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Effect of high pH column regeneration on the separation performances in reversed phase chromatography of peptides

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ABSTRACT

Caustic regeneration procedures are often used in chromatographic purification processes of peptides and proteins to remove irreversibly bound impurities from the stationary phase. Silica-based materials are the most commonly used materials in reversed phase chromatography of peptides. Their limited chemical stability at high pH can be, however, problematic when high pH column regeneration (i.e. cleaning in place) is required. The effect of cleaning in place on the surface chemistry of the stationary phase has been investigated using the Tanaka test. It has been shown that the high pH treatment does not significantly affect the hydrophobicity of the material, but it strongly increases its silanol activity. A representative peptide purification process has been used to investigate the impact of cleaning in place on the separation performance. It has been shown that the caustic regeneration increases the peptide retention at high pH (pH 6.5), due to the interactions between the peptide and the negatively charged silanol groups. These unwanted interactions reduce the separation performances by decreasing the selectivity between the late eluting impurities and the main peptide. However, it has been shown that the effect of the silanol groups on the peptide adsorption and on the separation performance can be minimized by carrying out the purification process at low pH (pH ~ 2). In this case, the silanol groups are protonated and their electrostatic interactions with the positively charged analyte (i.e. peptides) are suppressed. In these conditions, the peptide adsorption and the impurity selectivity is not changing upon high pH column regeneration and the separation performance is not affected.

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1. Introduction

Among the chromatographic techniques, reversed phase high-performance liquid chromatography (RP-HPLC) is the method of choice for peptide purification. In preparative chromatography, repetitive injections of large amount of crude peptide may lead to decreasing separation performances due to the irreversible adsorption of certain impurities or peptide aggregates on the stationary phase [1,2]. This phenomenon is well known, for example, in the human insulin purification process, because human insulin tends to aggregate at low pH and clog the pores of the stationary phase [1,3].

In order to degrade and desorb the irreversibly adsorbed peptides and aggregates, column regeneration procedures, usually referred to as cleaning in place (CIP) are applied [4]. This procedure consists in washing the stationary phase with a mobile phase containing sodium hydroxide. While the chemical stability of polymeric packing materials at high pH is uncontested [4,5], it is well known that the pH stability of silica-based material is limited [3,6]. NaOH treatments at pH > 10 bring along a risk of dissolution of the silica matrix [3,6]. The degradation of the silica matrix upon high pH treatment may lead to a reduced column efficiency, especially when positively charged compound are separated [3,6].

CIP procedures have been developed to desorb impurities irreversibly adsorbed during the purification of polypeptides. In this paper, the effect of multiple CIP cycles on the purification of a polypeptide from its crude mixture has been investigated. The results have been then correlated to the modification of the surface properties.

2. Column characterization

The separation performance of a reversed phase column strongly depends on the column properties [7]. Several chromatographic tests have been developed to characterize the properties of a stationary phase. The most commonly used test are the Engelhardt test [8,9], the Walters test [10], the SMR test [11], and the Tanaka test [12]. The main parameters evaluated with these tests are the hydrophobicity, the hydrogen bonding capacity (i.e. silanol

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activity), the ion exchange capacity, the metal impurities and the steric selectivity.

2.1. Hydrophobicity

The absolute hydrophobicity of a stationary phase can be characterized by the retention factor of an aromatic hydrocarbon [10,12]. The retention factor of amylbenzene and toluene has been used to characterize the absolute hydrophobicity of various reversed phase stationary phases [11–13,17]. The absolute hydrophobicity depends mainly on the surface coverage (i.e. density of functional groups) and the surface accessible for adsorption.

According to Lesellier and West [17], the measurement of the selectivity between two compounds measured in the same conditions eliminates the influence of the specific surface area. By measuring the selectivity between non-polar alkylbenzenes differing by one methylene group (i.e. the hydrophobic or methylene selectivity), it is possible to remove the influence of the surface area and surface coverage and to characterize the functionalization of the material (i.e. the length of the carbon chain) [8,12]. The selectivity between toluene and ethylbenzene and the selectivity between butylbenzene and amylbenzene have been often used to evaluate the hydrophobic selectivity [11–13,17].

2.2. Steric selectivity

The selectivity between two aromatic hydrocarbons which have similar hydrophobicity, but different molecular shape: one twisted and one planar is called the steric or shape selectivity. This measurement evaluates the capacity of a column to separate compounds of identical elemental composition but different three-dimensional structure.

The selectivity factor of triphenylene/ortho-terphenyl is widely used to characterize the steric selectivity of a stationary phase [14]. The hydrophobicities of triphenylene and ortho-terphenyl are identical but they do not adsorb onto the stationary phase to the same extent due to their different molecular shapes [12,14]. Triphenylene is a planar molecule. It generally penetrates more easily into the alkyl layer of the stationary phase than ortho-terphenyl, which is a twisted molecule. Its adsorption is therefore stronger than the ortho-terphenyl adsorption. Kimata et al. has shown that the selectivity between triphenylene and ortho-terphenyl can be correlated to the ligand density [12].

2.3. Silanol activity

The silanols groups can be negatively charged or neutral depending on the pH of the mobile phase. Méndez et al. have shown that typical silica-based C18 materials have two different types of silanol groups with different acidity (pK_a values about 3.5–4.6 and 6.2–6.8, respectively) [15]. The origin of these two types of silanol groups is however not clear. It could be due to the heterogeneous structure of the silica surface or to the presence of metal impurities in the silica matrix [16]. Neutral silanol groups interact via hydrogen bonding with hydrogen-bond acceptors (e.g. OH group of caffeine) and negatively charged silanol groups interact electrostatically with positively charged analyte (e.g. protonated amine). It has been shown that these two modes of interaction work independently from each other [12].

Poor peak symmetry and strong retention of basic compounds indicate the presence of negatively charged silanols on the stationary phase surface [8–14]. Pyridine, aniline, aniline derivatives and basic drugs like diphenhydramine and amytriptiline have been extensively used to determine the ion exchange capacity [8–17]. The ion exchange capacity can also be characterized by measuring the relative retention factors between a base and a slightly acidic or neutral compound at low and high pH values [10,12]. At high pH values (pH 7.6) the ion exchange interactions are strong since most of the residual silanol groups are dissociated. At lower pH values (pH 2.7) only the most acidic silanol groups are still active and are able to establish electrostatic interactions [12]. The selectivity factor of benzylamine and phenol has been widely used in the literature to evaluate the ion exchange capacity of a stationary phase [11–14].

The hydrogen bond capacity can be characterized by the selectivity of two compounds, which have different acid/base characters. Basic analytes are generally strongly retained in RP-HPLC because of the interactions with the acidic residual silanol groups, whereas acidic analytes are only retained by the hydrophobic interaction with the surface [12,17]. The selectivity between caffeine and phenol has been often used to characterize the hydrogen bonding capacity [12,17]. Caffeine strongly interacts in fact with protonated silanol groups via hydrogen bonding, whereas phenol does only interact with the hydrophobic alkyl chains.

3. Experimental

3.1. Chemicals

HPLC grade acetonitrile, triethylamine, butylbenzene, amylbenzene, o-terphenyl, triphenylene, phenol and benzylamine were purchased from Sigma–Aldrich (Buchs, Switzerland). Orthophosphoric acid 85% and ammonium acetate were purchased from Merck (Darmstadt, Germany). Tri-fluoroacetic acid was purchased from ACROS (Geel, Belgium). Caffeine was purchased from Alfa Aesar (Karlsruhe, Germany). All the chemicals were used without further purification. The deionized water was purified with a Simpak2 unit (Millipore, MA, USA) before use. The polypeptide crude mixture used in this work is a 1.2 kDa polypeptide and is representative of an actual industrial product. It was kindly donated by Genzyme pharmaceuticals (Liestal, Switzerland).

3.2. Experimental setup and columns

The experiments were carried out on an Agilent 1100 Series HPLC, equipped with an auto-sampler, a diode array detector, an online-degasser and a quaternary gradient pump. A Gilson FC 203B fraction collector (Middleton, WI, USA) was connected at the outlet of the HPLC to collect fractions during the peptide elution.

The stationary phases used to perform the cleaning in place study were obtained as powder and were given for packing to HiChrom Ltd. (UK). The packed columns have 25 cm length and 0.46 cm internal diameter. The ZEOsphere 100 A 10 μm C18 was obtained from Zeochem AG (Uetikon, Switzerland). The Kromasil 100 A 10 μm C18 and the Kromasil 100 A 5 μm C18 were obtained from EKA chemicals AB (Bohus, Sweden). The ZEOsphere 100 A, 10 μm, C18 and the Kromasil 100 A, 10 μm, C18 were used to study the effect of the cleaning in place. The Kromasil 100 A 5 μm, C18 was used to analyze the fractions collected during the chromatographic experiments (see Section 3.5).

The buffers for the analytical test are presented in Table 1. A gradient from 34% B3 to 64% B3 was carried out in 40 min on the Kromasil 100 A 5 μm C18 column at a temperature of 55 °C. The UV response at 215 nm was recorded and calibrated using samples of known peptide concentration. The purity and concentration of

Table 1
Analytical buffer composition.

Buffer	Composition
A3	Triethylamine phosphate, pH 2.25
B3	B3/acetonitrile, 40/60 (v/v)

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