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Edited by Stavros Kromidas

Gradient HPLC for Practitioners

RP, LC-MS, Ion Analytics,
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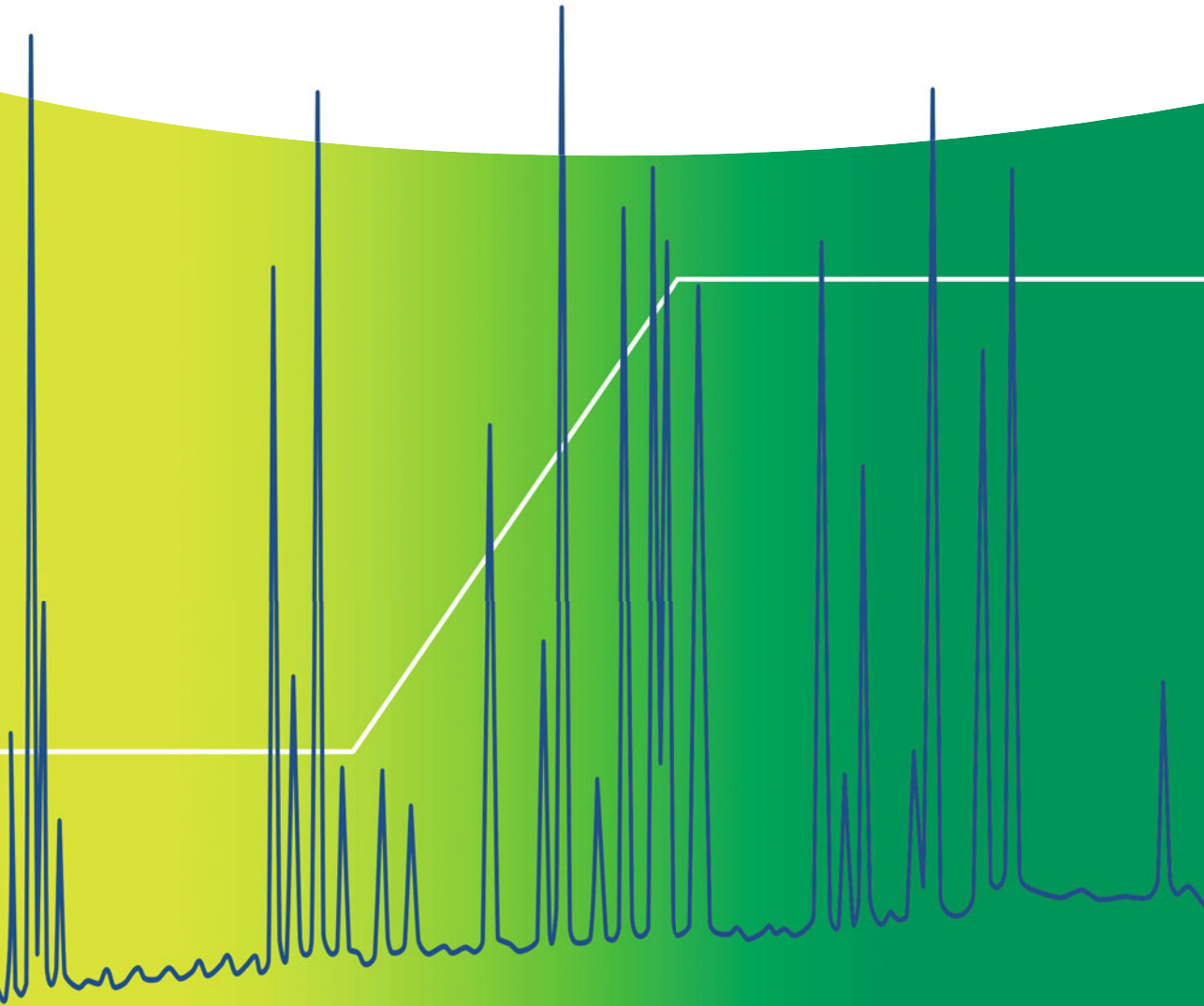

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Contents

Preface

Approximately 80% of the liquid chromatographic methods are gradient methods. In this book, we have tried to shed light on the "whole" world of the gradient in a detailed and practical way. Thus, the use of gradients is discussed in ion analysis and in biochromatography, apart from classical applications such as RP and LC-MS coupling: the salt and the pH gradient. Newer separation techniques such as HILIC and SFC as well as flow and temperature gradients round off the discussion. The book is intended for the experienced user and the practice-oriented supervisor. Although the discussion is in depth in many places, we have endeavored to always keep practice in view. We hope the reader finds useable information and tips on this widely used separation mode. I thank Wiley-VCH and especially Stefanie Volk and Frank-Otmar Weinreich for the good and trusting cooperation.

Blieskastel, January 2019 *Stavros Kromidas*

The Structure of the Book

The book consists of two parts: Part 1 provides the basic information on the gradient technique, while Part 2 presents the specifics of the gradient in different modes and separation techniques.

Part 1: The Principles of Gradient Elution

In Chapter 1 (*Aspects of Gradient Optimization*) Stavros Kromidas discusses in a compact fashion what is important in gradient optimization and presents simple "to-do" rules. Frank Steiner explains in Chapter 2 (*Instrumental Influences on the Quality and Performance of Gradient Methods and Their Transfer Between Different HPLC Devices*) to what extent even the smallest differences between HPLC systems can strongly influence chromatography. Part 1 ends with Chapter 3 by Hans-Joachim Kuss (*Optimization of a Reversed-Phase Gradient Separation Using EXCEL*), which shows one way to predict gradients using EXCEL.

Part 2: The Specifics of the Gradient in Different Separation Modes

Chapters 4 and 5 deal with the separation of ionic or ionizable components. In Chapter 4 (*Gradient Elution of Ionic Compounds*) Joachim Weiss deals with both the separation of small molecules such as inorganic ions and the separation of large molecules such as monoclonal antibodies and shows the specifics of pH and salt gradients. In Chapter 5 (*The Gradient in Biochromatography*) Oliver Genz deals with the different separation modes in biochromatography, which should also be noted here in particular for gradient runs. In Chapter 6 (*Specifications of Gradients in Hydrophilic Interaction Liquid Chromatography (HILIC)*) Thomas Letzel discusses all applicable gradients in HILIC, including temperature gradients. In Chapter 7 (*Specifications of Gradients in Supercritical Fluid Chromatography*), Stefan Bieber and Thomas Letzel present the three possibilities of gradient elution in SFC in condensed form. In Chapter 8 (*Aspects of Gradient Elution in LC-MS Analysis*) Markus Martin deals in detail with gradients in LC-MS coupling. Here, instrumental aspects of the LC and MS parts as well as the prob-

lem of quantification of gradients are discussed. Finally, in Chapter 9, Egidijus Machtejevas describes some rare gradient modes (*Additional Tools for Method Development: Flow and Temperature Gradients*).

The book does not have to be read linearly. The individual chapters have been written in such a way that they represent completed stand-alone modules – "jumping" between them is possible at any time. We have tried to do justice to the character of the book as a reference work. Let the reader benefit from this.

Notes on Contributors

Stavros Kromidas
Stavros Kromidas studied Chemistry in Saarbruecken, Germany, completing his PhD thesis on the development of new optically active phases for HPLC. He subsequently worked as a Sales Manager for Waters, when he founded 1989 NOVIA GmbH, an independent consultancy company for analytical chemistry. Since 2001, he has been working as a consultant and has given lectures and training courses on HPLC and Validation. Stavros Kromidas has authored, coauthored and edited numerous books on HPLC, validation, and quality in analytical chemistry.

Joachim P. Weiss
After his graduation in Chemistry in 1979 from the Technical University of Berlin, Germany, he worked in the field of Liquid and Gas Chromatography at the Hahn-Meitner Institute in Berlin and received his PhD in Analytical Chemistry in 1982 from the Technical University of Berlin. Weiss habilitated in Analytical Chemistry at the Leopold-Franzens University in 2002. He currently holds the position of International Technical Director for Dionex Products within the Chromatography and Mass Spectrometry Division (CMD) of Thermo Fisher Scientific, located in Dreieich (Germany). Dr. Weiss is recognized as an international expert in Analytical Chemistry (especially in the field of Liquid/Ion Chromatography). The 4th edition of his *Handbook of Ion Chromatography* was published in 2016.

Markus M. Martin
Markus M. Martin works as Manager, Product Management UHPLC Systems at Thermo Fisher Scientific in Germering (Germany). He joined the former Dionex Corporation, now part of Thermo Fisher Scientific, in 2010 as Solutions Manager for LC/MS, being responsible for UHPLC and LC/MS solutions marketing. He received his Doctorate in Analytical Chemistry from the Saarland University in Saarbruecken, Germany, in 2004 for capillary electrophoresis investigations on polyelectrolytes in the research group of Prof. Heinz Engelhardt. Before his Thermo Fisher Scientific engagement, he worked as Analytical Lab Head at Sanofi-Aventis and as a Research Fellow at the Saarland University; his scientific work has been focused on UHPLC, HPLC-MS, CE, and CE-MS techniques as well as integrated sample preparation.

Thomas Letzel
Thomas Letzel is a habilitated analytical chemist with almost 20 years of experience in the field of analytical screening techniques using LC and GC with mass spectrometric detection. He is Head of the Analytical Research Group at the Chair of Urban Water Systems Engineering at the Technical University of Munich (TUM), Germany. He holds a Diploma and PhD in Chemistry and the license to teach analytical and bioanalytical chemistry from TUM. Currently, the key aspects in his research cover technological, analytical-methodological, and analytical-chemical properties and can be applied in water and wastewater analysis as well as in other relevant environmental matrices, such as food analysis, beverage and plant extract analysis, among others. A special focus of his work is on chemical analysis with simultaneous functionality analysis using mass spectrometric detection. He is the author and coauthor of more than 150 journal papers, book contributions, conference proceedings, and four books.

Stefan Bieber
Stefan Bieber studied Pharmaceutical Bioprocessing Engineering at the Technical University of Munich, Germany. He received his PhD at the Chair of Urban Water Systems Engineering, where he investigated the occurrence of trace organic compounds in the aquatic environment and evaluated innovative separation techniques. Since 2018, he has been Director of AFIN-TS GmbH. His research focuses on the basics of SFC separations, aiming to achieve a better understanding of this technique and to improve the applicability of SFC.

Frank Steiner
Frank Steiner heads the marketing application lab in the HPLC organization of Thermo Fisher Scientific and serves as a Scientific Advisor for HPLC. In this function he coordinates scientific collaborations with external partners to advance UHPLC technologies and applications. Frank received his PhD degree in Chemistry in 1995 from Prof. Dr. Dr. Heinz Engelhardt at the Saarland University in Saarbruecken, Germany, working on the development of stationary phases for IC. He then became a postdoctoral research fellow at the CEA, Saclay in France focusing on elementary and isotopic analysis by IC and IC-ICP/MS in 1996. Frank returned to Saarland University in 1997 to conduct research on electro-driven separation (nonaqueous CE and CEC), LC purification, and MS coupling technologies and became an Assistant Professor in 2003. In 2005, Frank joined Dionex Softron GmbH in Germering, Germany, now a part of Thermo Fisher Scientific and held different roles in marketing as product manager, manager of LC hardware marketing, and manager of solutions marketing before he became a Scientific Advisor. Frank played a significant role in developing and launching the UltiMate 3000 UHPLC systems and solutions, as well as the new Vanquish UHPLC platform.

Oliver Genz
Oliver Genz studied Biology and Chemistry in Krefeld, Mainz and Freiburg (Germany). He worked for about 10 years at Pharmacia Biotech (today GE Healthcare) in sales, technical support, and the application lab and was responsible for

running international training courses in theory and hands-on-training in analytical, preparative and process chromatography. After that he spent many years in sales, marketing, and technical support for chromatography instrumentation and stationary phases for preparative and process scale at YMC, Grace Davison (today GRACE) and Labomatic. He is the author of several publications related to preparative- and process-scale chromatography. Since 2000, he has been a freelance consultant for preparative- and process-scale chromatography and downstream processing with separation technologies.

Hans-Joachim Kuss

After studying Chemistry in Karlsruhe (Germany), he graduated in the field of Spectroscopy (PhD). He was engaged in HPLC, GC, and GCMS for 34 years at the University of Munich. Hans-Joachim has held some hundreds of courses on chromatography and implementation of weighted regression, prediction of gradients, and integration problems in EXCEL.

Egidijus Machtejevas

Egidijus studied Organic Chemistry and Biotechnology at Kaunas University of Technology, Lithuania. He completed his PhD in Analytical Chemistry (dissertation title "Design of chiral adsorbents and enantioseparations by means of HPLC") in 2001. From 2001, he worked as a post-doc with Prof. Klaus Unger at Mainz University, Germany. He joined the R&D Department at Merck KGaA in Darmstadt, Germany in 2008 and worked on applications of silica monolithic columns. Currently, he is a global chromatography specialist. Egidijus Machtejevas has more than 20 scientific papers and ten book chapters to his name and his major research focuses include multidimensional liquid chromatography, proteomics, and the development of monolithic stationary phases for chromatography.

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Part 1

Principles of Gradient Elution

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Chapter 1
Aspects of Gradient Optimization

Stavros Kromidas (translated from German by Steve Ross)

1.1 Introduction

Gradients are versatile and therefore find wide application. For example, gradients are just as essential in method development of unknown samples as for quantification at trace levels. The theoretical background of gradient elution is quite complex, because what happens in the column during gradient elution, compared to isocratic separations, is affected by more factors; these sometimes act in opposite directions or are multiplicative.

Herein, we will focus on selected aspects of the optimization of gradient separations in RP chromatography in deliberately simple form. Other important aspects of the gradient such as theory, equipment, and troubleshooting are left to other sources [1–4]. First, we briefly describe the action of a gradient in the column, then using some basic formulas we discuss the characteristics/features of the gradient. On the basis of this, possibilities for optimization of the following objectives will be shown: low detection limit, high peak capacity, sufficient resolution, and the shortest possible retention times. Finally, there is a summary with some basic rules and recommendations.

1.2 Special Features of the Gradient

In HPLC, interactions of different strengths between the analytes on the one hand and eluent components and the stationary phase on the other usually occur during separation. In the case of isocratic separations there is a predetermined, constant eluent composition, consequently during chromatography an interaction of constant strength takes place between the eluent molecules and the phase material.

What happens now in a gradient run? During gradient separations the strength of the mobile phase increases, consequently its interaction with the stationary phase also increases during the gradient run. The rule in RP chromatography is: the more organic, nonpolar/hydrophobic the eluent becomes during the separation (more % B, ACN or MeOH), the stronger its interaction with the organic, nonpolar surface of an RP material becomes – it is indeed "like with like," that

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means the nonpolar ACN or MeOH molecules naturally "like" for example nonpolar C18 alkyl groups.

In the course of a gradient, because of the ever increasing concentration of ACN/MeOH molecules, the substance molecules become subject to increasingly strong competition in their interactions with the C18 alkyl groups. Because of this, the substance molecules are increasingly forced to leave the stationary phase faster, go into the mobile phase earlier and thus elute earlier compared to isocratic separations. With 100% MeOH or ACN at the end of the gradient even the very hydrophobic components of the sample elute, maybe even persistent organic contaminants that may have accumulated on the surface of the stationary phase – as a side effect the column is flushed at the same time.

Focusing on the peak form, with gradients we have two opposing trends. On the one hand, the later the peaks elute, the more the substance zone is subject to dispersion processes in the column and thus band broadening initially increases – analogous to isocratic separations. On the other hand, the acceleration of the migrating substance zone increases to the same extent, since the elution strength of the eluent permanently increases from the beginning to the end. As a result, these effects compensate each other and with a gradient we usually have narrow peaks. Note that with a gradient the concentration of the elution band constantly increases leading to lower band broadening in comparison with isocratic separations, consequently resulting in low detection limits.

This is true both for the front and for the end part of the chromatogram, in the ideal case the peak width remains constant. For this reason, in conjunction with the gradient speaking of a "plate number" is not allowed. The plate number, a measure of band broadening, is defined only for isocratic conditions. The phenomenon described here means, among other things, that in practice a reduction in packing quality and a suboptimal hardware (system dead space), which with isocratic separations leads to broad peaks, is not as noticeable with gradient separations. Even with "poor" equipment and "poor" columns chromatograms from a gradient elution look good, especially if the gradient is steep and ACN is used as the organic content of the eluent – a welcome fact for sample chromatograms in manufacturer's brochures …

Positive from the user perspective is, that simple gradient separations using 20–50 mm columns on conventional equipment generally prove to be no problem, at least as far as the peak shape is concerned. Also the advantage of smaller particle sizes, for example 2 or 3 µm particles compared to 5 µm particles, is less relevant in many applications. In the case of a difficult matrix, 3.5–5 µm material should therefore initially be considered. Unless one has to separate a large number of very similar analytes – then of course the separating efficiency of $\leq$ 2 µm particles also becomes relevant for gradients. In this context, it is also pointed out that as the eluent permanently becomes stronger (= nonpolar), the migrating substance molecules at the end of a peak, i.e., at the trailing edge, move faster than those at the beginning of the peak as the later eluting molecules of the substance band are always pushed "forward" faster. This fact, known as "peak compression," has the effect that in gradient separations tailing is rarely observed. Peak

symmetry is about 10% better compared to an isocratic run with equivalent eluent composition (H.-J. Kuss, personal communication).

1.3 Some Chromatographic Definitions and Formulas

Let us now consider some chromatographic definitions which are known from theory – which, by the way, was developed originally for GC and much later for isocratic LC separations. The derivation of the formulas used below is omitted, they are only used to elaborate the consequences for practical optimization. For a more detailed discussion, see references [2–4] and in particular [1].

The resolution R is, in simplified form, the distance between two peaks on the baseline. The retention factor k (formerly the capacity factor k') is the ratio of the time a component spends in/on the stationary phase and in the mobile phase, that is the quotient of the net retention time t'_R (time spent in the stationary phase) and the flow-through or dead or mobile time t_0 and t_m (time spent in the mobile phase). It thus represents a measure of the strength of the interactions of *these* components on *this* column under *these* conditions: $k = t'_R/t_0$. However, the retention factor is not constant for a gradient. Very high at the beginning (with 100 or 95% water/buffer the substances literally "stick" to the beginning of the stationary phase), it becomes less during the separation and at the end of the gradient is very small. With 90 or 100% MeOH or ACN, the substance molecules hardly have a chance to stay on the stationary phase, because the competition for the "attraction" of the C18 group has now become huge. Put simply, with a gradient from 100% water/buffer to 100% MeOH/ACN, the k value at the beginning is virtually infinite – in some references numbers between 3500–4000 are given – and at the end almost zero. Since the k-value changes during gradient elution, a k^* value (or $\overline{k}$) was introduced to take account of this particular feature [1]: this is the k-value of a component when it is just in the middle of the column.

Although the need for such a term to describe the gradient may be questioned, the k^* value is used here because it has advantages for our deliberations. And that the interactions, and therefore a measure for them, a retention size, is important for optimization considerations, is clear – however such a term may be defined.

The separation factor α is the quotient of the retention factors of two components that one wishes to separate, k_1 and k_2, and describes the ability of the chromatographic system to separate these two components. In the literature, different formulas are used for R and k^*. However, they are quite similar and ultimately lead, when the focus is on the practice, to similar numerical values and thus to similar propositions.

Here is an example: in Eq. (1.1) for the second term (the selectivity term), in addition to $(\alpha - 1)$ the terms $\ln \alpha$ or $\alpha - 1/\alpha$ are also to be found in the literature. Assuming a α-value of 1.05, the following numerical values for the selectivity term are found: 0.048, 0.049, and 0.050. However, these different numbers affect the value of the resolution only in the second decimal place.