overloading phenomena, and the possible lack of standards of highest optical purity (*K24*).

Williams et al. compared liquid and supercritical fluid chromatography for the separation of enantiomers on CSPs bearing three different types of chiral selectors. Although column equilibration and parameter optimization were generally accomplished more rapidly and with better resolution in SFC than in LC, analysis times were not always lower (*K25*). Wolf and Pirkle reviewed the applications of several types of CSPs in sub- and supercritical fluid chromatography with carbon dioxide mobile phases and the effects of temperature and the nature of polar modifiers added to the mobile phase (*K26*). Majors reviewed alternatives to HPLC for separation and resolution of enantiomers, with emphasis on gas chromatography, supercritical fluid chromatography, capillary electrophoresis, and capillary electrochromatography column packings; flow charts for typical analyses were provided (*K27*).

Following a summary of the distribution of D-amino acids in the body, their origin, metabolism, and possible roles in human diseases, Imai et al. discussed the methodologies for the analysis of D-amino acids in biological materials, including the use of chiral stationary phases and diastereomer formation via derivatization with chiral reagents followed by GC or HPLC separation (*K28*). Peter and Toth briefly outlined the syntheses of β-alkyl amino acids and then reviewed the different chromatographic methods available for the chiral separation of these amino acids and peptides (*K29*).

Two interesting alternatives to chiral column chromatography were reviewed. Pickering and Chaudhuri described the use of emulsion liquid membrane technology to separate low-molecular-weight enantiomers, using the selective extraction of phenylalanine enantiomers with copper(II) *N*-decyl-L-hydroxyproline as an example (*K30*). Zhong et al. reviewed recent contributions to the theory of simulated moving bed (SMB) chromatography, which is an implementation of preparative chromatography that is attracting much interest in the pharmaceutical industry; experimental results are well explained by the theory (*K31*).

In terms of positional isomers and closely related compounds such as carbohydrates, Wise and Sander reviewed their extensive studies on molecular shape recognition for polycyclic aromatic hydrocarbons in RPLC, providing a retention and solute shape parameter database of more than 200 PAHs and alkyl substituted PAHs (*K32*). El Rassi summarized recent progress in the reversed-phase and hydrophobic interaction chromatographic separations of carbohydrate species, including oligosaccharides, glycopeptides, and glycoproteins (*K33*). Koizumi (*K34*) reviewed the chromatographic behavior and separation of a wide variety of carbohydrates (monosaccharides, disaccharides, cyclodextrins (CDs), branched CDs, oligosaccharide alditols, chitooligosaccharides, N-linked oligosaccharides, glycopeptides) on graphitized carbon columns. Elution patterns were based on the size and planarity of the molecule (position and configuration of linkage). Churms reviewed the evolution of size-exclusion chromatography into a high-performance separation technique and its application, utilizing significant developments in detection systems, to the separation and molecular weight distribution analysis of carbohydrates (*K35*). Ross and Knox reviewed carbon-based packing materials (especially porous graphitic carbon) and their applications for the LC separation of geometric isomers and closely related compounds, including sugars, carbohydrates, and glucuronides; the separation of ionized and other highly polar compounds was also discussed (*K36*). Nishida reviewed the determination of the absolute configuration and the conformation of carbohydrate molecules based on NMR and HPLC. For the conformational analyses, stereoselective deuteration was coupled with NMR methods, while for the configurational analysis, a new fluorescent chiral derivatizing agent, (*S*)-TBMB carboxylic acid, was developed and combined with the HPLC analysis (*K37*). Lee described carbohydrate analyses utilizing high-performance anion-exchange chromatography with a pulsed amperometric detector (PAD); in addition to the number of hydroxyl groups, separations can be based on anomers and positional isomers (*K38*).

New Chiral Stationary Phases. Hyun and Lee prepared a cyclohexylamide derivative of (*S*)-naproxen which showed greater enantioselectivities for the two enantiomers of *N*-(3,5-dinitrobenzoyl)-α-amino esters and amides than the CSP derived from the 3,5-dimethylanilide of (*S*)-naproxen; the latter was superior in resolving *N*-(3,5-dinitrobenzoyl)-α-arylalkylamines, however (*K39*). Lin et al. prepared a two chiral center-CSP derived from (*R*/*S*)-phenylalanyl- and (*S*)-1-(1-naphthyl)ethylamino-disubstituted cyanuric chloride. Compared to a one chiral center-CSP made by replacing a substituent group attached to the asymmetric center with hydrogen, the two chiral center-CSP exhibited significantly lower enantioselectivity for the enantiomers of dinitrobenzoyl (DNB) derivatives of amino acid methyl esters and amino alcohols; the phenyl ring in the phenylalanyl moiety seems to exert steric effects instead of acting as a π-interacting group (*K40*). Cuntze and Diederich covalent bound an optically active molecular cleft incorporating a 9,9′-spirobi[9*H*-fluorene] spacer and tow *N*-(5,7-dimethyl-1,8-naphthyridin-2- yl)carboxamide [CONH(naphthyl)] moieties as H-bonding sites to obtain a CSP capable of complexing optically active dicarboxylates and 1,1′-binaphthalene-2,2′-diols. The order of enantiomer elution under normal-phase chromatographic conditions was rationalized by computer modeling of the association between the solute enantiomers and the immobilized molecular cleft (*K41*).

Chassaing et al. prepared three new CSPs based on heterogeneously derivatized cellulose with multiple derivatives: the first CSP has a 3,5-dimethylphenyl carbamate at the 2 and 3 positions and a phenylethyl carbamate at the 6 position; the second has a benzoate at the 2 and 3 positions and a meta-substituted phenyl carbamate at the 6 position; and the last has a 3,5-dimethylphenyl carbamate at the 2 and 3 positions and a para-substituted benzoate at the 6 position. The substituent present at the 6 position is very important for chiral recognition; enantioselectivities often exceeded that of commercially homogeneously derivatized cellulose (*K42*). The new CSP prepared by Hyun and Min from (*R*)-*p*-hydroxyphenylglycine exhibited unusually high enantioselectivity for the enantiomers of *N*-(3,5-dinitrobenzoyl)-α-amino acid amides, with greater retention observed for the *S* enantiomers (*K43*). Hyun et al. also made a new CSP by changing the direction of a connecting tether from the support particle to the chiral selector and observed much greater enantioselectivities than with the original CSP (*K44*).

Stalcup et al. separated the enantiomers of several benzodiazepines on a new CSP based on maltooligosaccharides and discussed the role of organic modifier, ionic strength, pH, and temperature. In general, selectivity and retention were found to

decrease with increasing organic modifier concentration, while resolution and enantioselectivity were found (counterintuitively) to improve with increasing temperature (*K45*). They also introduced a new sulfated β-CD CSP with a degree of substitution of approximately 13−14/cyclodextrin. This CSP was used to successfully resolve 33 enantiomeric pairs, including antihistamines, antidepressants, and phenylhydantoins, all but six of which had some amine functionality. Generally, the best separations were obtained for analytes in which the stereogenic center was either incorporated in a ring system or positioned between two aromatic rings (*K46*). Liu et al. attached a new chiral selector based on L-valine-3,5-dimethylanilide to monodisperse poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) beads. The polymeric separation medium provided greatly enhanced enantioselectivities (α's up to 7) and reduced retention times compared to the analogous silica-based chiral stationary phase in the separation of the enantiomers of 3,5-dinitrobenzamido derivatives of α-amino acids under normal-phase HPLC conditions (*K47*).

Armstrong et al. reported a new CSP made from the covalent attachment of the macrocyclic glycopeptide antibiotic teicoplanin to silica gel. It exhibited good stability and enantioselectivities in the reversed-phase, normal-phase, and "polar-organic" modes for native amino acids, peptides, α-hydroxycarboxylic acids, and a variety of neutral analytes including cyclic amides and amines (*K48*). Piette et al. immobilized *tert*-butyl carbamoylated quinine on nonporous silica for the separation of several N-protected α-amino acids, including 3,5-dinitrobenzyloxycarbonyl amino acids (DNZ-AAs). In combination with DryLab RTM software, the effect of mobile-phase pH, buffer concentration, and percentage of methanol or acetonitrile on enantioselectivity was examined and optimized, permitting resolution of the set of test compounds within several minutes (*K49*). Aldrich-Wright et al. developed a covalently bound DNA stationary phase for the separation of optically active $[Ru(dipyrido[6,7\text{-}d\text{:}2',3'\text{-}f]quinoxaline)_3]^{2+}$ and $[Ru(1,10\text{-phenanthroline})_3]^{2+}$ complex ions; retention was dependent on both the pH and size of the aromatic ligands (*K50*). Machida et al. synthesized a novel CSP having a (*R*,*R*)-tartramide derivative as a chiral moiety for the enantiomeric separation of diols and β-amino alcohols. The driving force of the enantiomeric separation was assumed to be the dual hydrogen-bonding associations and $\pi-\pi$ interactions (*K51*). The new CSP Sudo et al. prepared via the covalent binding of (*S*)-2,2′-dihydroxy-1,1′-binaphthyl to silica gels showed enantioselectivity mainly for secondary and tertiary amines; the enantioselectivity and peak shape were significantly affected by the addition of HO_2CCF_3 or diethylamine to the mobile phase (*K52*). Lynch et al. prepared a new (−)-menthyl bonded silica phase by hydrosilation of a hydride silica intermediate and used it for enantiomer separations in the reversed-phase mode (*K53*). Finally, Zhou et al. separated *N*-3,5-dinitrobenzoyl-DL-amino acid methyl esters with enantioselectivities of 1.04−1.18 on a novel CSP derived from L-proline (*K54*).

Other Studies. Roussel et al. developed a comprehensive molecular database (CHIRBASE) which contains over 35 000 chiral separations by liquid chromatography. Using this database, they performed an analysis of 5000 solutes separated on 25 stationary phases using 15 empirical molecular descriptors (*K55*). Booth et al. described a new method for the prediction and description of enantioselective separations on HPLC CSPs based on the combination of multivariate regression and neural networks. The method was successfully applied to the separation of 29 aromatic acids and amides, chromatographed on three amylosic CSPs. Combinations of charge-transfer, electrostatic, lipophilic, and dipole interactions, identified by multivariate regression, describe retention and enantioselectivity, with highly predictive models being generated by the training of back-propagation neural networks (*K56*). Durham employed force field calculations to model the inclusion complexes of enantiomeric brompheniramine, ephedrine, pseudoephedrine, ibuprofen, mandelic acid, methylphenobarbitone, and hexobarbitone with β-cyclodextrin and compared the difference in enthalpy of complex formation between enantiomeric pairs with literature chromatographic data (*K57*). Stringham and Blackwell noted that, for many chiral separations, as the temperature is raised, enantioselectivity decreases until enantiomers coelute at an isoelution temperature. Above this temperature, however, the elution order should reverse and enantioselectivity will increase with increasing temperature, along with column efficiency. The key to exploiting these favorable entropy-driven separations is the selection or design of the right chiral selector and the control of the operational variables (mobile-phase composition, etc.) so that the isoelution temperature is in a practical temperature range for the sample of interest, as well as a minimization of nonspecific retention (*K58*). Pirkle and Welch pointed out that although the coupling of dissimilar chiral columns can sometimes be useful for the separation of enantiomers in complex mixtures, from first principles their coupling to achieve a broad-spectrum screen for the separation of otherwise pure enantiomers cannot be recommended (*K59*). Using QSRR and thermodynamic approaches, Booth and Wainer identified two separate retention mechanisms for mexiletine-related compounds on an amylose tris(3,5-dimethylphenyl carbamate) chiral stationary phase. Highly statistically significant regression equations were derived which describe the retention of the first- and second-eluting enantiomers in terms of nonempirical molecular descriptors (*K60*).

Loun and Hage used plate height measurements to investigate the kinetics of (*R*)- and (*S*)-warfarin binding to an immobilized human serum albumin (HSA) column. From the dissociation data, it was found that an increase in temperature led to a large decrease in plate height for both enantiomers due to improved stationary-phase mass transfers, although the relative proportions of enthalpic and entropic contributions to the activation energy for binding were very different for the enantiomers. Their results supported an earlier model of warfarin binding, in which (*R*)- and (*S*)-warfarin were proposed to interact with regions on the interior and exterior of HSA, respectively (*K61*). Yang and Hage showed with their model single-site binding system of D- and L-tryptophan with HSA that changes in retention due to changes in the temperature, pH, ionic strength, or 1-propanol content of the mobile phase can be explained by changes in not only the binding constant but, in some cases, the number of available binding sites (*K62*).

Messina et al. described a new procedure for ergot alkaloid-based CSP preparation based on bonding the allyl derivative of terguride to mercaptopropylsilanized silica gel. The packing exhibited higher content of chiral selector, stability, reproducibility, and enantioselectivity toward amino acids compared to that previously studied. The influence of pH, ionic strength, content

and nature of organic modifier, temperature, and other factors were examined (*K63*). Kempe described a novel approach for making molecularly imprinted polymers by copolymerization of the branched, trifunctional cross-linkers pentaerythritol triacrylate and 2,2-bis(hydroxymethyl)butanol trimethacrylate with methacrylic acid. Compared to previously reported noncovalent molecularly imprinted polymers, they exhibited considerably higher loading capacity, increased enantioselectivity, and better resolving capability (*K64*). Stalcup et al. investigated the chiral selectivity of a heparin affinity column for chloroquine. The (+)-enantiomer eluted first, consistent with results reported previously using heparin as a chiral additive in capillary zone electrophoresis. The effects of pH, ionic strength, and organic modifier on the enantiodiscrimination were explored. A combination of electrostatic and hydrophobic interactions seems to play an important role in the enantiodiscrimination exhibited by this novel phase (*K65*).

Fukushima et al. described the enantiomeric separation and detection of 2-arylpropionic acids derivatized with [(*N*,*N*-dimethylamino)sulfonyl]benzofurazan reagents on cellulose tris(3,5-dimethylphenyl carbamate)-coated silica gel (*K66*). Bargmann-Leyder et al. systematically compared the chiral recognition mechanisms of cellulose- and amylose-derived chiral stationary phases in LC and SFC using pharmaceutical compounds. The presence of polar functions, such as primary or secondary hydroxyl or amine functions, may result in marked discrepancies in selectivity between LC and SFC, particularly on these carbohydrate phases, in contrast to other types of CSPs whose behavior is more consistent (*K67*). O'Brien et al. reported mechanistic aspects of chiral discrimination for a diol intermediate on tris(4-methylbenzoate)-derivatized cellulose. At low temperatures the enantioselectivity is entropy driven whereas at higher temperatures the separation is enthalpy driven (*K68*). Enomoto et al. described two methods for chemically bonding amylose to silica gel, followed by a conversion of the hydroxy groups of amylose to carbamate residues. Significant differences in the resolving power and durability against solvents such as THF were observed for the resulting CSPs. The influence of the spacer length between the amylose and silica gel, and the mobile-phase composition, on chiral recognition was studied (*K69*). Grieb et al. coated PGC with cellulose tris(3,5-dimethylphenyl carbamate) and assessed its chiral chromatographic properties with 15 chiral analytes (neutral, basic, acidic) and different mobile-phase additives (*K70*). They later described the preparative resolution of some chiral basic analytes on a flash chiral chromatographic column packed with a cellulose carbamate-coated ODS phase; compared with separations obtained onto underivatized silica of a similar diameter (40−63 μm), a substantial improvement was observed (*K71*).

By varying key mobile-phase parameters (pH, flow rate, buffer strength, organic concentration), column temperature, and sample loading, Risely and Sharp optimized the separation of seproxetine ((*S*)-norfluoxetine) from (*R*)-norfluoxetine using a new pepsin enzyme chiral stationary phase (*K72*). Kimata et al. separated a racemic mixture of the *R* and *S* isomers of phenyl(*phenyl*-d_5)-methanol using cellulose tribenzoate-coated silica as a stationary phase and a 2-propanol/hexane (5/95) mixture as the mobile phase (*K73*). Iida et al. prepared a series of five phenyl carbamoylated β-CDs and used them in tandem with an RPLC column to achieve a complete enantiomeric separation of phenylthio carbamoylated amino acids (*K74*).

Geometric and Positional Isomers. In a normal-phase separation of retinal and retinol isomers, Noell showed that former identifications in the literature are inconsistent or wrong (*K75*). Sidelmann et al. reported the first demonstration of directly coupled, continuous-flow HPLC/proton NMR spectrometry for the characterization of the positional isomers and anomers of various glucuronides (*K76*−*K78*). Lenz et al. reported similar results for drug glucuronides in whole urine (*K79*).

Emenhiser et al. achieved partial to complete resolution of a photoisomerized mixture of several geometrical isomers of α-carotene on a polymeric C30 column in the nonaqueous reversed-phase mode (*K80*). Strohschein et al. reported a stopped-flow HPLC NMR method for the structure elucidation of five β-carotene isomers after separation using a C30-bound phase (*K81*). More recently, they achieved complete separation of α-, β-, γ-, and δ-tocopherol and α-tocopherol acetate by RPLC within 14 min; detection was performed by UV and ^{1}H NMR spectroscopy (*K82*) and showed a C30 stationary phase to be superior to a C18 phase for the separation of thermally isomerized retinoic acids (*K83*).

Monde et al. developed a branched-chain, fluorocarbon-bonded silica gel column for the HPLC separation of geometrical isomers of halogenated and nonhalogenated phenols. Polyphenols such as flavonoids were separated with a sharp peak shape and excellent durability under extreme eluting conditions, in contrast with ordinary hydrocarbon-bonded silica gel columns (*K84*). Kalman et al. used RPLC to isolate and low-temperature NMR to identify the conformational states of di-, tri-, tetra-, penta-, and hexapeptide isomers (*K85*). Lin et al. used gradient elution, nonaqueous RPLC to separate 45 synthetic triacylglycerols and diacylglycerols. For both classes of glycerides, the elution corresponded closely with chain length, degree of unsaturation and presence of polar groups (*K86*).

MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY AND COLUMN SWITCHING

In keeping with tradition from past reviews, we restrict the term "multidimensional" to LC systems in which there is a distinct difference in retention mechanisms in the columns used. "Column switching" refers to multiple-column systems in which the mode of retention does not change. Only on-line (directly coupled) techniques will be discussed because of their greater accuracy and precision, decreased analysis times, and amenability to automation—a feature that seems to be a growing component of multiple-column separations. As in past years, a significant number of on-line sample enrichment columns coupled directly to LC analytical columns were described, and in general, these are not included here. Many of the more interesting applications of these on-line enrichment and LC separations can be found in the Trace Analysis section of this review.

Fundamental principles and important applications of multidimensional column liquid chromatography were the subject of a number of reviews. Wu and co-workers descibed an automated multidimensional HPLC screening approach for rapid method development in drug screening (*L1*). Brinkman reviewed multidimensional techniques useful for environmental analysis (*L2*), and Schoenmakers et al. did the same for analytical separations

important to the oil industry (*L3*). Lough and Noctor focused on bioanalytical chiral separations in their review (*L4*). Regnier and Huang discussed the potential of multidimensional LC combined with mass spectrometry for targeted component analysis (*L5*), and Hogendoorn et al. outlined the feasibility of coupled LC with selective detection in trace analysis (*L6*). Finally, the concept of solvent modulation for serially coupled columns was demonstrated with β-cyclodextrin columns and isomeric polyaromatic hydrocarbon solutes (*L7*).

The combination of chiral and traditional reversed-phase LC columns continues to be a popular approach for separating enantiomers in complex mixtures. Tanaka and Yamazaki used a cellulose chiral column after cleanup on an RP column for analysis of the enantiomers of pantoprazole in human serum (*L8*). Direct coupling of achiral−chiral columns with column switching was described for the separation of enantiomers of a metabolite of bupropion in human plasma (*L9*), enantiomers of the major metabolite of tipredane in rat urine (*L10*), and montelukast, a leukotriene receptor antagonist, and its *S* enantiomer (*L11*).

Size-exclusion and reversed-phase modes are also easily combined on-line, and this combination was used for a number of large-molecule separations. Opitek and co-workers in Jorgenson's group described an SEC−RPLC combination with MS detection for the analysis of protein digests (*L12*), and Amari and Mazsaroff combined SEC and RPLC in an assay for human interleukin-11 fusion protein in *E. coli* cells (*L13*). Complex polymer mixtures were the subject of separations with SEC columns coupled to a variety of secondary analytical modes (*L14*).

Affinity columns were also coupled directly with RPLC columns. Benkestock used an α-1-acid glycoprotein covalently bound to silica coupled to an RPLC column for the analysis of propiomazine in rat plasma (*L15*). On-line immunoaffinity chromatography−RPLC was described for dexamethasone in horse urine (*L16*), and D-9-tetrahydrocannabinol (THC) in human saliva (*L17*).

Cation-exchange chromatography followed on-line by an RPLC analytical separation and electrospray ionization mass spectrometry detection was used by Opiteck and co-workers to analyze protein mixtures (*L18*). Glyphosate and its main environmental metabolite aminomethylphosphonic acid were separated, after precolumn derivatization with a fluorophore, with a weak anion-exchange column coupled to a C18 analytical column (*L19*). Asai et al. reversed the traditional column order, using an RPLC first column and anion-exchange analytical column to assay orotidine in urine (*L20*). Ion-exchange columns were also coupled to size-exclusion columns for the analysis of poly- and oligosaccharides (*L21*). Finally, an internal-surface phenylboronic acid column was used for the selective extraction of β-blockers from biological fluids and coupled on-line with a RPLC analytical column (*L22*).

One of the most powerful but technically difficult combinations is HP-column LC and electrophoresis, and Jorgenson's group has contributed significantly to the development of this multidimensional approach. Jeffery et al. reviewed the use of on-line RPLC and capillary zone electrophoresis and discussed several applications (*L23*). Lewis et al. described an on-line RPLC−CZE-MS system for the analysis of peptide mixtures and tryptic digests of RNase (*L24*). The LC column was coupled to the CZE capillary column by a transverse flow gating interface. Moore et al. described three approaches for coupled RPLC−CZE analysis of peptides and proteins (*L25*). A three-dimensional separation of peptides was developed which combined SEC/RPLC/CZE, coupling the RPLC and CZE columns with optical gating (*L26*).

Other interesting combinations used to develop two-dimensional separations included ion-pair chromatography columns with column switching for the analysis of total iodine in biological material after ashing (*L27*). Hogendoorn et al. described a unified LC−LC screening method for polar pesticides in water that was supported by GC−MS confirmation (*L28*). The effect of various parameters associated with the second dimension on two-dimensional separations of low-molecular-weight polymers and surfactants was discussed by Murphy et al. (*L29*). Polymer separations were also the object of a two-dimensional system that combined LC adsorption chromatography with SEC (*L30*). Finally, Seki et al. developed a multidimensional separation of uric acid and creatine in biological fluids that involved sophisticated switching and six columns (*L31*).

PREPARATIVE LC

During this review period, preparative separations of pharmaceuticals, especially chiral compounds and proteins, were dominant. A technique called simulated moving-bed chromatography for preparative separations, described below, attracted considerable interest because of its increased throughput and mobile-phase savings.

Reviews. Wisniewski et al. (*M1*) reviewed process design considerations for large-scale chromatography of biomolecules. Preparative HPLC for enantiomeric separations using chiral stationary phases was presented by Han (*M2*), Ichida (*M3*), and Aboul-Enein (*M4*). Reviews were presented on large-scale HPLC of enzymes for food applications (*M5*), food-derived peptides (*M6*), and egg proteins (*M7*).

Packings/Columns/Hardware. Majors (*M8*) highlighted trends for preparative HPLC columns and packings. Ohnishi et al. (*M9*) reviewed the development of chiral stationary phases consisting of cellulose derivatives. Cellulose phenyl carbamates were developed and evaluated by Chankvetadze et al. (*M10*) for preparative-scale enantioseparations of chiral pharmaceuticals. A novel chiral stationary phase consisting of a chiral selector attached to poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) packing was shown to be applicable under overload conditions (*M11*).

A new polymer resin, PolyFlo, was described for large-scale purification of synthetic oligodeoxyribonucleotides varying in length from 18 to 41 bases (*M12*). Burton et al. (*M13*) compared the performance of Sepharose and Perloza cellulose matrixes for the purification of chymosin. Performance characteristics of membrane adsorbents were compared to Fast Flow Sepharose for the purification of human serum albumin (*M14*). Polymer-coated high-carbon C18 packing materials were developed and evaluated for preparative LC (*M15*). A polymeric C30 stationary phase was assessed and applied to the semipreparative isolation of α-carotenes isomers (*M16*).

Rodrigues et al. (*M17*) studied elution behavior of Poros Q/M particles for the separation of proteins and showed improved performance due to better mass transfer from enhanced intrapar-

ticle convection. Gruene (*M18*) developed a perfusion chromatography system and workstation which enables automation of methods development and scale-up.

Stanley et al. (*M19*) reported on the mechanical properties of packings in dynamic axial compression columns for preparative HPLC. These investigators also studied the reproducibility of column performance and the role of packing density including preparative columns packed under dynamic axial compression (*M20*). A spring-loaded packing apparatus was described and compared to a hydraulic ram system, but was found to have decreased column efficiency (*M21*).

A patent was awarded for an instrument capable of delivering high flow rates to generate turbulence for preparative separations (*M22*). An HPLC system was described by Hatch et al. (*M23*) for linear scale-up from an analytical to a preparative mode. A discontinuous cyclic countercurrent chromatographic process and apparatus was given by Grill (*M24*) for preparative separations. Lukin (*M25*) reported on an automated pilot-plant scale system for the production of pure isomeric forms of carotenes. A completely automatic chromatographic system was presented by Mellor et al. (*M26*) for the isolation of natural products. This method consists of a combination of HPLC and solid-phase extraction columns in which 1−5 g of a natural product can be fractionated into 200−300 pure compounds within 24 h. A preparative supercritical fluid chromatograph was discussed by Heaton et al. (*M27*) in which the liquid supercritical fluid modifier was used as a trapping fluid. Yokouchi et al. (*M28*) described a multistage fractional extraction device.

There was been increased interest in a technique termed simulated moving-bed chromatography for preparative separations. With SMB chromatography, large amounts of mobile phase are saved and throughput is increased. The usefulness of this technique was demonstrated by Francotte and Richert (*M29*) on the separation of enantiomers of chiral drugs. Schulte et al. (*M30*) compared the specific productivity of different chiral stationary phases used for SMB chromatography. This group also described a study to verify the rigorous dynamic SMB process model using experimental data from an HPLC column for the optimization of chiral separations (*M31*). Pais et al. (*M32*) developed a model for predicting the cyclic steady-state performance of SMB chromatography and determined the effect of several operating parameters. A continuous countercurrent chromatographic separation was carried out in an SMB system and software was developed to simulate the behavior of the separator (*M33*). Cavoy et al. (*M34*) designed a laboratory-based SMB system and applied it to the separation of chiral drug enantiomers. Mazzotti and coworkers (*M35*) developed a general theory for choosing SMB operating conditions. Zhong et al. (*M36*) reviewed their contributions to the theory of SMB chromatography under linear conditions.

Theory/Optimization/Methodology. Farkas and Guiochon (*M37*) determined the radial distributions of the local linear velocity of the mobile phase, the local efficiency, and the local analyte concentration at the column outlet. This was accomplished by simultaneously recording the elution bands at different radial locations over the column exit cross section. In a separate study, these investigators used optical fibers in a fluorescence detection scheme to determine the radial distribution of the transit time, column efficiency, and analyte concentration (*M38*). Felinger and Guiochon (*M39*) studied the optimization of preparative separations by overload gradient elution chromatography on a theoretical basis. Jandera et al. (*M40*) reported on the effects of the gradient profile on the production rate in reversed-phase gradient elution overload chromatography.

Mass overloading in elution LC was reviewed by Kowalczyk and Wrobel (*M41*). They stressed that the applicability of all existing models is limited to a single compound or binary mixtures when Langmuir isotherms are valid. Choi et al. (*M42*) investigated the effects of operating conditions on HETP in preparative LC and concluded that HETP was mainly affected by resistance to mass transfer in the intraparticle section of packings at high velocities. Kaltenbrunner et al. (*M43*) demonstrated the influence of extracolumn effects on band broadening in small preparative columns.

Gallant et al. (*M44*) employed a steric mass action model to describe the behavior of concentrated bands of protein under gradient elution conditions. The model provides insight into the process of gradient elution for preparative and large-scale separations. Miron and co-workers (*M45*) presented a mathematical model, based on displacement preparative LC, to help support preparative LC processes. Asplund and Edvinsson (*M46*) described a method for the rapid simulation of preparative LC which can be applied to the optimization of industrial-scale LC. A strategy was reported by Altenhoener et al. (*M47*) for modeling and estimating parameters for production-scale LC using the dynamic simulation tool SPEEDUP. Truedinger and Kupka (*M48*) described a software package, ChromSim, for simulation and modeling of preparative LC and process optimization. A computational algorithm and the Prep-Lear program was described for calculating peak profiles in nonlinear preparative HPLC using adsorption isotherm equations (*M49*).

Using two mathematical models, Row (*M50*) investigated the effect of sample size on peak shape in preparative LC. One of the models was based on a linear kinetic approach, and the other on a nonlinear adsorption isotherm method. Heuer et al. (*M51*) applied a concept based on measuring the adsorption isotherms to the scale-up of preparative separations. Delayed adsorption on the tailing portion of a peak was analyzed over a wide range of flow rates by Ohkuma et al. (*M52*). A model for blending aqueous buffers for gradient preparative LC of proteins, based on a continuous stirred-tank reactor model, was given by Kaltenbrunner and Jungbauer (*M53*). A modified version of the volume-averaged continuum theory for multiphase processes was reported by Colby et al. (*M54*) to predict the pressure drop across compressible packed beds of Sepharose Big-Beads SP.

Guiochon's group (*M55*) reported on the influence of the heat of adsorption on elution band profiles in nonlinear LC. Their results suggest that the influence of the heat of adsorption on actual separations is small and could be neglected in most cases. The influence of temperature gradients in preparative HPLC was presented by Brandt et al. (*M56*) in which columns operating at high flow rates are subjected to viscous heat dissipation. Axial and radial temperature profiles were measured within 60-mm-diameter columns to show the influence of temperature on the separation process.

For optimizing the design and operating conditions in preparative LC, the product of the production rate and the recovery yield was maximized (*M57*). This new objective function leads to optimum experimental conditions. Kirkland (*M58*) found that separation selectivity and resolution can be significantly increased and often optimized by modifying the nonpolar mobile phase with certain aprotic solvents. A systematic scheme is proposed for optimizing the separation of enantiomeric drugs. Using chemical engineering concepts, Luo and Hsu (*M59*) optimized gradient profiles in ion-exchange chromatography for protein purification. For the purification of scopolamine, Wirth and Hearn (*M60*) were able to increase the amount of injected sample by >10-fold using an optimized sample displacement procedure.

Gagnon and Grund (*M61*) reported on factors that affect capacity in preparative hydrophobic interaction chromatography. Their data suggest that although capacity in HIC responds to the same factors as with other forms of chromatography, high salt concentrations exert unique effects with important ramifications for large-scale process design. Cramer et al. (*M62*) disclosed a method for the purification of proteins by displacement chromatography on ion-exchange media using low-molecular-weight displacers. Chen and Scouten (*M63*) used hydrolyzed carrageenan as an efficient displacer for protein purification in IEC. Vogt and Freitag (*M64*) investigated anion-exchange and hydroxyapatite displacement chromatography for the isolation of whey proteins.

Selected Applications. Preparative LC separations have been reported for fullerenes (*M65*−*M67*), saponins (*M68*), natural products (*M69, M70*), human interleukin-2 (*M71*), modified H1 histones (*M72*), supercoiled plasmid DNA (*M73*), biotin (*M74*), vancomycin (*M75*), and gramicidin A (*M76*).

PRE- AND POSTCOLUMN DERIVATIZATION

This section of the review will first list relevant review articles and fundamental studies and will then group articles according to solute type or functional group. The searches revealed literally hundreds of articles published during this two-year period. Only those of a more fundamental nature are included here.

Derivatization is a necessary evil for many chromatographic analyses to add a detectable group, or even a more sensitive detectable group, to the solute(s) of interest. Chemistries have been developed for virtually every type of compound, and advances are now coming in the design of faster reactions, more selective reactions, and the synthesis of highly sensitive derivatizing agents. As in the past reviews, most interest is still in precolumn derivatization.

Four reviews of particular derivatization types appeared this period. Mukherjee and Karnes reviewed UV and fluorescence derivatization reagents for carboxylic acids (*N1*), and Liu et al. (*N2*) reviewed the use of postcolumn photochemical derivatization. Meguro and Hiroshi (*N3*) reviewed the strategy and design of reagents for fluorometric analysis of biomolecules, and Fink et al. (*N4*) discussed the evolution of a specific fluorogenic derivatization method for ivermectin.

An area of surprisingly little interest is in derivatization to enhance detection by mass spectrometry. Zhou et al. (*N5*) reported a study of electrospray mass spectrometry of phenylthiohydantoin (PTH) amino acid derivatives, with subfemtomole detection limits being shown for selected reaction monitoring.

The use of polymerized, solid-phase derivatization schemes offers great advantage in reproducibility, ease of automation, and reusability of reagents, yet this has been slow to be adopted. Krull's group continued their study of these methods, with a report of *size-selective* derivatizations (*N6*). Several other reports of on-line solid-phase derivatization schemes also appeared. Breckenridge et al. (*N7*) reported simultaneous sorption and derivatization from aqueous solution for volatile and hydrophilic carbonyls and reported speed enhancements of the derivatization of at least 6 times for reactive aldehydes and a 36−72-time improvement for slower reacting ketones. Emara et al. (*N8*) used cerium trihydroxyhydroperoxide as an oxidant in a packed column for on-line derivatization of methotrexate. Jain et al. (*N9*) determined bromide in complex matrixes using solid-phase extraction and derivatization with 2-iodosobenzoic acid. Herraez-Hernandez et al. (*N10*) designed an on-line system for derivatization of drugs, using a precolumn for sample cleanup and enrichment, with subsequent injection of the derivatizing agent and elution onto an analytical column. They showed the feasibility of this method by determining amphetamine and methamphetamine in untreated urine. Campins-Falco et al. (*N11*) further described this system for the determination of these two drugs.

Two other novel approaches were shown for removal of the derivatizing agent from solution. Sasamoto et al. (*N12*) used a new coumarin-based amine for derivatization of carboxylic acids using a two-phase system of water and ethyl acetate. Wu et al. (*N13*) developed a new sulfonate derivatizing agent with a fluorophore for sensitive detection and a tertiary amino function that can be removed by acid treatment after derivatization.

On-line photolytic reactions continued to be of interest, and Di Pietra et al. (*N14*) used on-line photochemical reactions before diode array detection for the unambiguous identification of analytes in pharmaceutical creams.

The use of micellar media for derivatization reactions also has certain advantages; specifically, they can be useful for solubilization of hydrophobic reagents and can speed reaction kinetics. Hutta et al. (*N15*) used a micellar system for solubilization of dithizone and subsequent reaction with mercury(II) and alkylmercury for sensitive photometric detection. Gautier et al. (*N16*) showed a surfactant-sensitized postcolumn reaction of xylenol orange for the determination of lanthanide ions by ion chromatography.

Two other ion-based derivatization schemes appeared. Ye and Lucy (*N17*) described the ion chromatographic determination of chelating ligands based on the formation of postcolumn ternary fluorescent complexes, and Hoshino et al. (*N18*) used H-resorcinol for complexation with Be(II), allowing highly sensitive visible detection at 500 nm.

Three new fluorescent derivatizing reagents were reported. Takechi et al. (*N19*) developed 7-(dimethylamino)coumarin derivatives for the detection of carboxylic acids, and Motte et al. (20) studied the use of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate for the determination of alcohols in aqueous media. Rahavendran and Karnes (*N21*) studied the use of a reagent synthesized from rhodamine 800, a far-red dye, as a precolumn derivatizing agent for amino-containing analytes. The use of this

dye/reagent allows the use of visible diode laser-induced fluorescence detection.

Amines are likely the most heavily studied group for chemical derivatization because of their biological, industrial, and environmental importance. There are many classic reactions, and virtually every detection technique has been applied to the analysis of these compounds. There was a great deal of activity in this area during this review period, and for convenience, we have grouped these reports approximately by detection technique.

Irvine (*N22*) gave a general discussion of the use of phenyl isothiocyanate for the derivatization of amino acids, and Guitart et al. (*N23*) reported an optimization of this reaction.

Morley et al. (*N24*) determined residual amines in bulk drug synthesis by precolumn derivatization with 3,5-dinitrobenzoyl chloride, Woo et al. (*N25*) used butyl isothiocyanate for the precolumn derivatization and determination of amino acids in food, and Kirschbaum and Brueckner (*N26*) used 4-phenylazobenzyloxycarbonyl chloride for precolumn derivatization.

Fluorescence detection generally gives better limits of detection, simplifies the separation process by eliminating those compounds that do not fluoresce, and is subsequently much more studied for amines and amino acids.

Quinoline derivatives continue to be useful as well, Saleh and Pok (*N27*) used 8-quinolinesulfonyl chloride and 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate was used by Reverter et al. (*N28*), Diaz et al. (*N29*), Merali and Clarkson (*N30*), and Busto et al. (*N31*). Crimmins and Cherian reported an improvement in sensitivity of this reagent (*N32*) using simplified procedures.

The other highly popular fluorescent agent is 9-fluorenylmethyl chloroformate. Two reports of improvements in the use of this reagent appeared. Bank et al. (*N33*) reported that derivatization in borate buffer at pH 11.4 for 40 min yielded more stable derivatives and Qu et al. (*N34*) reported improved chromatographic conditions for the separation of these derivatized amino acids. Campins-Falco et al. reported using this reagent for reaction in solid-phase extraction cartridges (*N35*), which seems to be a highly promising area for further study.

Busto et al. (*N36*) reported on-column derivatization of biogenic amines in wine with *o*-phthalaldehyde and used the method with several Spanish red wines (and the remaining wine could have proved helpful during the preparation of this review!).

Other novel fluorescent agents for amines and amino acids also appeared. Chen and Novotny reported a new dihydrofuran derivative useful for determination of primary amines by LC, CE, and MALDI/MS (*N37*). Other reagents reported were formamidines (*N38*), thiazoles (*N39*), aromatic glycinonitriles and methylamines (*N40*), (benzothiazolyl)benzoyl fluoride and -benzenesulfonyl chloride (*N41*), naphthoquinone sulfonate (*N42*), and 4-aminofluorescein and aminotetramethylrhodamine (*N43*).

Electrogenerated chemiluminescence continues to be useful for these compounds as well. Skotty et al. (*N44*) and Chen and Sato (*N45*) both used bipyridine ruthenium complexes for this purpose.

As noted in the last review, *after 10 years of writing this review, it appears it would be highly useful for a comprehensive, thorough experimental comparison of the many methods that have been proposed for amino acids to be performed and published.*

Saccharides continue to be of much interest, and here there were two unique reports that combined novel analytical determinations along with new derivatization schemes. Okafo et al. (*N46*) reported a coordinated LC, CE, and MS approach for the analysis of oligosaccharide mixtures derivatized with 2-aminoacridone, and Anumula (*N47*) reported procedures for the determination of monosaccharides typically found in glycoproteins along with sialic acids and amino sugar alcohols of glycoproteins.

Other reports include nonreducing sugars by postcolumn derivatization (*N48*), disaccharides separated on a porous graphitic carbon column followed by postcolumn derivatization with benzamidine (*N49*), precolumn derivatization of mono- and oligosaccharides by a pyrrolidine-aminosulfonyl-benzoxadiazole compound (*N50*), and aminopyrazine as a precolumn reagent for monosaccharides (*N51*).

Several new reagents were also reported for the derivatization of carboxylic acid compounds. Most notable among those is the report by Rahavendran and Karnes of nile blue, an oxazine dye, which reacts with carboxylic acid-containing analytes and allows the use of visible diode laser-induced fluorescence (*N52*). Other reagents used include a bromomethoxynaphthalene (*N53*), bromomethylfluorescein (*N54*), a dimethoxybenzothiazole (*N55*), a highly functionalized benzoxadiazole (*N56*), a functionalized aminobenzofurazan (*N57*) and a diphenylimidazole (*N58*).

New reactions and reagents were also reported for a variety of other compounds or functional groups. In the interest of space, only the analyte group will be mentioned; interested readers should then see the original article for the derivatizing group and conditions. These include glyphosates (*N59*, *N60*), thiols (*N61*, *N62*), isocyanate (*N63*, *N64*), alcohols (*N65*, *N66*), serotonin (*N67*), 5-hydroxyindoles (*N68*), aldehydes (*N69*), genamicin sulfate (*N70*), β-carboline alkaloids (*N71*), estrogens (*N72*), homocysteine and cysteine (*N73*), and methylcarbamates (*N74*).

MICROCOLUMN AND OPEN TUBULAR LC

The rapid growth in capillary electrophoresis, including capillary electrokinetic chromatography, does not appear to have stifled interest in microcolumn LC. Indeed, much of the fundamental development in micro-LC, such as mass spectrometer interface design, seems to be proceeding in parallel with CE, since the two techniques share many of the same advantages (e.g., very low solvent flow rates). Most authors seem to be adhering to the general convention that restricts the term "microcolumn" to open-tubular or packed capillary columns with internal diameters less than ~0.5 mm, while "microbore" refers to columns with internal diameters between 0.5 and 2.0 mm. Microbore LC will not specifically be reviewed here, but the practical advantages of microbore columns are now firmly established, as evidenced by the number of citations in other sections of this review such as Multidimensional LC and Column Switching.

The steady interest in micro-LC is reflected in the number of reviews which have appeared during the past two years. Vissers et al. discussed microcolumn LC research over the past 10 years, focusing on developments in column technology and the demands that microcolumns put on instrumental components such as detectors (*O1*). These authors also discussed the potential of using micro-LC in hyphentated techniques, including muldimensional LC and LC−MS. Swart et al. focused on more recent

developments in open tubular LC (OTLC), particularly in 5−10-μm-i.d. columns (*O2*). These authors argue that upgrading conventional HPLC instrumentation is easy and demonstrate this with protein−peptide mapping studies using OTLC coupled to electrospray mass spectrometry. Luque de Castro and Gamiz-Gracia included micro-LC in their review of trends in miniaturization (*O3*). Yates et al. discussed the future of micro-LC and electrospray tandem mass spectrometry in studies of complex biological problems such as those encountered in immunology (*O4*). Takeuchi and Ishii reviewed instrumentation and efficiency in micro-LC and OTLC (*O5*). Jinno and Fujimoto focused on micro-LC in hyphenated techniques such as LC/FT-IR (*O6*). Finally, Novotny reviewed micro-LC techniques useful in biochemical analysis (*O7*).

The unique characteristics of microcolumns and OTLC columns bring advantages and disadvantages. Peak broadening due to relatively large injection volumes can be overcome to some extent using on-column focusing. Vissers et al. optimized injection loop volumes for use with 300-μm packed capillary columns using focusing (*O8*). Malavasi and Asacalone demonstrated that large-volume injections with on-column focusing could result in 3−5-fold increases in sensitivity relative to conventional HPLC for certain drugs in biological fluids (*O9*). Rezai et al. also used this focusing technique to enhance the sensitivity of particle beam mass spectrometry detection used with 250-μm packed capillary columns (*O10*). Horka et al. reported enrichment factors up to 30 for model compounds by focusing the analyte in the tail of a moving solvent front in 200-μm OTLC columns (*O11*).

While the small internal volumes of micro-LC columns make injections somewhat problematical, their large surface area-to-volume ratios offer a great advantage; the ability to use temperature, including temperature programming, to optimize separations. Ryan et al. studied temperature programming in both normal- and reversed-phase OTLC and were able to obtain a 40% reduction in analysis times in a reversed-phase separation of chlorobenzenes compared to an isothermal separation (*O12*). Nyholm et al. coupled atmospheric pressure ionization mass spectrometry to high-temperature (150 °C) OTLC and used temperature gradients as well (*O13*). These authors also suggested that many thermally labile analytes were stable under the conditions they used. Houdiere et al. combined temperature *and* solvent programming and were able to reduce analysis times by 50% without loss of separation efficiency (*O14*).

Column preparation and stationary-phase loading and/immobilization are again the subjects of several reports. Hsieh and Jorgenson slurry-packed capillary columns of between 12- and 33-μm internal diameters with 5-μm octadecyl-silica and observed that the eddy diffusion (*A*) van Deemter term was the most significant contributor to lowered plate heights (*O15*). Flow patterns were monitored during slurry packing of PEEK and stainless steel columns by Zimina et al. (*O16*). Column efficiency and column resistance was found to be most sensitive to slurry media and column tubing material, the latter being attributed to wall effects. Glass-lined stainless steel packed microcolumns of 300-μm were described by Cheong et al. (*O17*), while Ruban et al. packed 530-μm fused-silica columns with various reversed-phase sorbents (*O18*). Both groups attributed most peak broadening to extracolumn effects, particularly in component connections. In a particularly interesting approach, Guo and Colon prepared C8 reversed-phase columns using a sol−gel process (*O19*). The sol−gel process allowed preparation of a coated, porous silica layer on the walls of the tube in one step, with greater retention and efficiencies than conventionally prepared OTLC columns. Other stationary phases immobilized in open tubular columns included a Chirasil-Dex polymer coated on Nucleosil 300-5 (*O20*) and modified cyclodextrin (*O21*), both for enantiomeric separations, and a polyacrylate phase for both reversed- and normal-phase separations (*O22*). Swelling of this polyacrylate phase in pure organic solvents is a problem, but when used with moderately polar solvent mixtures, the phase appears suitable for both normal- and reversed-phase separations.

Micro-LC places intense demands on components such as injectors, couplings, and particularly detectors. Miniaturization and sensitivity enhancement of detectors for use with microcolumns is thus a recurring theme. Swart et al. reported on their use of a frequency-doubled argon ion laser operated at 257 nm for fluorescence detection in OTLC (*O23*). Ring-disk and split-disk dual electrodes based on carbon films were described for detection of catecholamines in low-volume flow cells suitable for use with micro-LC (*O24*). Universal solute detection in nanoliter volumes at the picogram level was accomplished with a refractive index detector based on interferometric backscatter (*O25*). This system's utility was demonstrated with a reversed-phase separation of a test mixture. Specific improvements in mass spectrometry detection for use with micro-LC columns included postcolumn addition of Na, K, Rb, and Cs to form charged adduct ions for electrospray ionization (*O26*). Modified cyclodextrins, oligosaccharides, bafilomycins, and 18-crown-6 ethers were the target analyte molecules in that study. Elemental analysis with an ICP mass spectrometry was coupled to packed-capillary LC for the determination of tetramethyl- and tetraethyllead (*O27*).

Other instrumental advances included a microflow gradient system using stainless steel microcolumns (*O28*). The use of conventional HPLC instrumentation for capillary LC was reported in a review (*O29*). A "unified" chromatograph that can be used for capillary GC, SFC, or LC was described (*O30*). This chromatograph employs a single switching valve to accomplish sequential analyses using any of the capillary modes.

The maturing of microcolumn LC is reflected in the number of applications appearing in the literature since the last Review. Some of the more interesting applications noted include the determination of microcystins in cyanobacteria using 0.32-mm-i.d. columns with UV detection (*O30*). Detection limits of 10 pg of microcystins were obtained. The direct on-line coupling of capillary columns with high-field proton NMR was reported by Albert et al. (*O31*). Using a 180-μm capillary mounted in a microprobe on a 600-MHz NMR spectromter, the authors were able to acquire 2-D spectra and determine coupling constants to within 1.5 Hz by a stopped-flow method.

Some of the most impressive applications of micro-LC involved detection by various mass spectroscopy techniques. Rezai et al. used large-volume injections into a reversed-phase capillary column to enhance the sensitivity of particle beam mass spectrometry for a variety of pesticides (*O10*). Vissers et al. used on-line microcolumn switching to remove SDS from tryptic digests of proteins before electrospray ionization mass spectrometry

(*O32*). Cardenas et al. also coupled electrospray ionization and tandem mass spectrometry with microcolumn switching, incorporating an on-line derivatization step in a precolumn, for sequence analysis of peptides (*O33*).

TRACE ANALYSIS

Trace analysis by liquid chromatography invariably requires the use of a highly sensitive detector, derivatization to produce an enhanced response in a conventional detector, or sample enrichment of some form. The best methods combine these approaches, and many articles describing the combination of efficient and selective sample concentration with liquid chromatography separations coupled to sensitive detectors for trace analysis of analytes in complex matrixes have appeared since the last review. This section will focus on reports of the coupling of sensitive detection techniques with LC for trace analysis, as well as some novel sample enrichment strategies. No attempt has been made to provide a comprehensive review of the use of LC for trace analysis, and the reader is referred to the *Application Review*s for specific examples.

The continued development in ionization sources suitable for interfacing LC effluents and mass spectrometers has propelled LC−MS into the forefront of trace analysis techniques. In a timely review, Careri et al. discussed applications of different LC−MS techniques for determinations of xenobiotics in foods and outlined the advantages and disadvantages of different interfaces, including particle beam, thermospray, atmospheric pressure ionization, and electrospray (*P1*).

Electrospray ionization (ESI) has made a particularly significant impact in this area, and a number of trace analytical applications of LC−MS coupling through an ESI interface were noted. Giraud and co-workers combined solid-phase extraction and LC−ESI-MS for the determination of six pesticides in water (*P2*). They noted that, even with acceptable MS performance, current drinking water regulations require a concentration step such as solid-phase extraction. Corcia et al. used ESI and a single-quadrupole mass spectrometer to confirm the reversed-phase separation and UV detection of commonly used sulfonylurea herbicides in surface waters (*P3*). This method included off-line solid-phase extraction on a graphitized carbon black cartridge, followed by fractionation of the herbicides from humic acids by selective elution. Crescenzi and co-workers developed an LC−ESI-MS method for 45 widely used pesticides that also included solid-phase concentration on carbon black (*P4*). Cai et al. analyzed agricultural runoff for atrazine and its hydroxylated product by LC−ESI-MS (*P5*). This method compared favorably with GC−MS and LC−FAB-MS techniques previously used for this same purpose. Shahgholi et al. measured muramic acid, a marker for bacterial cell walls, in environmental dust by LC−ESI-MS−MS (*P6*).

A significant number of reports of the use of atmospheric pressure ionization (API) interfacing for trace analysis by LC−MS were noted. Ferrer et al. combined immunosorbent solid-phase extraction on-line with LC−API-MS to determine triazine and phenylurea herbicides at ppt levels in water and sediments (*P7*). The system they described was fully automated. Puig et al. described a similar on-line extraction and LC−API-MS system for the measurement of priority phenol pollutants (*P8*). Lacorte and Barcelo described on-line extraction and LC separation for analyzing ppt levels of organophosphorus pesticides in groundwater with both positive and negative modes of API (*P9*). Slobodnik and co-workers also used an on-line extraction and LC separation, but compared ESI and API with MS and MS−MS for structural confirmation (*P10*). The ionization methods gave similar detection limits, although API ionization showed much less sodium and acetonitrile/water cluster adducts than ESI, and several organophosphorus pesticides could only be detected in the negative ion ESI mode. These authors also built an SPE-LC-API-MS-MS library that was used for searching spectra and identifying atrazine in surface water samples. Finally, the use of a quadrupole-time-of-flight mass analyzer with reversed-phase LC for LC−MS−MS analysis of drug impurities was described (*P11*).

The combination of high-performance LC separations and inductively coupled plasma mass spectrometry (ICPMS) for trace metal determinations continues to attract the attention of researchers in numerous fields. Coni and co-workers reviewed the use of LC−ICPMS for determining trace metals in milk (*P12*) and discussed the nutritional value of the data this method can generate (*P13*). The speciation of metals in pond water was studied by LC−ICPMS after ultrafiltration to separate metals bound to large organic molecules such as humic acids (*P14*). Rivaro and Frache combined UV−visible detection with ICPMS after reversed-phase LC for the analysis of vanadyl porphyrins in mussel tissues (*P15*). A non-MS system was described which used postcolumn reaction to generate volatile arsenic hydrides which were then detected by atomic absorption spectroscopy (*P16*).

Trace measurements by LC were by no means restricted to systems with MS detectors. Somsen et al. used postcolumn liquid−liquid extraction for solvent elimination in order to couple reversed-phase LC with FT-IR spectroscopy and analyze herbicides in river water (*P17*). A dual electrochemical LC detector was described by Cheng et al. for the determination of dopamine and serotonin in rat brain tissue (*P18*). This dual detector uses the cathodic signal for dopamine measurements in order to avoid the irreversible electrochemical behavior of some of the interfering peaks. Nonionic poly(ethylene oxide)−surfactant mixtures were analyzed by solid-phase extraction and RP-LC on an octyl column (*P19*). Since these compounds have no chromaphores, differential refractometry was used and detection limits down to 0.25 mg/L obtained. Even conventional diode-array UV−visible detection yielded detection limits of ∼0.1 mg/L for pesticides and their degradation products after on-line concentration and RP-LC gradient elution separation (*P20*).

Some of the more interesting enrichment methods that were combined with an LC separation included a supported liquid membrane for concentration of alkylthio-*s*-triazine herbicides in water (*P21*). Martin-Esteban et al. described an on-line membrane extraction disk and RP-LC separation with diode array UV−visible detection for determination of polar pesticides in water (*P22*). These same workers developed an immunosorbent enrichment precolumn for phenylurea herbicides and combined it on-line with RP-LC with diode array detection (*P23*). Cai and Hennion also developed an immunoaffinity enrichment column, in this case for β-agonists in bovine urine, and combined it on-line with packed column capillary liquid chromatography and electrospray ionization-tandem mass spectrometry (*P24*). Microextraction in fused-silica capillaries was combined with RP-LC for measurement of

herbicides in water (*P25*). Polar pesticides in water were concentrated on Baker's yeast that was immobilized on silica, and the yeast−silica combined on-line with RP-LC and diode array detection (*P26*). Finally, dialysis enrichment was combined on-line to RP-LC for the determination of the drugs levosimendan (*P27*), and verapamil (*P28*) and norverapamil (*P28*) in human plasma.

A number of other articles relevant to this topic appeared since the last review, including a review of porphyrins as ligands in HPLC systems for trace metal analysis (*P29*). Malavasi and Ascalone discussed the use of large-volume injections combined with on-column focusing for capillary and microcolumn LC for trace analysis of drugs in biological fluids (*P30*), as did Rezai et al. for packed-capillary LC for pesticides (*P31*). Finally, Hogendoorn and co-workers reviewed the use coupled-column LC and selective detection for trace analysis of polar pesticides (*P32*).

PHYSICOCHEMICAL MEASUREMENTS

This section deals with the use of HPLC for studying physicochemical properties and behavior of solutes. Readers interested in this topic may wish to consult the corresponding section in the Size Exclusion Chromatography review in this issue of *Analytical Chemistry*.

Linear Free Energy Relationships/Hydrophobicity/Partition Coefficients. Abraham et al. (*Q1*) applied a linear free energy equation to the capacity factors of 25 series of solutes on six different C18 stationary phases with aqueous methanol and acetonitrile mobile phases. Parameters used in the equation were excess molar refraction, solute polarizability, solute hydrogen bond acidity and basicity, and solute volume. Nasal et al. (*Q2*) studied the retention properties of polyethylene-coated silica and polyethylene-coated zirconia HPLC packings using liner free energy relationships. Li and Carr (*Q3*) described the use of linear solvation energy relations to thermodynamically characterize the retention behavior on a polybutadiene-coated zirconia. Vailaya and Horvath (*Q4*) used a simplified version of the solvophobic theory to reexamine retention data with nonpolar and weakly polar solutes in reversed-phase HPLC.

To understand the types and relative strengths of various intermolecular forces between β-cyclodextrin and the guest solute, a linear solvation energy relationship relationship was used by Nah and co-workers (*Q5*). Lowrey and Famini (*Q6*) described the use of theoretical linear solvation energy relationships with respect to HPLC capacity factors of compounds used in explosives. This approach provided a reasonable interpretation of retention times in terms of molecular volume and quantities associated with acidity and basicity.

Helweg et al. (*Q7*) found different free energy relationships between retention and octanol/water partition coefficients of polar polycyclic aromatic compounds. He and Wang (*Q8*) measured and correlated octanol/water partition coefficients, adsorption coefficients for soils and sediments, and acute toxicities to *Daphnia magna* of 28 different alkyl (1-phenylsulfonyl)cycloalkanecarboxylates by linear solvation energy relationships and capacity factors using reversed-phase HPLC.

Yamagami and Katashiba (*Q9*) determined the relationship between octanol/water partition coefficients and capacity factors of phenyl *N*-methyl and phenyl *N*,*N*-dimethylcarbamates from which hydrophobicity parameters were determined. Yang et al. (*Q10*) obtained hydrophobic parameters of 20 substituted ferrocene derivatives from capacity factors and used these data to interpret the relationship between the structure of ferrocene derivatives and their retention. The hydrophobicity indexes of hydroxybenzoate esters, as determined by HPLC, were compared by Yoo and Jung (*Q11*). Montanari et al. (*Q12*) compared the HPLC lipophilicity parameters, the capacity factor, and the partition coefficient to the hydrophobicity index φ_0.

Valko et al. (*Q13*) described a new chromatographic hydrophobicity parameter that can be used as a high-throughput physicochemical property for profiling 50−100 compounds per day, such as in drug design. Forgacs and Cserhati determined the relationship between the physicochemical parameters of steroidal drugs and their retention properties on polyethylene-coated silica (*Q14*) and C18 (*Q15*) and monoamine oxidase inhibitory drugs on porous graphitized carbon (*Q16*). These authors also reported on the relationship between the hydrophobicity and specific hydrophobic surface area of pesticides and compared HPLC data to those results obtained by reversed-phase TLC (*Q17*). Yamaki (*Q18*) studied the relationships between the hydrophobicity of peptides and their capacity factors on a microspherical carbon column and a C18 packing.

Marina and Garcia (*Q19*) reviewed the use of micellar HPLC for determining distribution coefficients. This group also studied the correlation between the capacity factor and the octanol/water partition coefficients of aromatic compounds in micellar electokinetic chromatography (*Q20*). Medina-Hernandez and Sagrado (*Q21*) presented an empirical model that describes the relationship between retention in micellar HPLC and the partition coefficient for a series of neutral compounds. Using micellar mobile phases, Medina-Hernandez et al. (*Q22*) established a hydrophobicity scale with retention data for *o*-phthalaldehyde-*N*-acetyl-L-cysteine amino acid derivatives using the glycine derivative as a reference point. These investigators also used micellar HPLC to study quantitative retention−structure and retention−activity relationships of local anesthetics (*Q23*) and β-blockers (*Q24*). Szczepaniak and Szymanski (*Q25*) applied micellar HPLC to investigate the quantitative relationships between the structure of a number of biologically active compounds and their biological activity.

Barbato et al. (*Q26*) determined capacity factors of a set of 4-phenyldihydropyridine calcium channel blockers on an immobilized artificial membrane (IAM) column which represents a solid-phase model of fluid membranes. These membranes are chromatographic surfaces prepared by covalently immobilizing cell membrane phospholipids to solid surfaces. Receptor binding values from rat cortical brain preparations correlated with partition coefficients determined by this method. Ong and co-workers (*Q27*) measured partition coefficients of drugs on IAM columns and were able to correlate these values to those determined in fluid liposome systems. Salminen et al. (*Q28*) used IAM columns to study the relationship between retention and brain penetration of structurally diverse drugs. Turowski and Kaliszan (*Q29*) described a keratin immobilized membrane column for chromatographically modeling skin permeation.

Collantes et al. (*Q30*) developed a quantitative structure−retention (QSAR) relationship from HPLC retention data of unsubstituted polycyclic aromatic hydrocarbons using a three-

dimensional QSAR method known as comparative molecular field analysis (CoMFA). Kim et al. (*Q31*) also used the CoMFA approach to correlate hydrophobicity parameters of a mixed set of compounds from their three-dimensional structures.

The relationship between capacity factors and octanol−water partition coefficients were determined for monosubstituted thiophenes (*Q32*), barbiturates (*Q33*), progesterone and corticosteroid derivatives (*Q34*), glucuronides (*Q35*), local anesthetics (*Q36*), 2-amino-2-oxazolines (*Q37*), explosives (*Q38*), dipiperazine diquaternary ammonium derivatives (*Q39*), lanostanoid triterpenes (*Q40*), 1,2-dithiole-3-thiones and 1,2-dithiol-3-ones (*Q41*), aromatic sulfur-containing compounds (*Q42*), and 6-fluoroquinolones (*Q43*).

Nakamura et al. (*Q44*), using Sep-Pak columns, related octanol−water partition coefficients to solid-phase extraction behavior of agricultural chemicals. Ritter and co-workers (*Q45*) compared three different reversed-phase HPLC methods for determining octanol−water partition coefficients. Berger and colleagues (*Q46*, *Q47*) used HPLC to determine partition coefficients of Re coordination compounds. Jenke (*Q48*) evaluated the use of nonporous, 1.5-μm C18 packings to determine octanol−water partition coefficients of model compounds.

Association and Stability Constants. Nakagawa and coworkers (*Q49*, *Q50*) described a technique called high-performance frontal analysis (HPFA), which is used to determine unbound drug concentration in drug−protein binding equilibrium. When an excess volume of a drug−protein solution is injected into an HPFA column packed with a restricted-access phase that excludes protein but retains the drug in the micropores, the drug is eluted as a zonal peak with a plateau region. The authors developed a theoretical plate height model which confirms that the drug concentration in the plateau agrees with the unbound drug concentration in the sample solution. This method was used to determine unbound concentrations of semotiadil and levosemotiadil in human serum albumin (*Q51*) and racemic warfarin and phenylbutazone with human serum albumin (*Q52*, *Q53*).

Hage's group (*Q54*) used plate height measurements to investigate the kinetics of warafin binding to an immobilized human serum albumin column from which association rate constants were calculated. These researchers also used frontal analysis to examine changes in the association constant and moles of binding sites for tryptophan on an immobilized human serum albumin column (*Q55*, *Q56*). Wang et al. (*Q57*) developed a method for determining drug−protein interaction parameters, including the association constant and number of binding sites using a microdialysis sampling technique combined with HPLC. This approach was applied to the study of carbamazepine−human serum protein (*Q57*) and sulfamethoxazole−human serum albumin (*Q58*).

Bertucci and Wainer (*Q59*) reviewed the applications of human serum albumin-based stationary phases for the characterization of drug−protein binding and drug−drug binding interactions. Soltes and Sebille (*Q60*) investigated reversible binding interactions between tryptophan enantiomers and albumins using achiral and chiral stationary phases and also employed a column switching technique using both columns. Immobilized serum albumin stationary phases were used for binding studies with warafin (*Q61*), nonsteroidal antiinflammatory drugs (*Q61*, *Q62*), benzodiazepines (*Q61*), and indolocarbazole derivatives (*Q63*).

Oravcova et al. (*Q64*) compared the Hummel−Dreyer method in capillary zone electrophoresis with that of HPLC to study the interaction of racemic carvediol and its individual enantiomers with human plasma proteins. Sun and co-workers (*Q65*) used the Hummel−Dryer method to investigate the binding of sulfinpyrazone to bovine serum albumin. Koeplinger and Zhao (*Q66*) developed an SEC method for determining drug−protein interaction of HIV protease inhibitor−serum albumin association. In this approach, serum albumin is added to the mobile phase, and a shift toward shorter retention time was indicative of drug−protein interaction.

Liu et al. (*Q67*) developed a domain binding model for explaining multiple peaks obtained with fibrinogen on a DEAE anion-exchange HPLC column. The different peaks resulted from the binding of either the D or E domain of fibrinogen to the packing. Mano et al. (*Q68*) reported on the relationship between the association constant and enantioselectivity of enantiomers with a protein-conjugated chiral stationary phase using a flavoprotein-conjugated stationary phase, with ketoprofen as a model drug.

Feng et al. (*Q69*) described the reversed-phase ion-pair HPLC behavior of aluminum complexes with 5-sulfoquinoline-8-ol, in which the equilibrium and rate constant of the interconversion between two complexes was determined. In a subsequent paper (*Q70*), the equilibrium and kinetic rate constants was measured for the ligand-exchange reaction of aluminum(III)-5-sulfoquinoline-8-ol complex with fluoride ion. Deng et al. (*Q71*) determined the stepwise stability constants of nickel ion with 1,10-phenanthroline and the protonation constant of the ligand. Wang and Fu (*Q72*) reported on a model for determining the stability constants of metal complexes with on-column derivatization using reversed-phase HPLC.

Mehra and colleagues (*Q73*) used optical spectroscopy and reversed-phase HPLC to investigate the binding of Hg(II) to plant metal-binding peptides (phytochelatins). Leopold and Gunther (*Q74*) used HPLC coupled on-line with ICP-MS to determine heavy metal binding properties of phytochelatins in plant cell cultures. Sutheimer and Cabaniss (*Q75*) used cation-exchange HPLC to study aluminum binding to humic substances.

Karikas et al. (*Q76*) employed an HPLC method to measure the binding of polyamines and lipidic amines to DNA. Lukulay and McGuffin (*Q77*) studied solute−solute interactions for a number of steroids and its influence on retention behavior in reversed-phase HPLC.

Tang and Love (*Q78*) studied the effects of molecular size and shape on the formation constants of polynuclear aromatic compounds and β-cyclodextrin inclusion complexes in a β-cyclodextrin-modified mobile phase. Moeder et al. (*Q79*) determined stoichiometric coefficients and apparent formation constants for α- and β-cyclodextrin complexes of terpenes using reversed-phase HPLC. Langourieux and Crouzet (*Q80*) used dynamic coupled LC for determining the formation constants of β-cyclodextrin complexes of 2-methoxynaphthalene, ethyl salicylate, and 1-menthol. Sadlej-Sosnowska (*Q81*) measured the association constants of steroid hormones with β- and γ-cyclodextrin using the Hummel−Dryer method. Ferguson et al. (*Q82*) described a systematic approach to quantitatively link the binding constants determined in CE and HPLC for anionic methylbenzoates with β-cyclodextrin.

Garcia-Alvarez-Coque et al. (*Q83*) reported on the partitioning behavior of solutes and data treatment in micellar LC. Argiles et al. (*Q84*) used capacity factors of solutes at zero micellar concentrations and solute−micelle association constants as a hydrophobicity index and related these values to QSAR studies. Garcia and Marina (*Q85*) studied the influence of alcohol organic modifiers on association constants and retention behavior for benzene derivatives and polycyclic aromatic hydrocarbons by micellar LC. San Andres and Vera (*Q86*) determined micellar binding constants of Ni(II), Co(II), and Cu(II) diethyl dithiocarbamate complexes using micellar LC. Li and Fritz (*Q87*) calculated solute−surfactant binding constants using novel surfactant additives.

Barbosa et al. (*Q88*) used HPLC to determine dissociation constants of a series of tripeptides and correlated these values to solvent properties using linear solvation energy relationships. In a subsequent study, this group measured dissociation constants of fluoroquinolones and correlated these values with Taft and Kamlet solvatochromic parameters (*Q89*). Bosch et al. (*Q90*) used reversed-phase HPLC to study solute properties of ionizable solutes, including acid dissociation constants. Jano and coworkers (*Q91*) described a general equation for calculating the dissociation constants of polyprotic acids and bases from measured retention factors and applied this approach to polyprotic leukotrienes (*Q92*). HPLC was used to measure pK_a values of polybasic acids (*Q93*), 2-amino-2-oxazolines (*Q94*), phenolic and nitrogen-containing compounds (*Q95*), substituted phenols (*Q96*), and cytosine, cytidine, and their synthetic analogues (*Q97*).

Thermodynamic Studies. Guillaume and Guinchard (*Q98*) determined the separation factor of *p*-hydroxybenzoic esters as a function of temperature, from which Gibbs Helmholtz parameters were calculated. This group (*Q99*) also measured enthalpic and entropic interactions of benzodiazepines in reversed-phase HPLC over a wide range of mobile-phase compositions. A theory was also presented to describe the variation of the retention factor of alkyl benzoate esters and was used to determine the enthalpy, entropy, and Gibbs free energy for (1) the transfer of these solutes from mobile phase to stationary phase and (2) the solute solvation by acetonitrile clusters (*Q100*).

Basuik and Gromovoy (*Q101*−*Q103*) estimated thermodynamic parameters for amino acid, peptide, and 2,5-piperazinedione adsorption onto silica from water using HPLC. Akapo and Simpson (*Q104*) investigated the influence of temperature and mobile-phase composition on retention properties of oligomeric-bonded phases in reversed-phase HPLC. Thermodynamic properties of the transfer of polynuclear aromatic hydrocarbons from the mobile phase to the stationary phase were determined by Delgado et al. (*Q105*). McGuffin and Chen (*Q106*) measured thermodynamic properties of methylene and benzene homologues in reversed-phase HPLC. Li and Carr (*Q107*) described the effect of temperature on the thermodynamic properties of polybutadiene-coated zirconia packings.

Geng (*Q108*) reported on enthalpy of adsorption and desorption in reversed-phase HPLC.

Zimmerman and Kwan (*Q109*) compared enthalpies of formation in solution and enthalpies for HPLC retention for hydrogen-bonded host−guest complexes from normal-phase HPLC. John and Schlegel (*Q110*) calculated the equilibrium constant and Gibbs free energy of 11-hydroxy anomers of thromboxane B_2. Ringo and Evans (*Q111*) studied the role of pressure perturbations on enantiomeric complexation with β-cyclodextrin in LC experiments.

Miyabe and Takeuchi (*Q112*) investigated surface diffusion in reversed-phase HPLC and reported on the enthalpy−entropy compensation effect, the linear free-energy relation. Also studied were correlations between surface diffusion coefficient and mobile-phase composition and between the ratio of surface diffusion coefficient to molecular diffusivity and mobile-phase composition. In a subsequent study (*Q113*), these authors proposed a model for surface diffusion based on molecular diffusion.

Kinetic Studies. Boulkanz et al. (*Q114*) examined the kinetic behavior of human serum albumin adsorbed on a reversed-phase support. In the presence of 20% acetonitrile in a phosphate buffer, the apparent adsorption rate of human serum albumin was about 60 times lower as compared to the buffer alone. Measurements with FT-IR show that acetonitrile induces structural changes of the protein and competes with alkyl chains for the interaction with human serum albumin. Sojo et al. (*Q115*) studied sorption kinetics of pesticides in carrot tissue slurries using a novel on-line HPLC microextraction procedure.

Mallat et al. (*Q116*) used HPLC to study photolytic degradation of benomyl and carbendazim in water. Lowendahl and Allenmark used chiral normal-phase HPLC method to investigate the lipase-catalyzed ester hydrolysis reaction of methyl 2-(octylsulfinyl)-benzoate (*Q117*). The reaction kinetics of glycols and phenyl isocyanate in different solvents were measured using HPLC (*Q118*).

HPLC coupled with photodiode array detection was employed to determine the formation kinetics of hemorphins during peptic hydrolysis of bovine hemoglobin (*Q119*). The rate of phosphodiester cleavage of an oligoribonucleotide substrate by an RNA ribozyme with catalytic activity was measured using anion-exchange HPLC (*Q120*).

Conformational Studies. Turula and de Haseth (*Q121*) used particle beam LC/FT-IR to investigate the effect of chromatographic conditions on protein secondary structure in reversed-phase HPLC. This approach was also applied to β-lactoglobulin digests (*Q122*) and ribonuclease A and α-chymotrypsin (*Q123*). The retention behavior of methionine and methionine sulfoxide replacement analogues of 18-mer model peptides and neuropeptides was investigated by Rothemund et al. (*Q124*, *Q125*). Their results show that potentially α-helical peptides become helical on binding during chromatography. In a subsequent study (*Q126*), this research group also reported on hydrophobically induced conformation in ovine corticotropin-releasing hormone.

Blondelle et al. (*Q127*) investigated the induction of peptides into β-sheet structures during reversed-phase HPLC using a newly developed CD spectroscopic technique. This detector is based on C18-coated quartz plates that permit the detection of induced peptide conformations at the aqueous/lipid interface. A continuum of conformations was found to be induced at different equilibrium stages during the HPLC elution process. Liu and Anderson (*Q128*) found that fibrinogen gave multiple peaks on an anion-exchange HPLC column. These peaks resulted from the native and denatured forms binding to the packing through different domains on the molecule. During reversed-phase HPLC,

de Collongue-Poyet et al. (*Q129*) observed that acetonitrile in the mobile phase, as well as the packing, induced different structural changes for interferon γ and the analogue II.

Greve et al. (*Q130*) used HPLC and CE to examine intact and degraded fusion protein CTLA4I_g. Also, CE was used to study the conformational kinetics between two forms of CTLA4I_g and to estimate the activation energy of the conformer−conformer transition. Dong et al. (*Q131*) used reversed-phase HPLC for measuring the dynamic unfolding of bovine insulin which was denatured in dithiothreitol.

Nishikawa et al. (*Q132*) used reversed-phase HPLC to study cis−trans isomerization of proline dipeptides as a function of temperatrure. O′Neal and colleagues (*Q133*) determined the kinetics of cis−trans isomerization of prolactin receptor proline-rich motif peptides. Cis−trans isomerization of a small cyclic peptide was reported using both HPLC and NMR (*Q134*).

De Frutos et al. (*Q135*) determined the influence of reversed-phase and hydrophobic interaction chromatography on the conformation of whey proteins using CD spectroscopy. Zhou (*Q136*) used a phenylSuperose HR5/5 hydrophobic interaction column to measure surface hydrophobicities and conformational changes of proteins. Gilpin et al. (*Q137*) investigated the elution properties of D,L-tryptophan and L-kynurenine on silica-immobilized bovine serum. Their results suggest that solvent entrapment in the interior hydrophobic region of the protein may lead to small changes in conformation and/or dynamics which influences site-specific binding of the protein and hence changes in chromatographic retention.

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