SESSION I WEDNESDAY, JUNE 8 th, 2011

CHAIRPERSONS: D. Agbaba and R. Kaliszan

SESSION II WEDNESDAY, JUNE 8 th, 2011

CHAIRPERSONS: M. Waksmundzka-Hajnos and H. Kalasz

SESSION III THURSDAY, JUNE 9 th, 2011

CHAIRPERSONS: W. Lindner and A. Voelkel

SESSION IV THURSDAY, JUNE 9 th, 2011

CHAIRPERSONS: B. Chankvetadze and Y. Vander Heyden

SESSION V FRIDAY, JUNE 10 th, 2011

CHAIRPERSONS: I. Vovk and K. Kaczmarski

1.

New effects with new chiral stationary phases for liquid-phase enantioseparation techniques

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In this presentation the results of our recent studies on development of novel chiral stationary phases (CSP) for enantioseparations using high-performance liquid chromatography (HPLC), capillary liquid chromatography (CLC) and capillary electrochromatography (CEC) are summarized. In the first part of the presentation the emphasis will be made on novel phenylcarbamate derivatives of cellulose and amylose as useful CSPs for analytical and preparative scale enantioseparations. These novel materials are applicable for HPLC enantioseparations in combination with normal phase-, polar organic mobile phase and reversed-phase eluents, as well as for SFC enantioseparations at higher pressure. In the second part of the presentation the screening results on 5 commercially available representatives of this series of chiral columns will be discussed with major emphasis on the complementary properties of various chiral selectors and mobile phases. The effect of fine tuning of the properties of these materials and separation conditions on resolution of enantiomers of various compounds will be discussed in detail using the examples of challenging separations. In the final part of the presentation newly observed reversal of the enantiomer elution order of some chiral drugs and amino acid derivatives by variation of separation temperature and composition of the mobile phase will be discussed [1].

[1] L. Chankvetadze, N. Ghibradze, M. Karchkhadze, L. Peng, T. Farkas, B. Chankvetadze, *J. Chromatogr. A*, submitted.

2.

Application of monolithic chromatography in drug discovery and development

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Monolithic columns have a continuous separation bed which is prepared by in situ polymerization inside the column tubing. The stationary phase itself has a sponge-like structure with numerous flow-through pores. Due to the presence of these pores, relatively low back pressures are obtained when these columns are operated at higher flow rates. Therefore, monoliths allow working at higher linear velocities compared to classical particle-packed columns. Hence, higher efficiencies can be achieved using longer or coupled columns.

The introduction of monolithic supports as stationary phases in liquid chromatography started around the beginning of the 1990's. At that time, polymer-based monoliths were the dominating type of monolithic stationary phases. The introduction of commercially available silica-based monoliths followed about ten years later. Since then, the applications of monolithic columns extended to different fields of separation science, among which drug development.

In this presentation, applications in drug development are discussed using monolithic supports with liquid chromatographic techniques, such as high-performance liquid chromatography, capillary liquid chromatography and capillary electrochromatography. A distinction is made regarding applications on polymeric monoliths on the one hand, and on inorganic monoliths on the other.

3.

The impact of stationary phase backbone on HILIC selectivity

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In recent years the research on and the application of HILIC (hydrophilic interaction chromatography) regained popularity as it provides methodologies to separate and analyze highly polar compounds which can hardly be retained on reversed phase LC columns. Generally speaking, the retention mechanisms of HILIC is dominated by partition of the analytes between a water rich (very polar) hydro-organic layer adsorbed on a polar sorbent surface and a (less polar) organic rich stagnant layer in the pores and thus of the mobile phase. However, in reality there are besides partition often also direct adsorption related increments responsible for retention of the analytes. These are mainly driven by electrostatic interactions encompassing ion pair formation, hydrogen bonding and polar interactions, respectively, depending on the chemical structure of the polar analytes and the polar surface of the "adsorbents". Most of the time plain silica or polar modified silica are used as "adsorbents". With other words, the observed HILIC retention mechanism refers inherently to mixed modal retention characteristics which are difficult to de-convolute into the individual parts responsible for the overall observed retention and selectivity parameters. Integral to this discussion will also be the contribution of the mobile phase characterized by the polar solvents, the buffer salts and the pH thus driving the composition of the water and buffer salt rich stationary phase layer.

Given this situation it becomes imperative that besides the mobile phase composition also via the covalently modified adsorbents the HILIC type selectivity will be tuneable. The polar stationary phase backbone can be of so-called neutral, acidic, basic or zwitterionic nature.

In this contribution we will describe and discuss novel silica based and polar modified HILIC type phases in terms of their unique selectivity pattern. These phases are overall of (i) neutral, (ii) weakly basic, or (iii) weakly acidic character. In addition they all will contain polar functional groups suitable for (multiple) hydrogen bonding. Based on a broad and systematically selected set of test analytes we will try to set up a protocol to characterize HILIC phases and to discuss retention models.

4.

QSRR: Extrathermodynamic *vs.* thermodynamic modeling of chromatographic retention

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The retention prediction performance was tested of the well thermodynamically founded solvophobic theory of Csaba Horváth of the reversed-phase HPLC in comparison to the extrathermodynamic, statistically derived Quantitative Structure-Retention Relationships (QSRR). The model derived when observing the rules of classical thermodynamics appeared to be clearly interpretable in physicochemical terms but of limited retention prediction ability. An improvement was attained when applying an extrathermodynamically derived correction to that model based on thermodynamic hermeneutics. The simple QSRR model, relaying on chemical intuition and employing analyte structural descriptors from calculation chemistry, produced similar retention predictions as the combined thermodynamic/extrathermodynamic model. Both the thermodynamic and the QSRR models accounted well for abilities of analytes to participate in nonspecific, dispersive intermolecular interactions. Less reliable appeared descriptors of analyte polarity. The approach proposed can be further developed to search for appropriate descriptors of polarity which would allow a better prediction of physicochemical and/or biological properties of chemical compounds, and hence, would help to rationally design substances of requested quality.

5.

Analysis of the chromatographic column efficiency packed with the totally and superficially porous particles and their separation power

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In recent years the chromatographic columns technology was evolving in the direction of the use of sub-2μm adsorbent particles. The adsorbent particles can be totally porous or have solid central part and active outer layer (shell particles). The reduction of the size of adsorbent causes the decrease of mass transfer resistances and increases the column efficiency. Further reduction of mass transfer resistances is attained with introduction of superficially porous particles by reduction of diffusion path to active shell.

The performance of chromatographic columns, filled with different adsorbent, is conveniently compared on the basis of the values of their HETP. The theoretical equation of the HETP for columns packed with spherical particles, shell particles and for monolith column can be developed by application of the moment analysis to elution peaks, calculated with the General Rate model. This equation can be developed for linear adsorbtion isotherm or first order adsorption-desorption kinetic.

The more important measure of a chromatographic separation power is the resolution of the components. The resolution of the components can also be evaluated with the help of the moment analysis.

From analysis of HETP equations, for infinite adsorption-desorption kinetic, follows that the highest column efficiency can be obtained for shell thickness decreasing to zero. However, it does not mean that the maximum resolution of the components is also obtained for shell thickness decreasing to zero.

The aim of this work was to investigate the effect of the shell thickness on the column efficiency and the components resolution for infinite and finite adsorption-desorption rate. The optimal solid core radius was calculated to achieve minimum HETP or maximum resolution in different process conditions.

6.

Molecular rotors in thin layer chromatography

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While investigating chiral profens by means of TLC, some of us have serendipitously discovered an effect of lateral relocation of the analyte spots in planar chromatograms (LR-TLC) [1-4]. The investigated profens migrating upward with the solvent on the vertical TLC plates, deviated from the expected straight-line route. The preferential left or right-handedness of the choice interactions with a TLC plate could not be understood on the basis of the known chromatographic theories. Here we will discuss a hypothesis that rotoring ability can explain the LR-TLC effect.

Molecular and chiral rotors (CRs) are molecules able to produce a variety of special effects, due to their ability for the specific rotational motion, although these effects are not well recognized. Although lateral relocations (LR) have been theoretically predicted for the fluxes of specifically rotating molecules [5], such phenomena have never before been observed in the experiments. We will discuss in this presentation how this model can explain the LR-TLC effect.

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7.

Thin-layer chromatography with biodetection to screen plant extracts for the presence of potential drugs

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Numerous plant extracts as well as compounds isolated from them have been investigated for their potential use in various diseases. For example in the case of Alzheimer disease drugs belonging to a class of acetylcholinesterase (AChE) inhibitors are currently used to alleviate the symptoms of this ailment. However considerable body of evidence indicates that oxidative stress plays an important role in the development and progress of age-related neurodegenerative diseases. The continuous generation of reactive oxygen species (ROS) in living cells leads to cumulative damage to cellular organelle and finally to age-related pathology and other diseases such as: cancer, inflammation, asthma etc. Thus there is a need to screen different natural samples for the presence of antioxidants that may protect against the development of the aforementioned ailments. The important part of biological detection is connected with screening of plant samples for the presence of antibacterial and antifungal compounds.

Thin-layer chromatography coupled with biodetetction gives the possibility to screen plant extracts for the presence of AChE inhibitors, glucosidase inhibitors, antioxidants, free radical scavengers and antibacterial and antifungal constituents. In this presentation the possibility of using planar chromatography for the search of new substances with potential to be used as drugs in different diseases is discussed. Practical problems encountered while performing such analyses is addressed as well as some solutions are proposed. The future perspectives for the method development are outlined and discussed.

8.

Investigation of vegetable triterpenoids and phytosterols by chromatography and mass spectrometry

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Triterpenoids are secondary metabolites widely distributed in nature. Structures enriched with different functional groups, sometimes some of them derivatised to esters, glycosides etc. render a huge number of over 20.000 compounds belonging to this group. For many of them biological activity was proved. Reported beneficial effects of various triterpenoids for human health indicate the importance of this group of compounds in the diet. However, little is known about the content of triterpenoids in vegetables and about the triterpenoids intake by everyday diet. Determination of triterpenoids in plant extracts is rather difficult, since they contain a vast amount of various triterpenoid compounds, which differ in skeleton structure and polarity because various functional groups can be attached to the ring system. Furthermore, the presence of isomeric triterpenoids in plant epicuticular waxes and the fact that triterpenoids lack chromophores render the determination of triterpenoids even more difficult. Therefore, suitable modern analytical methods are highly desired.

The aim of our work was to investigate triterpenoids in epicuticular waxes of various vegetables like cabbage (*Brassica oleracea* L.), pepper (*Capsicum annum* L.), lettuce (*Lactuca sativa* L.), chicory (*Cichorium intybus var. foliosum*) and parsley (*Petroselinum crispum* L.). Screening of the vegetable surface extracts before and after hydrolysis was performed by silica gel and reversed-phase (C18 RP) thin-layer chromatography (TLC), TLC-MS and C18 RP high-performance liquid chromatography (HPLC) with UV and mass spectrometric (MS) detection using atmospheric pressure chemical ionization (APCI). TLC screening of triterpenoids on silica gel HPTLC plates revealed that all samples contained a triterpenol fraction. Lettuce and chicory extracts contained also esterified triterpenols, oleanolic and ursolic acid. Sterol fraction was present in all samples in trace amounts. The RP 18 layer gave much more accurate insight in the qualitative composition of isomeric triterpenoids in vegetable surface extracts, since some isomeric triterpenols (α-amyrin, β-amyrin, lupeol) and esterified triterpenols were separated. Glycosylated or/and esterified triterpenols were also present in the extracts. In order to detect them, sample preparation included basic hydrolysis (saponification) for detection of esters and acid hydrolysis together with saponification for detection of both types of derivatives.

9.

Hyphenated HPTLC for fast analysis of bee's products

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New hyphenated HPTLC approaches for fast analysis of bee's products (honey and propolis) were suggested for the first time.

The quantitation of 5-hydroxymethylfurfural (HMF) in honey is now possible using HPTLC with just 5 minutes migration time, and analyzing up to 24 samples simultaneously. The detection is performed by absorbance measurement at 290 nm. Other possible detection modes include fluorescence measurement after post-chromatographic derivatization and mass spectrometric detection. The reliability of the suggested approach was evaluated using 10 samples of honey with known quantities of HMF and it proved to be at least at the same degree suitable for honey analysis as the conventional methods, including HPLC and Winkler method. HPTLC-ESI/MS coupling can be used as an additional tool, when it is necessary to confirm the results of prior quantitation by HPTLC/UV. Due to the lower sensitivity, HPTLC-DART/MS could be recommended just for the initial screening performed, applying large volumes of honey solution to the HPTLC plate.

The other hyphenated approach was applied for analysis of a large number of propolis extracts, collected during several years in different regions of Germany. A new HPTLC-UV/Vis/FLD-MS method was developed based on the flavonoid profile and a recognizable pattern of biomarkers was detected after selective derivatization. Mass spectrometry employing electrospray ionization or 'direct analysis in real time' (DART) ionization was used for confirmation and verification of the identity of the biomarkers found.

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10.

TLC-DPPH TEST REVISITED

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Reactive oxygen species (ROS) are constantly produced under physiological conditions in human organism. Apart from their role in intracellular signaling they may react with DNA, proteins and lipids causing the change of their biological functions. It has been proved that ROS may be involved in the progress of many ailments, e.g.: atherosclerosis, Alzheimer's and Parkinson's diseases, asthma, cancer and still others. Antioxidants and free radical scavengers are currently the subject of an intensive research interest, as they may protect cellular organelle from damages caused by oxidative stress. In vitro screening is the primary selective tool for finding potential antioxidants and free radical scavengers. One of the techniques commonly applied for the screening of plant extracts for the presence of antiradical compounds is the TLC-DPPH test. After development under the optimized conditions a chromatographic plate is sprayed with a solution of a stable radical – 1,1-diphenyl-2-picrylhydrazyl (DPPH). Intense purple color of DPPH changes into pale yellow in a presence of a substance able to donate hydrogen atom or electron. The majority of the reported TLC-DPPH tests have been performed on silica gel layers, as normal phase systems usually enable satisfying resolution of polyphenolic compounds characterized with antiradical potential. In the experiments performed in our department we have observed that the results of the test are influenced by several factors. Free radical scavenging properties of polyhenolics are influenced by the chemical nature of the adsorbent used. The antiradical potential of the investigated phenolic acids and flavonoids was strengthened on silica and alumina and weakened on polar bonded stationary phases (DIOL- and CN-silica). The results also differed when methanol, used to dissolve DPPH, was changed to acetone or acetonitrile. Thus there is an urgent need to elaborate a standard TLC-DPPH procedure in order to obtain reliable results in the search for potent free radical scavengers.

11.

HPLC monitoring of blood-brain-barrier penetration of certain polar drugs

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Introduction: Lipophilic compounds can easily penetrate the blood-brain-barrier (BBB), however carriers may transfer not only hydrophilic, but even polar organic compounds from the blood to the central nervous system. Our aim was to study the BBB penetration of hydrophilic compounds determining the drug levels in the blood, brain and cerebrospinal fluid (CSF) using reversed-phase chromatography.

Materials and methods: Various bis-pyridinium mono-aldoximes (K-compounds) were synthesized having a variety of different alkyl bridges between the two pyridinium parts as published earlier. Compounds such as pralidoxime, K-027, K-048 and K-203 were studied. Rats were injected by different doses of the compounds, then the animals were sacrificed under anesthesia according to the animal ethical codex of Semmelweis University Budapest, Hungary (permission number of local authorities:22.1/609/001/2010). Blood and CSF were taken, and brain was dissected. The rat brain was homogenized, samples of brain homogenate, blood and CSF were subjected to clean-up using precipitation by perchloric acid and centrifuged at 14,000 rpm at 4 ^{o}C for 20 min. Following the centrifugation pH of the supernatants was adjusted to 2. The samples were subjected to reversed-phase HPLC on Zorbax Rx-C18 stationary phase.

Results: Brain homogenate and CSF samples contain low level of pyridinium aldoximes, therefore the use of electrochemical detector was required to detect the submicrogram/g (0.01 through 1 μg/g) level of pyridinium aldoximes. The corresponding blood samples contain higher level of pyridinium aldoximes, in the range of 1 through 200 microgram, so UV detection at 276 nm could be used. Moreover, the background peaks originated from either the brain or CSF samples required high concentration of ion-pairing agent (0.25% of octanesulfonic acid sodium), while blood samples could be chromatographed using 0.1% of the same ion-pairing agent. To work with the same HPLC method, all separations were performed using mobile phase containing 0.25% octanesulfonic acid sodium

Conclusions: Carefully adjusted ion-pairing agent concentration in the mobile phase helped us to avoid peak-overlapping of the samples of biological origin. The quantitative determination showed that BBB penetration of these highly polar compounds show dose-dependence in the respect of blood-brain and blood-CSF ratios.

12.

Tracking of the food-drug interactions including alcohol-drug interaction with different bioanalytical methods

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Food can alter the bioavailability of drugs either by direct physical or chemical interaction or by the physiological response. The physiological response to food intake, in particular gastric acid secretion, may reduce the bioavailability of certain drugs. Since the bioavailability and clinical effect of most drugs are correlated, the bioavailability is an important pharmacokinetic effect parameter. Such interactions are frequently caused by chelation with components in food or dairy products (ciprofloxacin and norfloxacin). For drugs belonging to the BCS (Biopharmaceutical Classification System) Class I (highly soluble, highly permeable) that rapidly dissolve from immediate release solid oral drug products, bioequivalence under fed conditions has been postulated. Alcohol can profoundly influence both drug metabolism and nutritional status.

The sensitivity and selectivity of HPTLC/OPLC-MS, Headspace-GC, GC-MS, LC-MS and LC-MS/MS applications are of high importance in the pharmaceutical food-drug and food-alcohol interaction research. Bioanalytical methods play an important role in the original and generic drug development. In the recent years it has come to the centre of attention that food intake exerts a complex influence on the biological availability of certain drugs. A number of *in vivo* studies have been published, however, only a few *in vitro* data are known. Fed and fasted conditions can be simulated *in vitro* with dissolution tests using dissolution media with appropriate food components according to the Ph. Eur. 5.

The primary aim of the present lecture is give a comprehensive view about the tracking possibilities of food-drug and alcohol-drug interactions with bioanalytical methods. Another purpose is to illustrate the possible implementation of novel simulated in-vitro method for the analysis of food-drug interactions with several examples. The presented method enables good estimation of IVIVC concerning the prediction of the type and mechanism of food interaction.

13.

Update of generic chiral separation strategies for pharmaceutical compounds using chromatographic and electrophoretic techniques

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Pharmaceutical drug compounds are often chiral, which can imply that different pharmacological and pharmacokinetic activities of the enantiomers are seen in the human body. Therefore, regulatory authorities demand that, if possible, a single enantiomer drug is developed, only containing the therapeutically active enantiomer. In the registration procedure of a drug molecule, methods for the separation and quantification of the enantiomers must be presented. However, in early drug development, industry mostly prefers to synthesize a racemate and to separate this afterwards. In this stage, a fast screening for separation conditions is already performed to reduce the method development time at later stages. Therefore, generic screening and optimization strategies can be very useful at this stage of drug development. A fast screening experiment gives an idea about the enantioselectivity, and the optimization steps can be used afterwards to enhance the obtained separation. The defined strategies are generic, meaning that they are applicable on large sets of structurally diverse molecules. It was seen that polysaccharide-based chiral stationary phases (CSP) were well suited to define such strategies, as they show a broad enantioselectivity range. Different strategies were already developed in normal-phase liquid chromatography [1,2], polar organic solvent chromatography [3], reversed-phase liquid chromatography [2,4] and capillary electrochromatography [5]. For supercritical fluid chromatography, only a screening step was defined [6].
A new challenge concerns the evaluation of newly introduced CSP with other types of polysaccharide selectors for their applicability in generic analysis. In a first step, the applicability of the screening step on the newly introduced CSP is evaluated for all considered techniques. When the enantioselectivity of the new CSP is better than the older ones, the screening step was altered by replacement of some CSP by other ones to achieve a higher success rate than before.

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14.

Disadvantages of the current methods of selectivity evaluation in TLC analysis

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The selectivity of the TLC system is a very important topic in quantitative analysis. The analyst must prove that the analyte is determined without influence from any other compounds. In the case of thin layer chromatography, a spot of analyzed substance cannot be contaminated by another one.

Currently, the most common practice of selectivity validation is to measure the spectrum of the spot by a densitometer. The spectrum can be compared between the analyzed spot and reference spot of pure standard; additionally spectra of different parts (start, middle and end) of the spot are often compared. The Pearson's correlation coefficient is most often used as a similarity measure, together with visual inspection.

Up to date, noone investigated the distribution of correlation coefficient between real spectra at different conditions. There is no reference data, what is a probability to achieve a particular correlation between spectra of different compounds (or the same compound contaminated by spectrum of another one). Our preliminary simulation showed that there is a high risk to obtain very high spectral correlations even if contamination is very high. Therefore, we have performed comprehensive simulation of correlation distribution, done on 170 UV spectra of real drug-like molecules. We have simulated:

[6] Correlation distribution between pure spectra of compounds

[7] Correlation distribution between spectrum of compound and the same spectrum contaminated by another random spectrum in many different ratios

[8] Influence of noise addition and different levels to experiments 1 and 2

[9] Correlation distribution between the same spectra when a random noise is added at different levels, together with experiments on real noisy spectra.

The distribution of all experiments overlap significantly in many cases. This led to conclusion, that current methods cannot be treated as a reliable tool for spot impurity detection and the use of such practice should be suppressed. Instead, a peak purity approaches should be developed in analogous manner to HPLC, which will be the subject of our further work.

15.

A validated reversed phase hplc method for simultaneous determination of aspirin and clopidogrel and their related substances in combined dosage forms

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The analysis of a pharmaceutical substance and its impurities to the desired level remains an analytical challenge. In a formulation, it is even more difficult due to possible chemical interactions of the ingredients. Impurity control is a continuing concern of regulatory agencies and pharmaceutical industries. This calls for the development of sensitive and selective analytical methods for the quality control of drugs' safety and efficacy. In this study, a LC method was developed and validated for the simultaneous determination of aspirin and clopidogrel and their related substances in combined dosage forms. For the analysis, we started from the Pharmeuropa monograph for clopidogrel hydrogen sulfate. Three different columns have been tested for the separation of the specified and unspecified impurities of both APIs from the principal peaks. The Luna column (150 x 4.6 mm, 3 µm) was used for further method development and optimization. The proposed method was validated based on the ICH guidelines. The validation results revealed that the method is specific, linear, sensitive, accurate and precise for the determination of aspirin and clopidogrel and their related substances in dosage forms. It is shown that separation of the impurities of both APIs, if present, was satisfactory with the optimized chromatographic conditions. The method was applied for the analysis of 11 commercial batches of clopidogrel and aspirin combination dosage forms (tablets and capsules) for their content and related substances. The developed LC method was also found to be suitable for simultaneous assay determination of aspirin and clopidogrel in pharmaceutical formulations in the presence of their potential impurities. As there is no official method for this purpose, the LC method can be applied for routine quality control of the APIs and their related substances in combined dosage forms.

Keywords:
Aspirin; Clopidogrel; Liquid chromatography; Impurities; Method development

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