Journal of Chromatography A, xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Development of optimized mobile phases for protein separation by high performance thin layer chromatography

Julia Biller[a], Lena Morschheuser[a], Maria Riedner[b], Sascha Rohn[a,*]

[a] Institute of Food Chemistry, Hamburg School of Food Science, University of Hamburg, Grindelallee 117, D-20146 Hamburg, Germany
[b] Institute of Organic Chemistry, Mass Spectrometric Facility, University of Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany

ARTICLE INFO

*Article history:*
Received 8 July 2015
Received in revised form 22 August 2015
Accepted 24 August 2015
Available online xxx

*Keywords:*
HPTLC
Protein separation
Optimization of chromatographic separation
Protein analysis

ABSTRACT

In recent years, protein chemistry tends inexorably toward the analysis of more complex proteins, proteoforms, and posttranslational protein modifications. Although mass spectrometry developed quite fast correspondingly, sample preparation and separation of these analytes is still a major issue and quite challenging. For many years, electrophoresis seemed to be the method of choice; nonetheless its variance is limited to parameters such as size and charge. When taking a look at traditional (thin-layer) chromatography, further parameters such as polarity and different mobile and stationary phases can be utilized. Further, possibilities of detection are manifold compared to electrophoresis. Similarly, two-dimensional separation can be also performed with thin-layer chromatography (TLC). As the revival of TLC developed enormously in the last decade, it seems to be also an alternative to use high performance thin-layer chromatography (HPTLC) for the separation of proteins. The aim of this study was to establish an HPTLC separation system that allows a separation of protein mixtures over a broad polarity range, or if necessary allowing to modify the separation with only few steps to improve the separation for a specific scope. Several layers and solvent systems have been evaluated to reach a fully utilized and optimized separation system.



## 1. Introduction

Protein analysis is one of the most important research fields in life sciences as proteins are involved in all kind of biological processes. On the one hand, the so-called shot-gun proteomics – proteolytic digestion of proteins prior to LC-MS analysis [1] – is well established enabling the identification of thousands of proteins and even whole proteomes in one single experiment [2]. On the other hand, gaining information about a structure-function relationship is getting back into the focus of protein scientists, as posttranslational modifications (PTMs) are well-known to possess a high impact on specific biochemical functions. This trend is depicted by the creation of the new term "proteoforms" in 2013 describing all molecular forms of protein derivatives deriving from only one gene, although providing different functions [3]. Besides the countless PTMs variations such as the well-known phosphorylations and glycosylations, there are further modifications with all kinds of nucleophiles, being able to react with specific protein side chains, significantly affecting polarity, solubility, and corresponding properties of the protein(s).

Nevertheless, the analysis of intact proteins, but especially proteoforms is challenging due to their high chemical variance making it complicated to find "one for all" – a method that fits for all analytes. With regard to modern mass spectrometric techniques permitting a deep look into the chemistry of intact proteins, the main issue of protein and proteome analysis is the preceding protein separation.

So far, two main approaches for the separation of these more or less complex mixtures of proteins are commonly used: (I) The electrophoresis-based approach, where especially 2D gel electrophoresis provides, so far, the highest resolution of all protein separation techniques. It allows to a certain extent the separation of proteoforms and became therefore one of the standard techniques in proteomics. However, the extraction/utilization of intact proteins from those gels is difficult, even though feasibility has been shown [4,5]. (II) The liquid chromatography (LC) based approach, being typically used for proteolytically digested proteins: Separation of the corresponding peptides is fast, and sensitivity and resolution are high. Online coupling to mass spectrometry as detection method makes the identification of known proteins very easy. However, due to the previous proteolytic digestion, some

* Corresponding author.
*E-mail address:* rohn@chemie.uni-hamburg.de (S. Rohn).

http://dx.doi.org/10.1016/j.chroma.2015.08.048
0021-9673/

properties of the proteoforms cannot be analyzed anymore. Moreover, liquid chromatography separation for intact proteins suffers from low resolution, and identification of the eluting intact proteins via mass spectrometry is still a challenge. So, it seems that further alternatives are necessary to overcome the above mentioned limitations.

An appropriate methodology would be an approach where influencing parameters of the separation system can be changed quite easily without too much interference/antagonism of each influencing parameter. High-performance thin-layer chromatography (HPTLC) could be such an alternative technique. It is a fast and simple procedure where samples can be applied with or without only few pretreatment steps. This reduces analysis time and decreases the possibility of contamination or loss of compounds during the preparation. HPTLC is also characterized by the possibility to change separation conditions easily by varying eluents and stationary phases.

Knowing that the nature of PTMs of proteins has a significant influence on their polarity, solubility and corresponding properties, the named advantages of HPTLC are applicable for analyzing different PTMs of proteins with very little effort. The high variety of those samples obliges a methodology where method development occurs easily as variation of influencing parameters of the separation system can be changed quite easily and independently. For example, different solvent systems can be used separately, therefore being able to cover the whole range of polarity. Additionally, many chromatographic stationary phases are available. In the last few years, their development in terms of quality (e.g. particle size, reproducibility of the production) is nearly as fast as for HPLC columns. Wannenmacher et al. demonstrated the utility of alternative inorganic oxides, compared to the traditionally used silica gel stationary phases, even applied as ultra-thin layers expanding the capabilities of planar chromatography [6].

Moreover, a second advantage in the use of HPTLC is the applicability of a diverse number of detection possibilities. One chromatogram can be analyzed by several detection methods: (I) simple staining with one or a combination of different coloring reagents, (II) use of bioactivity-guided, effect-directed analysis, or (III) hyphenations with mass spectrometry [7–9]. Even miniaturization approaches can be considered [10]. All these possibilities illustrate the high potential of HPTLC. Especially, the coupling to mass spectrometry brings this technique on a comparable level with liquid chromatography and electrophoresis.

With regard to protein analysis, only few efforts were taken to explore the possibilities of TLC. First results on this topic came up in 1964, when Morris et al. adapted gel filtration chromatography onto glass plates and being able to realize a separation [11]. Some researchers tried to use different Sephadex derivatives to achieve a separation of selected model proteins in order to use this approach for estimation of the protein's molecular weight, e.g., [12]. Further setups combined TLC with electrophoretic methods and were described in literature as thin-layer electrophoresis. A considerable high amount of equipment was required and the increase of separation power was limited [13,14]. Further, those methods are laborious and, regarded from today's view, not suitable for automatization. Also traditional approaches utilizing paper and thin-layer chromatography were only of limited success with regard to separation capacity and sharpness of the resulting protein bands. As those results were not satisfying, TLC was declined being helpful in the separation of proteins.

In recent years, one argument often simply used was that "because several other high-resolution techniques are available, e.g., HPLC; column liquid chromatography involving size exclusion, ion-exchange, and affinity phenomena; SDS-PAGE; capillary electrophoresis; and mass spectrometry as a detector" [15]. In the review of Bushan and Martens [15], the main challenging properties of the proteins for their analysis with TLC are mentioned: kinetics of diffusion, adsorption and desorption, denaturation, and conformational changes. One aspect to keep also in mind is that, depending on the development system (glass chambers vs. automated multiple development in closed devices), TLC is an open system, that might be susceptible to oxidative stress for the analytes. Proteins can undergo oxidative transformations leading to unwanted PTMs (e.g. oxidation of methionine, formation of carbonyls) [16].

With regard to the few studies successful, most of them only aimed at single (isolated and purified) proteins. It was not tried comprehensively to separate complex protein mixtures. Similar to the electrophoretic approaches, in protein analysis with HPTLC, proteins are usually proteolytically digested prior to their separation, as reviewed in [15,17].

The idea of using HPTLC and developing the plates in two dimensions with two different solvent systems (2D-HPTLC) further improves resolution in peptide separation. Tuzimski [17] stated in his review: "The application of multidimensional thin-layer chromatography combined with different separation systems and modes of chromatogram development is often necessary for performing the separation of more complicated multicomponent mixtures." In 2008, Pasilis et al. [18,19] used the 2D-approach for the separation of peptides on ProteoChrom® HPTLC cellulose aluminum foils. Tscherch et al. [20] investigated PTMs of proteins (following a tryptic digestion), trying to evaluate whether 2D-HPTLC-MALDI-TOF-MS enables the identification of binding sites inside the protein chain and might contribute to understanding the mechanism of polyphenol–protein interactions. Of course, it is further possible to enhance the detection by using labeling of the proteins/peptides. One very successful example is the phosphorus-32 labeling of proteins and subsequent tryptic phosphopeptide mapping by TLC on microcrystalline cellulose [21]. Unfortunately, such an approach is very 'dirty' and requires corresponding lab facilities. Also the electrochemical approach had a little revival when the combination of two-dimensional planar electrochromatography and thin-layer chromatography (2D PEC/TLC) were presented as novel strategy for the fractionation of complex peptide mixtures [22].

However, the separation and analysis of intact proteins by HPTLC was often only of subordinated interest, although, as mentioned above, some information about proteoforms and protein properties in general are not feasible anymore, when the proteins are digested prior to their analysis. The aim of this study was to establish a variable chromatographic separation system that allows a separation of protein mixtures over a broad polarity range, or if necessary modify the separation with only few steps to improve the separation for a specific polarity range. As the whole variability of proteins cannot obviously be covered, different model proteins were selected in order to follow the optimization strategy.

## 2. Materials and methods

### 2.1. Plates and reagents

HPTLC silica gel 60 glass plates (10 cm × 20 cm) [in the following abbreviated as NP] and HPTLC silica gel 60 glass plates RP-18 (10 cm × 20 cm) [RP-18], TLC silica gel 60 glass plates RP-8 (20 cm × 20 cm) [RP-8] and TLC silica gel 60 aluminum foil plates RP-18 (20 cm × 20 cm) [RP-Al], HPTLC cellulose glass plates (10 cm × 20 cm) [Cel] were supplied by Merck KGaA (Darmstadt, Germany).

Acetone and 2-propanol were purchased from Gruessing Analytika GmbH (Filcum, Germany). Acetonitrile, *n*-hexane, pyridine, and *tert*-butyl methyl ether were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany); methanol and 2-butanone from

Please cite this article in press as: J. Biller, et al., Development of optimized mobile phases for protein separation by high performance thin layer chromatography, J. Chromatogr. A (2015), http://dx.doi.org/10.1016/j.chroma.2015.08.048