



TOSOH THE CUSTOMER MAGAZINE

SCIENCE / SERVICE / SOLUTIONS

NO. #01
2009



TOSOH BIOSCIENCE

02 EDITORIAL DEAR READER

Dear reader, welcome to the second issue of the Tosoh Bioscience customer magazine. The motto of this issue is science – service – solutions. It is featuring some aspects of the scientific or technological background of our products for liquid chromatography. These topics are presented by the members of the Tosoh Bioscience technical support group. Three technical specialists, Dr. Werner Conze, Volker Noedinger and Achim Sprauer are covering the technical support for HPLC columns and process resins. The technical group, headed by P.D. Dr. Egbert Mueller, located in Stuttgart, Germany, offers technical support on the phone, by mail, in workshops, on customer site or at one of our tradeshow booths e.g. at the upcoming event HPLC 2009.

The HPLC application of this issue, the combination of non-denaturing HPLC modes like SEC with biochemical detection, was established at the Westfälische Wilhelms-University of Münster by Nils Helge Schebb, group member of Professor Dr. Uwe Karst. The group of Professor Karst is highly respected in the field of analytical chemistry, not only for the high quality of scientific output, but also for the perfect organisation of ISC 2008 and other conferences in Münster.

If you would like to read more about our products, search our application database or contact the technical support group, we invite you to discover our website: www.tosohbioscience.com. The website has been relaunched this year with a new layout and extended content.

ENJOY READING AND STAY INFORMED.

REGINA ROEMLING | MARKETING MANAGER
TOSOH BIOSCIENCE GMBH



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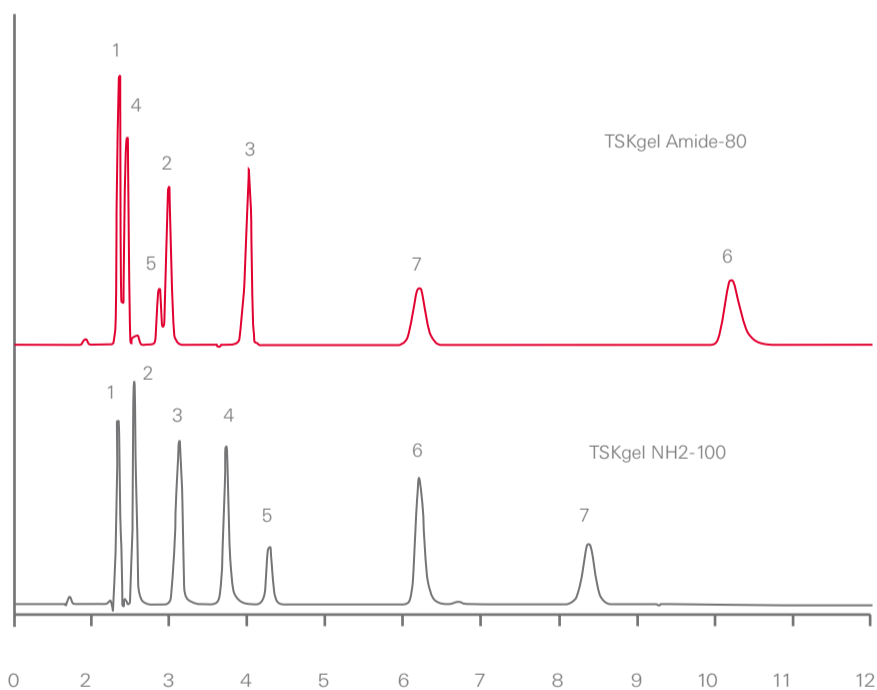
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03 WHAT'S NEW

NEW PRODUCTS

RECENTLY TOSOH BIOSCIENCE INTRODUCED SEVERAL NEW, INNOVATIVE TSK-GEL HPLC COLUMNS FOR THE ANALYSIS OF BIOMOLECULES. THESE COLUMNS EXPAND THE PRODUCT RANGE FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY [HILIC] AND ION EXCHANGE CHROMATOGRAPHY [IEC] MODE, RESPECTIVELY.

➤ **TSK-GEL HILIC COLUMNS:** Hydrophilic interaction liquid chromatography [HILIC] is the method of choice for the separation of very polar compounds. The TSKgel Amide-80 series is the industrial standard for the separation of Glycans by HILIC or HILIC-MS. Now, Tosoh Bioscience developed a new, robust amino phase: TSKgel NH2-100. The new amino columns expand the selectivity range of TSK-GEL HILIC solutions. It is well suited for all separations requiring amino functional groups. In contrast to many other amino phases the new column offers expanded stability under HILIC conditions. The new amino type HILIC phase is based on a 3 µm silica particle with 100 Å pores, which is treated with a proprietary endcapping procedure. Amino groups are introduced step wisely after endcapping and act as HILIC functional groups. Due to a high ligand density and large surface area TSKgel NH2-100V 3 µm columns show high retention for very polar compounds. For a comprehensive overview about the features of HILIC phases and applications the new brochure on TSK-GEL HILIC columns is available.

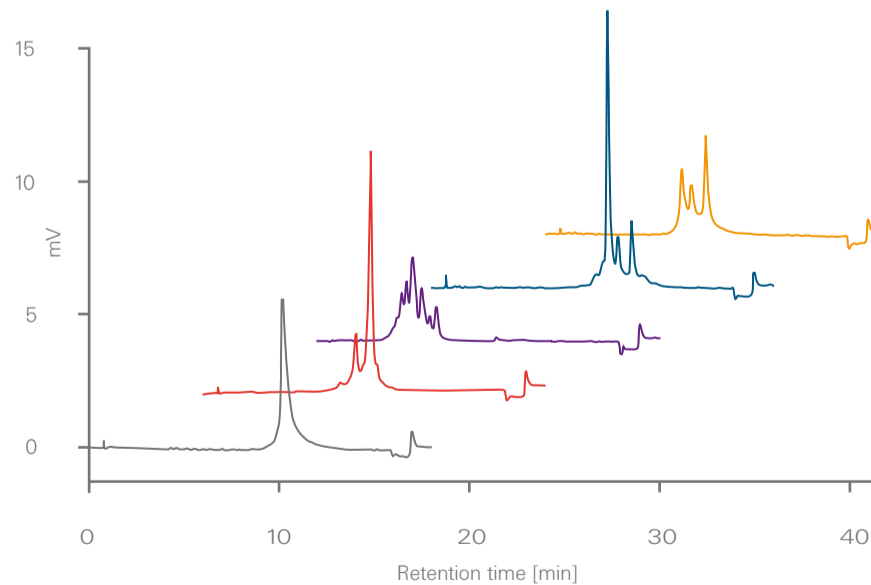
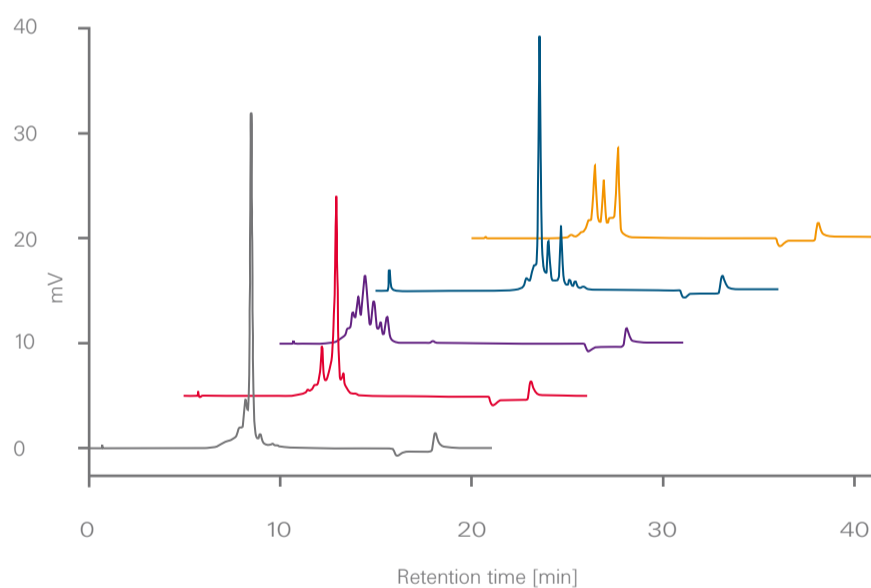


➤ **COMPARISON OF SELECTIVITY: ANALYSIS OF WATER SOLUBLE VITAMINS ON TSK-GEL AMIDE-80 AND TSK-GEL NH2-100**

1 = Nicotinamide | 2 = Vitamin B2 | 3 = Pyridoxine | 4 = Nicotinic acid | 5 = Vitamin C | 6 = Vitamin B1 | 7 = Vitamin B12

➤ **TSK-GEL IEX COLUMNS:** TSK-GEL STAT ion exchange columns, introduced in autumn 2008 turned out to be a very well accepted alternative to conventional or monolithic ion exchange columns currently used in quality control of biopharmaceuticals. The TSK-GEL STAT series comprises anion [quaternary ammonium] and cation exchange columns [carboxy & sulfo]. Dimensions and particle sizes are optimized either for highest throughput or for highest efficiency. Especially the 10 cm long high efficiency cation exchange columns [SP-STAT & CM-STAT] convinced users by offering at shorter analysis times a resolution similar to established methods. The first QC methods on TSK-GEL STAT columns are already under validation.

➤ **READ MORE ABOUT TSK-GEL COLUMNS ON**
WWW.SEPARATIONS.EU.TOSOHBIOSCIENCE.COM/PRODUCTS/HPLCCOLUMNS



➤ **RESOLUTION AND ANALYSIS TIME FOR FIVE ANTIBODIES ON TSK-GEL CM-STAT AND A COMPETITIVE WCX COLUMN. A: TSKGEL CM-STAT 7 µM, 4.6 MM ID X 10 CM L; B: COMPETITOR WCX, 10 µM 4.0 MM ID X 25 CM L**

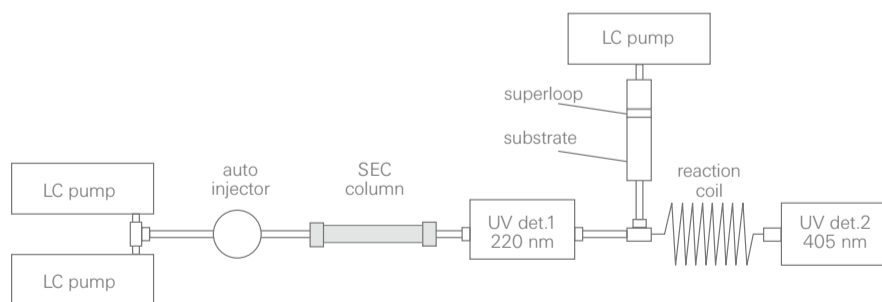
04 CUSTOMER APPLICATION

IDENTIFICATION OF PROTEASES IN CRUDE MIXTURES BY COMBINING AN ESI-MS/MS BASED ASSAY AND SEC WITH BIOCHEMICAL DETECTION

A NEW HPLC BASED METHODOLOGY FOR THE DETECTION AND ISOLATION OF PROTEASES IN COMPLEX MIXTURES HAS BEEN DEVELOPED AT THE INSTITUTE OF INORGANIC AND ANALYTICAL CHEMISTRY AT THE UNIVERSITY OF MÜNSTER/GERMANY. IT COMBINES THE CHARACTERIZATION OF CRUDE SAMPLES BY ELECTROSPRAY TANDEM MASS SPECTROMETRY (ESI-MS/MS) IN A MULTI-SUBSTRATE ASSAY AND THE DIFFERENTIATED SENSITIVE DETECTION OF THE RESPONSIBLE ENZYMES BY MEANS OF LIQUID CHROMATOGRAPHY HYPHENATED ONLINE TO A BIOCHEMICAL DETECTOR (BCD).

Natural sources like plant extracts are an almost unlimited pool of pharmacologically active compounds. Due to the complex nature of these samples, the identification and isolation of the individual active ingredients is a laborious task. Today, powerful LC- or LC/MS-based methods allow rapid screening of highly complex samples. However, it is still necessary to identify the active molecules among all separated compounds. A novel HPLC based method delivering both, separation of compounds and detection of biological activity facilitates the direct identification of active ingredients in biological samples.

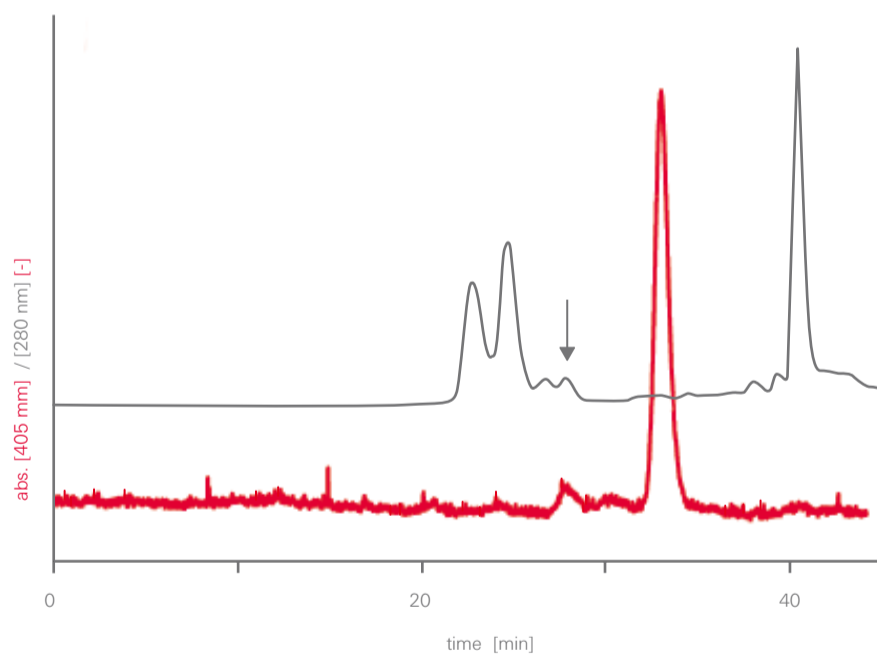
In this approach, HPLC is directly coupled to continuous-flow reactor serving as a BCD system as shown below. The biological activity is directly observed on-line after LC separation, allowing immediate identification of active fractions. Non-denaturing separation techniques as size exclusion chromatography (SEC) were applied to screen for enzymatic activities of proteases in complex mixtures. First, active samples were identified in a multi-substrate assay monitoring the conversion of eight substrates in multiple reaction monitoring in parallel within 60 seconds. Hereby, the product patterns are investigated and the suitable peptide as substrate for BCD analysis is selected. Subsequently, the active proteases are separated by SEC on a TSKgel G2000SWXL column and identified online in the continuous-flow reactor at 40°C. For biochemical detection, the selected p-nitroaniline (pNA) labelled peptide is added post-column. Eluting proteases cleave the peptide under release of the coloured pNA, which is detected at 405 nm with a second UV detector.



➤ **HPLC-BCD SYSTEM:** THE PROTEINS ARE SEPARATED UNDER NON-DENATURING CONDITIONS BY SIZE EXCLUSION CHROMATOGRAPHY [SEC]. AFTER UV-DETECTION [280 NM], THE ELUENT IS MIXED WITH THE SUBSTRATE SOLUTION DELIVERED BY A SUPERLOOP. IN THE FOLLOWING REACTION COIL, THE SUBSTRATE IS CONVERTED TO THE PRODUCT AS MONITORED BY UV/VIS ABSORBANCE DETECTION AT 405 NM.

The developed method was thoroughly characterized with the model proteases trypsin and chymotrypsin. For trypsin, a limit of detection of 0.1U/ml, corresponding to an injected amount of 0.4 ng protein (~ 18 fmol) was achieved. The method was also applied to analyze the proteases of pit viper venom and of a pathogenic amoeba. In both samples, fractions with proteolytic activity were identified. The chromatograms show the UV and BCD trace after SEC separation of the crude venom of the pit viper *Bothrops moojeni*. The arrow indicates the active fraction in the UV signal.

The BCD method allows the direct identification of bioactive fractions, which can be directly collected for further characterization of the contained enzymes.



➤ **HPLC-BCD ANALYSIS OF SNAKE VENOM:** UV [280 NM, GREY] AND BCD [405 NM, RED] CHROMATOGRAMS OBTAINED FOR THE CRUDE SAMPLES [1 MG/ML] AFTER SEC WITH SUBSEQUENT UV AND BCD DETECTION.

➤ **AUTHORS:** NILS HELGE SCHEBB & UWE KARST, INSTITUTE OF INORGANIC AND ANALYTICAL CHEMISTRY, UNIVERSITY OF MÜNSTER, GERMANY

➤ **REFERENCE:** : N. H. SCHEBB | T. VIELHABER | A. JOUSSET & U. KARST | J. CHROMATOGR. A [2009] 1216: 4407-4415

➤ **CONTACT FOR CUSTOMER APPLICATIONS AT TOSOH BIOSCIENCE:** REGINA ROEMLING [MARKETING MANAGER]

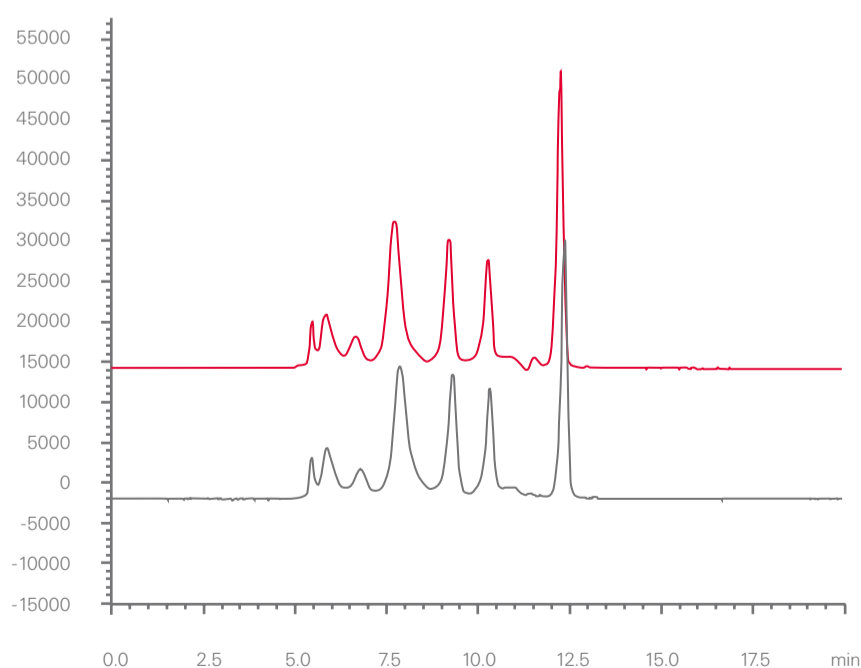
05 TIPS & ADVICE

ENHANCING COLUMN LIFETIME IN AQUEOUS SIZE EXCLUSION CHROMATOGRAPHY

AQUEOUS SIZE EXCLUSION CHROMATOGRAPHY [SEC] IS ONE OF THE MOST FREQUENTLY USED TECHNIQUES FOR ANALYSIS AND QUALITY CONTROL OF PROTEINS. ESPECIALLY FOR THERAPEUTIC PROTEINS A THOROUGH CHARACTERIZATION OF THE PRODUCT BY SEC AND COMPLEMENTARY METHODS IS A PREREQUISITE FOR CLINICAL USE AND MARKET APPROVAL. OUR SILICA BASED TSK-GEL SWXL COLUMNS ARE THE INDUSTRY STANDARD FOR HPLC SEPARATION OF PROTEINS BY SIZE EXCLUSION CHROMATOGRAPHY.

A frequently observed problem in SEC of proteins is their tendency to stick to the column matrix. This is leading to abnormal chromatograms and poor recovery and will finally shorten the lifetime of the column as well. Various approaches have been proposed to reduce non-specific binding of proteins to the stationary phase. Applying high ionic strength by using NaCl or phosphate at high concentrations is one option to reduce electrostatic interactions between protein and column matrix. The drawback of this approach is an increase in hydrophobic interaction, which may cause either non-specific adsorption to the column matrix or protein aggregation. The addition of urea or organic modifiers to the mobile phase inhibits hydrophobic interactions, but at the same time it increases ionic interactions.

Arginine has the ability to disrupt weak non-specific protein-protein interactions. It is often used as an additive assisting in protein refolding and solubilisation, suppression of protein aggregation and dissociation of antibodies from protein A. An aqueous arginine solution suppresses non-specific interactions between proteins and silica- or polysaccharide-matrices of SEC columns as well. The chromatogram below shows the separation of a common protein standard on a TSKgel G3000-SWXL column performed with 0.2 M arginine in 0.1 M PBS [red] compared to the same separation in 0.1 M PBS only [grey]. In addition to the suppression of non-specific binding of proteins to the stationary phase even slight increases in resolution and theoretical plates is achieved when adding arginine.



Arginine is able to suppress both ionic and hydrophobic interactions. Hence the lifetime of SEC columns will improve when applying arginine containing mobile phases. Adding 0.2 M arginine to the mobile phase was found to be sufficient to improve separation of various proteins and their soluble aggregates on a TSKgel G3000SWXL SEC column*.

Another recommendation of our technical specialists in order to improve lifetime of SEC columns is to use guard columns to protect the analytical column from early degradation. In systems consisting of analytical and guard columns a loss in performance is often related mainly to the degradation of the guard column. Hence, separation performance can often be retained after exchanging the guard column only.

* DAISUKE E. ET AL. | J CHROMATOGR. A [2005] 1094 1-2: 49-55

➤ FOR HPLC COLUMN RELATED TECHNICAL SUPPORT CONTACT
DR. WERNER CONZE OR VOLKER NOEDINGER
MAILTO: TECHSUPPORT.SEP@TOSOH.COM

➤ WWW.SEPARATIONS.EU.TOSOHBIOSCIENCE.COM/SERVICESUPPORT/TECHSUPPORT/MEETTHETECHSERVICETEAM



➤ SEPARATION OF PROTEIN STANDARDS BY SEC
UV@280 NM, 0.2 M ARGININE IN 0.1 M PBS [RED], 0.1 M PBS [GREY]

06 SIZE MATTERS

SEMI MICRO GPC AND MULTIPORE TECHNOLOGY: FAST POLYMER ANALYSIS AT LOW COSTS

TOSOH HAS A LONG HISTORY IN DEVELOPING GPC TECHNOLOGY. THE FIRST TSK-GEL HPLC COLUMNS EVER, INTRODUCED IN 1971, WERE GPC COLUMNS. THE FIRST GPC INSTRUMENTS WERE INTRODUCED IN 1972. EVER SINCE, TOSOH SCIENTISTS HAVE MADE IMPORTANT CONTRIBUTIONS TO ADVANCES IN POLYMER ANALYSIS BY GEL PERMEATION CHROMATOGRAPHY (GPC).

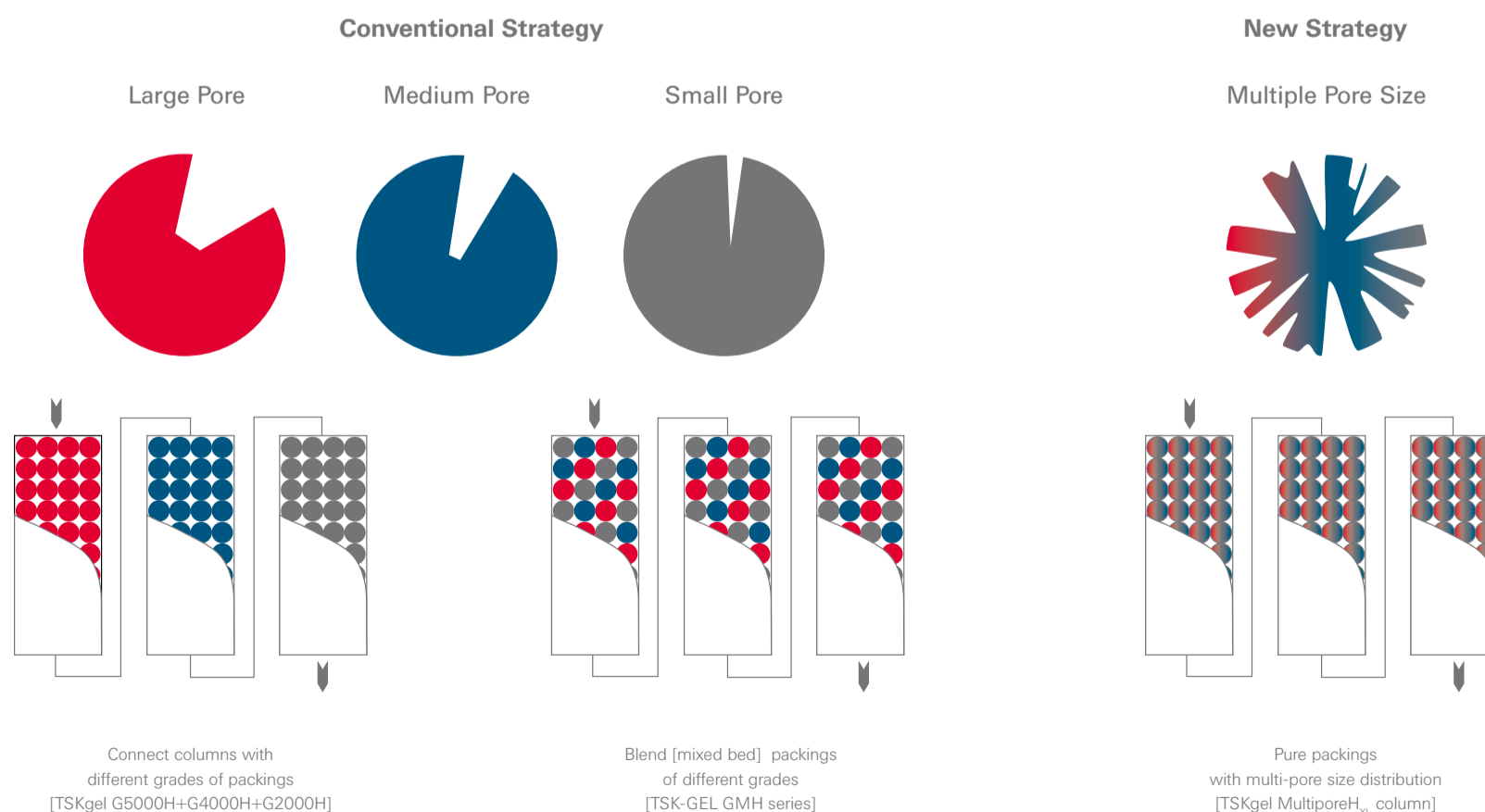
The latest progress in GPC column technology was the invention of multipore particle synthesis technology. Multipore stationary phases are used in GPC columns with extended linear calibration range. A linear molecular weight calibration curve can be obtained in three ways: [1] The simplest way is to couple different columns, each containing particles with distinct pore size distributions. It is inevitable that the resulting calibration curve will deviate from linearity between each pore size range, resulting in irregularities in the chromatogram. [2] These irregularities occur less frequently in the case of mixed-bed columns. Mixed-bed columns are prepared by mixing batches of particles each containing a narrow pore size distribution. However, inflection points in chromatograms can still arise due to a mismatch of pore sizes. [3] Inflection points are no longer observed with columns packed from particles prepared by multipore technology. Using this proprietary technology, Tosoh can manufacture particles containing a broad range of pore sizes. This innovative approach essentially creates a linear calibration curve within each particle. Recently Tosoh Bioscience introduced the EcoSEC GPC system, the 7th generation of dedicated GPC instruments. This all-in-one system delivers top performance, reliability and superior results when coupled with TSK-

GEL GPC or SEC columns. The columns of choice for use with the EcoSEC GPC system are referred to as semi-micro columns since their dimensions are smaller than conventional GPC columns in terms of internal diameter as well as in length: 4.6 mm or 6 mm ID x 15 cm length versus 7.8 mm ID x 30 cm L. Semi-micro column dimensions are advantageous in terms of shorter analysis time, lower solvent consumption and reduced solvent disposal cost. The EcoSEC GPC system is designed to achieve these benefits. Other GPC systems must be optimized with respect to delay or dead volume.

By integrating HPLC system design and semi-micro column dimensions with the innovative multipore particle synthesis technology the EcoSEC GPC system allows faster molecular weight determination at a lower cost per analysis.

➤ TO LEARN MORE ABOUT ECOSEC, SEMI-MICRO GPC AND MULTIPORE TECHNOLOGY VISIT OUR WEBSITE WWW.ECOSEC.EU

➤ STRATEGIES FOR SEC SEPARATION OF POLYMERS WITH WIDE MOLECULAR WEIGHT DISTRIBUTION



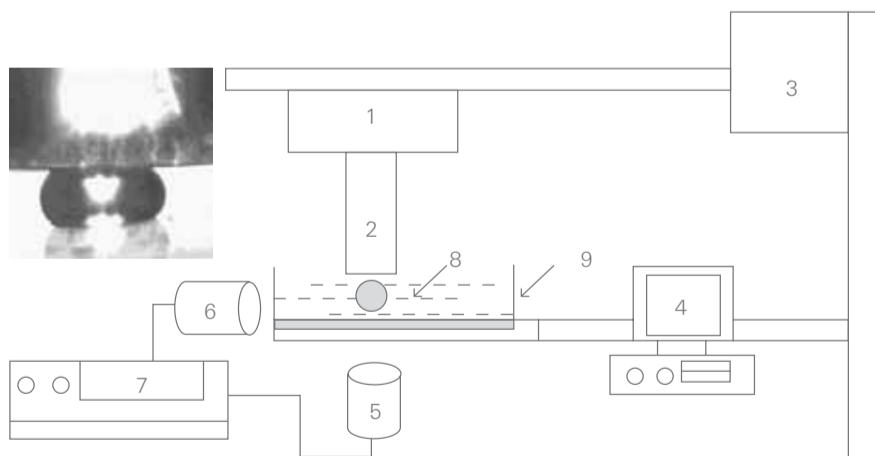
07 HOW FAR CAN YOU GO?

THE MECHANICAL STABILITY OF TOYOPEARL AND TSK-GEL PARTICLES

CHROMATOGRAPHIC PARTICLES SHOULD NOT ONLY POSSESS DESIRABLE SURFACE CHEMICAL PROPERTIES BUT ALSO GOOD MECHANICAL PROPERTIES. THE LATTER DETERMINE THE DEFORMATION OF THE PARTICLES UNDER HYDRODYNAMIC CONDITIONS OF PACKED BEDS AND FURTHER THE PRESSURE DROP. TOYOPEARL AND TSK-GEL RESINS FOR BIOPURIFICATION ARE MADE OF POLYMETHACRYLATE, A VERY RIGID POLYMER WITH PROVEN EXCELLENT PRESSURE FLOW CHARACTERISTICS. TOSOH IS FREQUENTLY ASKED, "WHAT IS THE MAXIMUM PRESSURE THAT CAN BE APPLIED TO TOYOPEARL RESINS WITHOUT ANY IRREVERSIBLE DAMAGE." IN COLLABORATION WITH THE UNIVERSITY OF BIRMINGHAM [UK] WE IDENTIFIED THE PRESSURE THAT IS NECESSARY TO IRREVERSIBLY DEFORM A SINGLE TOYOPEARL OR TSK-GEL PARTICLE*.

The primary motivation for setting the specified maximum pressure limit for Toyopearl to 3 bar was in order to protect the chromatographic system [including column hardware]. Experimentally, the pressure drop across a column of typical length 20 cm, filled with Toyopearl M grade particles [40- 90 μm] usually does not reach 3 bars pressure, even at high linear flow velocities. To estimate the mechanical stability of a packed chromatographic column, it is usual to record the pressure at various flow rates and so construct a pressure-flow curve. These curves are linear at low velocities but above a certain velocity, deviations from the linear pressure v. flow relationship occur up to the point where the resin fully collapses. The physical state of the particles at / near to collapse is not clearly understood. In order to better understand and predict the stability of chromatographic polymeric particles, the mechanical properties of single particles were characterized by a micromanipulation technique.

The principle of this technique is to compress single particles between two parallel surfaces in a micromanipulation rig as shown in the drawing. The force imposed on the particle and its deformation under compression are measured simultaneously in the following way: Single particles are placed on a glass slide and compressed between a probe connected to a force transducer and the glass slide. The slide is mounted onto the stage of an inverted microscope. Experimentally, 10–30 particles from each sample were compressed up to a particle deformation of minimum 70% in order to generate statistically representative data.



► MICROMANIPULATION RIG

[1] FORCE TRANSDUCER; [2] PROBE; [3] STEPPING MOTOR; [4] DATA ACQUISITION; [5] BOTTOM-VIEW MICROSCOPE; [6] SIDE-VIEW MICROSCOPE; [7] VIDEO RECORDER; [8] RESIN PARTICLE IN WATER AND [9] GLASS CHAMBER

The table below shows the pressures that are required to cause irreversible deformation of the particles. For the highly cross-linked TSK-GEL particle, a deformation of more than 90% is possible before the point of irreversible deformation is reached. This physical testing also indicated that hydrophobic interaction chromatography resins are physically more stable than ion exchange resins.

RESIN	MEAN PARTICLE SIZE [M]	IRREVERSIBLE DEFORMATION [ID] [BAR]	COMPRESSION [%]
TSK-GEL PHENYL-5PW	20	290	94
TOYOPEARL BUTYL-650M	65	180	81
TOYOPEARL DEAE-650M	65	180	70

A mechanically stable particle is easier to pack and does not shrink or swell during the chromatographic cycle. As a consequence, the life time of a packed bed column is also a function of the mechanical stability of a resin. This study has shown the exceptional rigidity of the methacrylate base materials of TSK-GEL and Toyopearl resins.

*E. MUELLER | J.-T. CHUNG | Z. ZHANG & A. SPRAUER | J. CHROMATOGR. A [2005] 1097 1-2: 116-123

► FOR MORE INFORMATION ABOUT THE PRACTICAL USE OF TOYOPEARL AND TSK-GEL RESINS CONTACT OUR TECHNICAL SPECIALISTS **ACHIM SPRAUER** OR **VOLKER NOEDINGER** AT TECHSUPPORT.SEP@TOSOH.COM OR REGISTER FOR ONE OF OUR CHROMATOGRAPHIC WORKSHOPS.

08 CONFERENCE REVIEW

6TH HIC/RPC BIOSEPARATION CONFERENCE 2009

IN THE PREVIOUS ISSUE OF THIS MAGAZINE WE ANNOUNCED THE 6TH HIC/RPC BIOSEPARATION CONFERENCE WHICH WAS HELD FROM MARCH 15-19, 2009 AT THE SILVERADO RESORT IN NAPA, CALIFORNIA. THE SCIENTIFIC PROGRAM COVERED BOTH, FUNDAMENTAL SCIENCE/ENGINEERING AND IMPORTANT INDUSTRIAL ADVANCES AND APPLICATIONS.

Internationally recognized speakers from academia and industry provided insights into their expertise in the area of hydrophobic interaction, reversed phase and mixed mode chromatographic purification steps as well as a range of related new technologies. A round table discussion of the scientific committee members addressed latest trends in HIC and mixed mode chromatography. This time the scientific committee was headed by Steven M. Cramer, Rensselaer Polytechnic Institute. The Journal of Chromatography A will publish a special edition on the conference.

In addition to the scientific program the conference offered several opportunities to network with colleagues and expanding the industry's knowledge base on hydrophobic techniques for process purification. The Californian landscape, the beautiful conference venue, tasty meals and famous Californian wines further contributed to making this conference an unforgettable event for all participants. Tosoh Bioscience is the sole sponsor of this conference series and provides support for logistics and organisation for the scientific committee. The biannual HIC/RPC Bioseparation Conference series alternates between Europe and the US. The 7th HIC/RPC Bioseparation Conference will be organized by Tosoh Bioscience GmbH and will take place in Estoril, Portugal, from 21-24 March 2011.



➤ THE CONFERENCE WEBSITE WWW.HIC-RPC.ORG WILL KEEP YOU UPDATED

NEWS & EVENTS | MEET TOSOH BIOSCIENCE

MEET TOSOH BIOSCIENCE AT TRADESHOWS AND CONFERENCES OR JOIN ONE OF OUR RENOWNED WORKSHOPS

UPCOMING EVENTS

- | | |
|---------------------------|---|
| ➤ JUNE 22 - 24 2009 | ➤ 16TH ARBEITSTAGUNG MIKROMETHODEN IN DER PROTEINCHEMIE MARTINSRIED [GERMANY] |
| ➤ JUNE 28 - JULY 2 2009 | ➤ HPLC 2009 DRESDEN [GERMANY] |
| ➤ AUG. 30 - SEP. 4 2009 | ➤ IMSC 2009 BREMEN [GERMANY] |
| ➤ OCT. 6 - 8 2009 | ➤ BIOTECHNICA 2009 HANOVER [GERMANY] |
| ➤ NOV. 8 - 10 2009 | ➤ 20TH JOINT GLYCOBIOLOGY MEETING COLOGNE [GERMANY] |
| ➤ NOV. 9 - 10 2009 | ➤ NOVIA HPLC TAGE 2009 FRANKFURT [GERMANY] |

TRAININGS | WORKSHOPS

- | | |
|-----------------------|--|
| ➤ JUNE 16 - 17 2009 | ➤ CHROMATOGRAPHY IN PROCESS DEVELOPMENT & PRODUCTION ADVANCED COURSE STUTTGART [GERMANY] |
| ➤ JUNE 23 - 25 2009 | ➤ CHROMATOGRAPHY IN PROCESS DEVELOPMENT & PRODUCTION BASIC COURSES STUTTGART [GERMANY] |
| ➤ JULY 7 - 9 2009 | |
| ➤ JULY 14 - 16 2009 | |