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Journal of Chromatography A, 880 (2000) 169–187

JOURNAL OF CHROMATOGRAPHY A

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Review

Perfusion chromatography: an emergent technique for the analysis of food proteins

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Abstract

Perfusion chromatography is a technique arised to overcome the problem associated with mass transfer in the separation of large molecules such as proteins by high-performance liquid chromatography (HPLC). Perfusion media are constituted by two set of pores: *throughpores* (6000–8000 Å) and *diffusive pores* (800–1500 Å) which enable better access of macromolecules to the inner of the particle by the combination of convective and diffusive flow. As a consequence, times required for a chromatographic separation are reduced. Perfusion media are available in different chromatographic modes: reversed-phase, ion-exchange, hydrophobic interaction, and affinity. From the theoretical models developed to explain the dynamic of retention of solutes in perfusive supports, it was derived that efficiency of a separation was independent of the flow-rate and only depended slightly on the particle diameter. Furthermore, loading capacity was also independent of the superficial velocity. All these advantages have promoted the use of this chromatographic technique for the separation of biomolecules both in analytical and preparative chromatography. Characteristics of perfusion chromatography make this technique very interesting for the analysis of food proteins. Perfusion chromatography enables the assessment of protein composition of a foodstuff at sufficient speed and low cost to be suitable in routine analysis.

Keywords: Reviews; Perfusion chromatography; Food analysis; Proteins

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00354-X

1. Introduction

Liquid chromatography has been the most important and used technique in the analysis of biomolecules (especially proteins) for more than three decades [1,2]. During this time, many advances have occurred related to chromatographic supports.

At the beginning, polysaccharides such as cellulose, agarose, and cross-linked dextrans were the chromatographic supports most used for the analysis of proteins. The principal advantages associated with the use of these gels were being cheap, easily derivatizable, and presenting high porosity and high surface area. In addition, this kind of chromatographic support did not suffer hydrolysis and seemed not to alter significantly the three-dimensional structure of biological macromolecules [1,2]. Nevertheless, their low mechanical strength constituted a limiting factor, being possible to observe deformation of sorbent particles even at low backpressures [3]. The use of larger particle sizes could mitigate this effect, but only at the expense of a great increase of the intraparticle mass transfer resistance which would lead to a low loading capacity [4].

Inorganic supports, such as silica, constituted an alternative to soft gels in the analysis of proteins, having higher mechanical strength. Nevertheless, the problems associated with silica-based materials were that they often showed cation-exchange effects from residual silanols (electrostatic interactions between amino groups in solutes and residual silanols on the sorbent) and presented limited stability at high pH values (>9), not being possible to carry out separations under basic conditions [1,2].

Organic resins were the next generation of chromatographic supports developed. Polymethacrylate and polystyrene–divinylbenzene resins presented higher mechanical strength than soft gels and they could support pH values ranging between 1 and 12 [1,2].

The fourth phase of chromatographic supports was focused on overcoming the problem related to mass transfer in the separation of large molecules such as proteins [1,2]. This search arose due to the need of accelerating all the aspects related to the research in biomolecules and, as a consequence of the great advances occurring in biotechnology. Large analysis times required to separate biomolecules were due to the difficulty in the diffusion of these molecules through the interior of the stationary phase particles. In order to make the intraparticle mass transfer easy, different possibilities have been proposed [5–7]:

(i) The reduction of the particle size, thereby reducing the length of the diffusion path and the time required for molecules to diffuse in and out of the particles. The main drawback associated with this proposal is that reducing particle size drastically increased column backpressure.

(ii) The use of pellicular packings consisting of spherical non-porous particles recovered with a very thin layer of porous adsorbent. In this case, binding occurs directly on the particle surface which is totally accessible for the solute molecule by convective flow. Both polymeric and silica materials have successfully been developed for this application and are very useful for rapid separations. Unfortunately, particles of low porosity present the disadvantage of limited loading capacity which may be overcome by using extremely small particles; however, the use of these small particles is not practical because of the high pressure needed to pump the mobile phase through the column [4,8–11].

(iii) The employment of chromatographic membranes consisting of hollow fibres and thick discs. Membrane chromatography is a separation technique which combines advantages of both column chromatography (selectivity and efficiency) and membrane technology (easy scale-up and low back-pressure). In this case, surface area is entirely accessible by convective flow, enabling rapid binding and elution. However, this kind of chromatographic support presents low loading capacity, large dilution factors, and high bandspreading [12–14].

(iv) The use of composite stationary phases comprising rigid porous particles whose pores are filled with a gel. These chromatographic supports based on the concept of ''soft gel in a rigid shell'', combine the advantages of a rigid open porous bead (providing high mechanical strength enabling operation at high flow-rates without compression) with the high binding capacity provided by a gel. This structure favors mass transfer of biomolecules at high flow-rates, maintaining a high loading capacity [4,9,15,16].

(v) Tentacular sorbents were designed to accelerate contact between biomolecules and interactive

groups. This kind of filliform stationary phase can be prepared by immobilizing a polymer onto silica support or by initiating polymerization at sorbent surface. Incorporating stationary phases into a hair-like border of polymeric filaments at the surface of a silica support increases the surface area and, consequently, loading capacity [9,17].

(vi) Other possibility to enhance mass transfer is to increase particle permeability by using wide pores connected to smaller diffusive pores. These chromatographic supports are called large pores supports. Different chromatographic supports corresponding to this structure are available: The called *superporous agarose beads* are characterized by presenting two sets of pores: the normal *diffusive pores*, characteristics of all agarose materials, and the very wide so-called *superpores* (ranging from 1/3 to 1/10 of the particle diameter) enabling part of the chromatographic flow to pass through each individual particle. These supports present the desirable properties of traditional agarose supports for the separation of biomolecules and the drawbacks associated to gels which have been previously cited [3,18]. The use of columns made of one piece of porous solid prepared by compression or by polymerizing monomers directly in a chromatographic column is another kind of chromatographic support presenting a biporous structure. The rods (monoliths of silica or an organic polymer) contain both flow-through channels and a system of conventionally sized pores which provide higher column efficiencies at much lower backpressures than particle-packed columns [19–24].

These large pore supports are prepared with porous materials (agarose, porous silica, etc.), presenting diffusive pores characteristics of these porous materials. The novelty presenting these supports is that besides its own diffusive pores, they have large flow-pores enhancing mass transfer.

During the period from 1989 to 1991, a new HPLC technique was introduced. This chromatographic technique is also characterized by presenting two set of pores, although in this case the starting supporting material is not porous. This new technique was called *perfusion chromatography* because perfusive particles used as stationary phase resembled the body's own system of arteries and capillaries which transport nutrients to cells [2,25,26]. Another difference of perfusion chromatography with the other large pore supports is the diameter of its pores (Table 1). In fact, convective pores in perfusive supports present diameters smaller than *superporous agarose beads* and polymeric rods and the diameter of diffusive pores is about 5 to 40 times bigger than the used in silica rods.

Table 1
Diameters of diffusive and convective pores in large pore supports [18–26]

Support	Diffusive pores (μm)	Convective pores (μm)
Superporous agarose beads	–	7–170
Porous silica rods	0.002–0.03	0.5–8
Porous polymeric rods	≈0.15	0.7–7
Perfusive particles	0.08–0.15	0.6–0.8

Perfusion chromatography constitutes an interesting alternative to the problem of intraparticle solute transport and supposes a great advance in the separation and purification of proteins, peptides, and nucleotides to analytical and preparative scale.

2. General characteristics of perfusion chromatography

In conventional liquid chromatography (diffusion chromatography) (Fig. 1), molecules move to the outer surface of the stationary phase particles by convection which constitutes a rapid step [25]. Nevertheless, transport of molecules through a stationary phase particle occurs by molecular diffusion which constitutes a very slow process, especially in the case of peptides and proteins that present high molecular weights and low diffusion coefficients [5]. As a consequence, a significant increase in bandspreading (loss in resolution) could occur, since molecules still in the convective stream or bound to the outside of the particles could elute before those diffusing to the inner of the particle. Furthermore, binding capacity could also decrease, since part of the sample may pass through the column before all of the binding sites deep within the particles were occupied [5,6,27–30].

In perfusion chromatography (flow-through particle chromatography), stationary phase particles used are designed to enable a better access of molecules to the inner of these through two classes

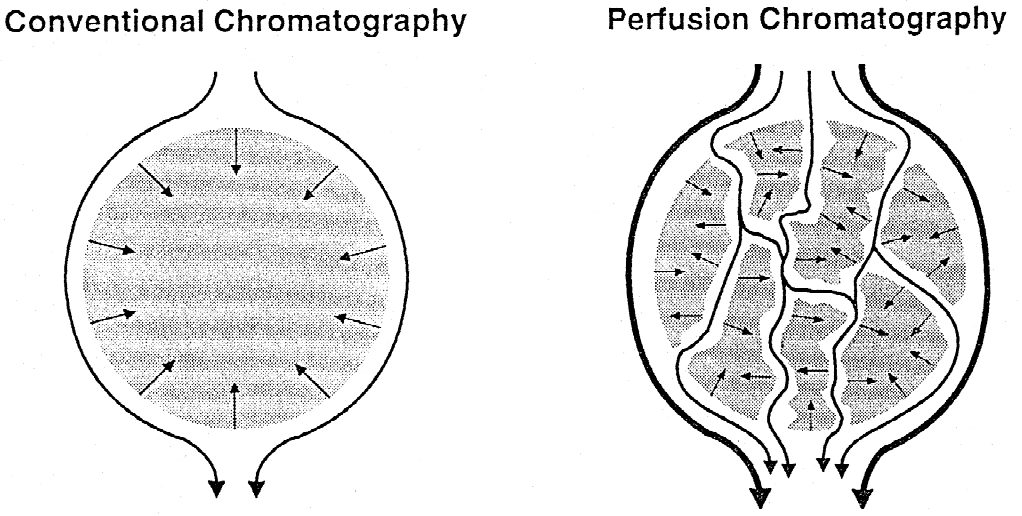

Fig. 1. Diagram of a conventional and a perfusion stationary phase particle. (From Ref. [25] with permission).

of pores: *throughpores* (6000–8000 Å), which cross the stationary phase particle from side to side and allow the transport of molecules into the interior of the particle by convective flow and *diffusive pores* (800–1500 Å), interconnecting the *throughpore* network and enabling the transport by diffusion (Fig. 1). In this way, molecules travel by convection through the column to the stationary phase particle, such as in conventional chromatography. Since there, molecules cross the stationary phase particles by means of a combination of convective and diffusive transport, thus, accelerating the transport of molecules through the particle. This singular bimodal structure significantly enhances mass transport of molecules, and as a consequence [5,6,25,26,28–30]:

(i) Separations are 10–100 times faster than in conventional chromatography,while resolution and column loading capacity are maintained.

(ii) The rapid transport within the *throughpores* and the ultra-short diffusion path lengths (<1 μm) of *diffusive pores* enables both resolution and loading capacity being independent of the flow-rate.

(iii) The reduction of the analysis times occurring in perfusion chromatography decreases the residence time of molecules inside the chromatographic column and, therefore, improves recovery of biological activity, given the fact that molecules inside the chromatographic column are exposed to potentially denaturing eluents, proteolytic degradation, and damage caused by the forces that bind them to the chromatographic support.

(iv) As a consequence of both the reduction in analysis times and the high loading capacity of perfusion supports, the cost of large-scale chromatographic processes decreases.

The benefits of perfusion chromatography are greater at high mobile-phase velocities (1000–5000 cm/h and beyond), where intraparticle convective transport greatly exceeds the rate of diffusion transport [26].

3. Perfusion media composition

Appropriate media for perfusion chromatography must possess outstanding chemical and mechanical stability. Although suitable media could consist of alumina, silica or even hydroxyapatite, most frequently, the matrix of perfusion media is constituted by highly crosslinked polystyrene–divinylbenzene [31]. This polymer presents a huge mechanical and chemical stability, being capable to put up with backpressures up to 200 bar and to resist a broad range of solvents, pH values ranging from 1 to 14, high ionic strengths (0–3 *M*), and temperatures up to 80°C [1,6,26]. The particle diameter of these media

can suit a given application: 10 μm (analytical applications requiring very high resolutions), 20 or 50 μm (preparative applications) [31]. Loading capacity of perfusion columns varies as a function of the chromatographic mode used. Loading capacity in reversed-phase perfusive supports, where this matrix not only acts as support but also as the own stationary phase [28], is approximately 50% of similar conventional packings, which is acceptable in analytical and semipreparative columns, but constitutes an important drawback for preparative works. In order to meet this demand, PerSeptive Biosystems has modified the pore morphology to incorporate a greater number of diffusive pores. The loading capacity of these new reversed-phase sorbents is about two to four times higher [26]. In the case of other surface chemistries (ion-exchange, affinity), loading capacity is 75–125% of conventional materials'. Table 2 groups all these characteristics of perfusive supports.

The use of a perfusion matrix in mode different from the reversed-phase chromatography requires a tightly crosslinked hydrophilic surface layer that masks the hydrophobicity of the matrix [26]. For this purpose, the matrix is coated with an hydroxylated polymer which is crosslinked to the inert backbone creating a neutral layer which presents a high concentration of hydroxyl groups. This layer consists of a co-polymer composed by at least three monomer species, every one having a different function. One of the monomers is used to anchor the copolymer to the surface of the matrix, preventing the erosion of the coating; the second monomer has the function of coupling stationary-phase groups to the support and the third monomer crosslinks individual polymer chains into an impermeable surface layer [26,30]. In this film, rich in hydroxylated groups, there are substituted functional groups forming different chromatographic modes: ion-exchange, hydrophobic interaction, and affinity [30].

Anion-exchange is used in the separation of many proteins because most of them present a net negative charge at physiological pH. Functional groups associated to commercially available perfusive anion-exchangers can be quaternary amines or polyethyleneimine. Cation-exchange, by other hand, is usually used for the separation of polypeptide hormones which are currently positively charged. In this case, functional groups associated to commercially available perfusive cation-exchangers can be sulfopropyl, sulfoethyl or carboxymethyl [28,29]. Nash and Chase [32] compared diffusion and diffusion–convection (perfusion) matrices for their use in ion-exchange separations of proteins. From these studies, it was found that at analytical level loadings, ion-exchange perfusion supports yielded higher resolution and were more suitable than conventional ion-exchange supports for the separation of proteins [32].

Hydrophobic interaction is an increasingly used chromatographic mode. It is suitable for the separation of proteins which exist in a high salt environment or when there is a high susceptibility to denaturation. Surface functional groups associated to commercially available perfusion supports can be phenyl, phenyl ether, butyl or ether [28,29].

Affinity chromatography constitutes an important tool in the separation and purification of biomolecules. This chromatographic mode is based on the different binding affinity that present biomolecules for immobilized biospecific ligands or heavy metal ions (metal chelate affinity). Thus, by capturing biomolecules on the pore surface, they can easily be separated away from all other sample components [33]. A wide selection of biospecific ligands (anti-

Table 2
Characteristics of perfusive supports [28,29]

Backpressure	$<$200 bars
pH range	1–14
Ionic strength	0–3 M
Temperature	5–80°C
Solvents	0–100% water, alcohols, acetonitrile, etc.
Buffer additives	All common agents: urea (up to 8 *M*), guanidine–HCl (up to 6 *M*), ethylene glycol, detergents, etc.
Loading capacity	75–125% of loading capacity in conventional columns.

bodies, proteins, peptides, nucleic acids, and carbohydrates) can be immobilized to the pore surface inside the particle by three different ways [28,33] in which the ligand is: (i) anchored with a crosslinking agent (dimethyl pimelimidate), or (ii) attached directly and covalently to the particle surface which is normally activated by aldehyde, epoxide or hydrazide derivatization, or (iii) immobilized in an avidin-activated surface.

In addition to be far more rapid than conventional enzyme-linked immunosorbent assay (ELISA) techniques, affinity chromatography is also extremely reproducible [5]. One variation of affinity supports are metal chelate affinity supports, having a metal ion as a ligand. In this kind of supports, the imidodiacetic acid acts as the metal chelating ligand. The metal ion bound to the metal chelating ligand modifies its selectivity being the metal ions from Zn, Cu, Co, and Ni the most used [28].

PerSeptive Biosystems has recently developed a novel immunoassay using perfusion chromatography technology. The ImmunoDetection (ID) assay consists of a flow-through cartridge-based immunoassay that operates in real-time on any chromatographic instrument. Whereas traditionally, immunoassays require hours to days to perform a single assay, ID assays can be performed in a time ranging between 30 s and 3 min [25,33–35].

There are also commercially available perfusion columns to work on hydrophilic interaction chromatography. The packing surface used in these cases is hydrophilic and it differs from conventional normal-phase in that the surface is uncharged. This chromatographic mode is used for the separation of biomolecules which are too hydrophilic to be separated by reversed-phase [28].

Perfusion media are not available for gel permeation chromatography because intraparticle convection could disturb the diffusional process necessary for a separation based on size [1,2,28,29].

4. Theory of perfusion chromatography

In order to explain in a theoretical way the dynamic of the retention of solutes in perfusive supports, several models have been developed. Afeyan, Rodrigues, and Liapis research teams are the three main groups which have dedicated part of their work to this purpose.

In the first years of the development of perfusion chromatography, Afeyan et al. [2] published a set of useful and simple expressions for describing linear velocity, mass transport, efficiency, and loading capacity in perfusion supports. They supposed that the driven force for perfusive flow-rate through the particle was the pressure gradient across the column, which imposed a pressure difference across each particle. As a consequence, flow-rate through a permeable particle could be modelized using the same equations used to determine the flow-rate through the column, although the exact flow-rate through a particle in a packed column was influenced by the pressure distribution around the particle which also depended on the neighbouring particles. They did a first approximation treating particles as a group of small packed particles (microspheres) in a way that channels were the interstices formed between microspheres. They also supposed that backpressure through the column was mainly due to the interstitial flow-rate around the particle. According to this, they obtained the following expression for the flow through the particle:

$$u_{\text{pore}} = \frac{K_{\text{p}}\, d_{\text{m}}^2 \left(\frac{\mathrm{d}p}{\mathrm{d}x}\right)_c (1 - \epsilon_{\text{b}})}{\eta\, \epsilon_{\text{p}}} \tag{1}$$

where K_{p} is the particle permeability, d_{m} the microsphere diameter ($d_{\text{m}} = r d_{\text{pore}}$), d_{pore} the throughpore diameter, r a constant related to the closeness of the packing (tortuosity), $(\mathrm{d}p/\mathrm{d}x)_c$ the pressure gradient across the column, ϵ_{b} the bed porosity or packed column void fraction (intraparticle volume divided by bed volume), η the mobile phase viscosity, and ϵ_{p} the intraparticle porosity or particle void fraction (pore volume divided by particle volume). The relationship between the flow through the particle and the superficial velocity through the column (u_{bed}) was given by:

$$\frac{u_{\text{pore}}}{u_{\text{bed}}} = \frac{K_{\text{p}}}{K} \cdot \left(\frac{d_{\text{m}}}{d_{\text{p}}}\right)^2 \cdot \frac{(1 - \epsilon_{\text{b}})}{\epsilon_{\text{p}}} \tag{2}$$

where K is the column permeability and d_{p} the particle diameter.

In order to adapt the equations ruling mass

transport in conventional chromatography to perfusive supports, Afeyan and co-workers deduced an expression for the effective diffusivity in the inner of the pore of a perfusion particle (D') starting from the diffusion coefficient of the solute in the pore for conventional chromatography (D) and from a factor which took into account the convection within the pore

$$D' = D + \frac{u_{pore}\, d_p}{2} \quad (3)$$

According to this equation, the diffusivity in perfusion chromatography depended on the pore velocity (u_{pore}) which, in turn, was related to superficial velocity of the mobile phase into the column (u_{bed}). Thus, at low flow-rates, mass transport in the perfusive particle was similar to that given in a conventional particle support, and $D' = D$; however, at very high flow-rates, $D' \approx u_{pore}\ d_p/2$. At these rapid velocities, in the classical Van Deemter equation, the predominant term is that corresponding to the bandspreading due to mass transfer. As a consequence, a new expression for the plate height in perfusive supports was given:

$$H \approx c' \cdot \frac{2\, d_p}{\kappa} = constant \quad (4)$$

where κ is a constant relating pore velocity to superficial velocity ($u_{pore} = \kappa\ u_{bed}$). From this expression, they observed that efficiency in the perfusion regime was independent of the superficial velocity. Furthermore, this equation also showed how the plate height only depended on the particle diameter in the perfusion regime, while in conventional supports, it depended on the squared particle diameter [2,5].

Fig. 2 compares the reduced plate height of insulin in reversed-phase columns as a function of the flow-rate for two different perfusion columns (presenting different particle sizes) and for a 300 Å (10 μm) conventional HPLC support. From this plot, the following features can be noted [2]

(i) Perfusive supports present much higher efficiencies at high mobile phase velocities than conventional supports.

(ii) Reduced plate height is only weakly dependent on the flow-rate in perfusive supports.

Finally, Afeyan et al. [2] obtained a new expres-

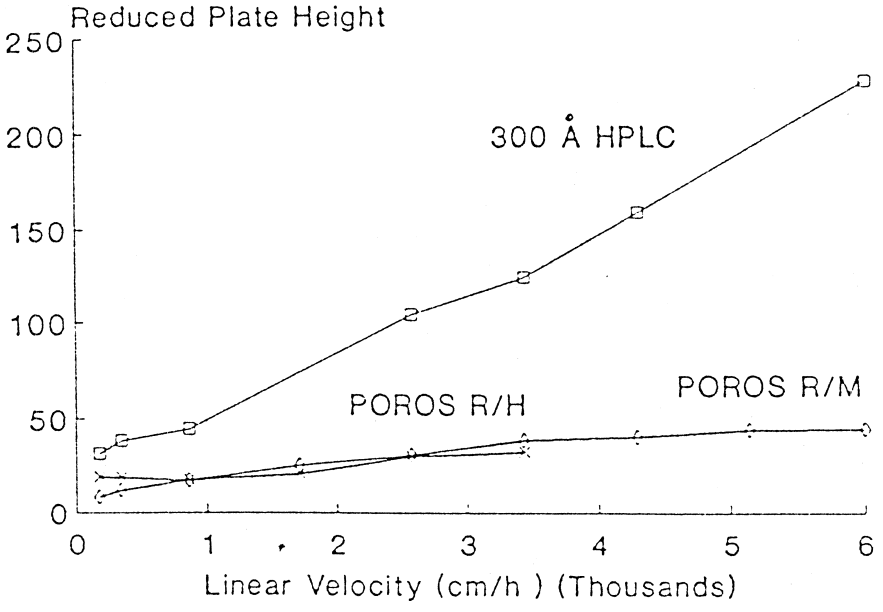

Fig. 2. Comparison of the reduced plate height of insulin in reversed-phase columns as a function of the flow-rate in two perfusive supports (Poros R/M, 30×2.1 mm I.D.; d_p, 20 μm; Poros R/H, 30×2.1 mm I.D.; d_p, 10 μm) and in a conventional 300 Å (30×2.1 mm; d_p, 10 μm) support. (From Ref. [2] with permission).

sion for the loading capacity (M) in perfusion media from the expression describing loading capacity in conventional chromatography and taking into account that mass transfer became independent of superficial velocity in the perfusion regime:

$$M = \frac{30\,(1-\epsilon_b)\,L\,\kappa}{d_p} = constant \tag{5}$$

where L is the column length.

In other way, Rodrigues research team dedicated their works to the theoretical study of large-pore materials such as catalysts and ceramic membranes [27,36]. They developed a mathematical model to explain the increase of the effective diffusivity with flow-rate occurring in these materials, concluding that this fact was due to intraparticle forced convection. As a consequence, they introduced the concept of ''diffusivity augmented by convection''. Later, they spread their work to others large-pore materials such as perfusion supports, modifying the mathematical model previously developed for its application to these supports. The mathematical model was based on the following two assumptions: (i) at the particle level, mass transport occurs by intraparticle diffusion and convection, and (ii) slab geometry for the support was considered to facilitate calculations.

These studies led to an expression for calculating the ''diffusivity augmented by convection'' (D') in these supports [36]:

$$D' \approx D \cdot \frac{1}{f(\lambda)} \tag{6}$$

where $f(\lambda)$ was:

$$f(\lambda) = \frac{3}{\lambda} \cdot \left(\frac{1}{\tanh \lambda} - \frac{1}{\lambda} \right) \tag{7}$$

being λ the Peclet number defined for spherical particles as:

$$\lambda = \frac{u_{pore}\, d_p}{3\,D} \tag{8}$$

which is a parameter relating intraparticle convective flow and diffusive flow.

Comparing Eq. (6) with Eq. (3) corresponding to the expression for D' given by Afeyan et al. [2], it could be observed in both cases, that at low flow-rates ($\lambda \ll 1$), $D' = D$ and that at high flow-rates (high λ), D' depended on the pore velocity (which in turn depended on the superficial velocity). Nevertheless, although at high flow-rates Rodrigues expression for D' ($\approx u_{pore}\, d_p/9$) was similar to that of Afeyan ($\approx u_{pore}\, d_p/2$), there were some differences probably due to the fact that Afeyan et al. [2] based on the assumption that the concentration term governing diffusion and convection could be treated as approximately equal [37].

As a consequence of the ''diffusivity augmented by convection'' (D'), the Van Deemter equation was modified inserting a term which showed the contribution of intraparticle convection at high bed superficial velocities (v) [38]:

$$H \approx 2\,d_p + \frac{3}{5} \cdot \frac{v}{(1+v)^2} \cdot \frac{\epsilon_p\, K}{\epsilon_b\, K_p}\, d_p \tag{9}$$

where

$$v = \frac{1-\epsilon_b}{\epsilon_b}\,\epsilon_p \tag{10}$$

This study for perfusive supports supposes, again, an important deviation from the classic Van Deemter's line. Analogous to Eq. (4) of Afeyan et al. [2] when increasing the superficial velocity (i.e., intraparticle convection), H decreased relative to the classic Van Deemter, tending to a plateau and being independent of this superficial velocity [27,38]. The kinetics of adsorption–desorption of proteins and the bidisperse nature of the particles were also taken into account by the Rodrigues research team [39,40]. This model was applied to elucidate the mass transfer mechanisms for different proteins (bovine serum albumin, myoglobin, and ovalbumin) in ion-exchange perfusive particles combining theory with experimental work [41–43]. In these works, Rodrigues [9] followed this methodology:

(i) Performing elution chromatography experiments of proteins under unretained conditions which enabled the understanding of mass transport mechanisms inside particles in the absence of extra effects related to protein adsorption. This step was performed by the calculation of H as a function of flow-rate. This study was complemented with particle characterization and direct methods to measure convective flow inside flowthrough particles.

(ii) Measurement of adsorption equilibrium isotherms by batch equilibration or from dynamic experiments using frontal chromatography to obtain

basic information on adsorbent capacity. In frontal chromatography experiments, a solution of protein with concentration C_o was continuously passed through the column under retained conditions. The plot of the concentration of the bed outlet as a function of time was the breakthrough curve, from which it was possible to measure the dynamic capacity of the adsorbent.

(iii) Finally, modeling/simulation of HPLC columns and model validation allowed to design chromatographic columns under conditions different from those at laboratory scale.

Liapis and co-workers developed other mathematical model to explain the dynamic of the process of adsorption of solute molecules in columns having adsorption perfusion particles [44,45]. First of all, they defined ''perfusion chromatography'' to refer to any chromatographic system in which the intraparticle velocity was non-zero. The model expressions for the adsorbent particles included the intraparticle mass transfer mechanisms of convection and diffusion, as well as the mass transfer step involving the interaction between the adsorbate molecules and the active sites on the surface of the porous adsorbent particles. This model was solved and used to study the dynamic behavior of single- [44] and multicomponent [45] adsorption systems for purely diffusion and perfusion chromatographic columns with different particle sizes, column lengths, column superficial velocities, and different values of the effective and total number of active sites per volume of adsorbent.

Next, tries for a better description of the dynamic behaviour of adsorption in perfusion particles by Liapis research team led to consider perfusion adsorbent particles as bidisperse porous structures having a macroporous region (through-pores) in which intraparticle convection and diffusion occurred, and a microporous region, made by spherical microparticles that were taken to be purely diffusive [46]. This mathematical model [47–51] was used to describe single- and multi-component systems in chromatographic columns packed with spherical perfusive or spherical purely diffusive adsorbent particles having a bidisperse porous structure. The model expressions for the bidisperse porous adsorbent particles now included the intraparticle mass transfer mechanisms of convection and diffusion in the macroporous region and the mass transfer step involving the interaction between the adsorbate molecules and the active sites on the surface of the macropores and micropores, which had been taken into account since the beginning, in addition to the intraparticle mass transfer mechanism of diffusion in the microporous region. Nevertheless, although this macroscopic model could successfully describe the dynamic behaviour of column systems, the parameters of this model characterizing intraparticle mass transfer mechanism could not be determined by direct measurements, but by matching dynamic experimental data from an adsorption system of interest with the predictions of the macroscopic model [52–54]. This is because this model groups the pore structure characteristics of the pore size distribution and pore connectivity into the empirical tortuosity factor in the equation describing mass transfer in perfusive particles. Therefore, one does not have an understanding of the relationship between the pore structure characteristics and intraparticle fluid flow and pore diffusion in these porous particles [55–57]. In this respect, Meyers and Liapis [55] constructed a model which presented the characteristics of porous particles (pore connectivity) as a function of the superficial velocity to determine the intraparticle velocity in porous chromatographic supports.

Another study carried out to model solute retention was that of Staby and Mollerup [58] which measured the solute retention data on semipreparative hydrophobic interaction perfusion chromatographic matrices to model solute retention of proteins in this kind of columns.

5. Experimental conditions used in perfusion chromatography

Table 3 shows the experimental conditions used for the analysis of biomolecules by perfusion chromatography.

5.1. Perfusion reversed-phase high-performance liquid chromatography

When using perfusion reversed-phase high-performance liquid chromatography (perfusion RP-HPLC), biomolecules such as proteins, peptides, and nucleotides are normally eluted by employing linear

Table 3
Analysis of biomolecules by perfusion chromatography[a]

Mode	Column	Flow-rate (ml/min)	Analysis time (min)	Elution. Mobile phases	Applications	Ref.
Reversed-phase	Poros R/M	5	0.2	Gradient: 20–50% B in 24 s. Mobile phases: (A) water–0.1% TFA; (B) ACN–0.1% TFA.	Very rapid separation of a mixture of five standard proteins (ribonuclease A, cytochrome c, lysozyme, ovoalbumin, and β-lactoglobulin).	[59]
Reversed-phase	Poros R2/H	1	<10	Gradient: 0–20% B in 8 min; 20–50% B in 1 min; 50% B for 1 min. Mobile phases: (A) 2 m*M* tetrabutylammonium hydrogensulfate 10 m*M* dipotassium hydrogen phosphate in water (pH 7.4); (B) 2 m*M* tetrabutylammonium hydrogensulfate in ACN–water (80:20).	Rapid separation and quantitation of dinucleotides.	[60]
Reversed-phase	Poros II R/M Poros R/H	5	5	Gradient: 18–28% B in 5 min. Mobile phases: (A) 0.1% TFA in water; (B) ACN.	Purification and analysis of a biological active peptide	[61]
Reversed-phase	Poros 20R2	1.5	15	Gradient: 0–15% B in 15 min. Mobile phases: (A) 10 m*M* sodium phosphate (pH 11.5); (B) 10 mM sodium phosphate–35% ACN (pH 11.5).	Development of a chromatographic method for the separation of a phosphorylated peptide from its nonphosphorylated form.	[62]
Reversed-phase	Poros R/H	4	3	Gradient: 0–4% B in 0.3 min; 4–10% B in 0.3 min; 10–15% B in 2.4 min. Mobile phases: (A) 20 m*M* disodium tetraborate in water; (B) 100% tetrahydrofuran.	Determination of chloroquine and desethylchloroquine in biological samples with fluorescence detection.	[63]
Reversed-phase	Capillary column packed with Poros II R/H media	0.045	10	Gradient: 0–30% B in 10 min. Mobile phases: (A) water–0.05% TFA; (B) ACN–water (90:10) with 0.05% TFA.	Evaluation of packed capillary perfusion columns for their ability to rapidly separate and sequence enzyme digests with detection by mass spectrometry using electrospray ionization.	[64]
Reversed-phase	Microbore column packed with Poros II media	0.025–0.125	4–11	Gradient: 20–90% B in different analysis times. Mobile phases: (A) water–0.1% TFA; (B) ACN–0.1% TFA.	Rapid separation and molecular mass determination of a mixture of standard proteins (myoglobin, cytochrome c and ubiquitin) with electrospray ionization mass spectrometry.	[65]
Reversed-phase	Poros R2/M	10	<1	Gradient: 15–30% B for 0.8 min. Mobile phases: (A) 0.1% HCl; (B) ACN.	Development of a fast assay to measure product purity and concentration of an *E. Coli* lystate.	[66]

Table 3. Continued

Mode	Column	Flow-rate (ml/min)	Analysis time (min)	Elution. Mobile phases	Applications	Ref.
Ion-exchange	Poros S/M Poros HS/M	5	5	Gradient: 0–40% B in 5 min. Mobile phases: (A) 20 m*M* Tris–HCl (pH 8.5); (B) 20 m*M* Tris–HCl with 0.5 *M* NaCl.	Purification and analysis of a biological active peptide.	[61]
Ion-exchange	Poros 20 HS	8.5	4	Loading solvent: buffer (pH 5). Eluting solvent: 500 m*M* NaCl.	Purification of an *E. Coli* lysate.	[66]
Ion-exchange	Poros Q	1	10	Gradient: 4 m*M* $LiClO_4$ for 2 min; 90 m*M* $LiClO_4$ for 2 min; 125 m*M* $LiClO_4$ for 1 min; 200 m*M* $LiClO_4$ for 3 min; 300 m*M* $LiClO_4$ for 2 min. Eluting solvent: 20 m*M* Bis-Tris (pH 6.0), 50 m*M* dodecyl maltoside (2.5%), 1% Triton X-100, and 4 m*M* $LiClO_4$.	Very rapid isolation of photosynthetic complexes from bacterial and chloroplast membranes.	[67]
Ion-exchange	Poros 50 HQ	6	6	Gradient: 5–200 m*M* of magnesium sulfate in 6 min.	Improvement of the chromatographic isolation procedure for a pigment–protein complex (photosystem 1).	[68]
Ion-exchange	Poros HQ/M	1.5	15	Gradient: 0–20 *M* NaCl in 20 m*M* Bis-Tris-propane (pH 7) in 15 min.	Purification of an exotoxin called leukotoxin, which is toxic for leukocytes from ruminant species.	[69]
Ion-exchange	Poros HQ	10–15		Gradient: 225–1500 n*M* NaCl in 25 m*M* Bis-Tris-propane/Tris–borate, pH 7.5	Purification of a chloroplast enzyme.	[70]
Ion-exchange	Poros HS/M	8.5	6	Gradient: 0–350 m*M* NaCl. Mobile phases: (A) phosphate buffer at different pHs; (B) 3 *M* NaCl.	Rapid purification of a murine antibody from ascites fluid.	[71]
Ion-exchange	Poros HQ/M	10	6	Gradient: 200–350 m*M* NaCl and pH from 8 to 6. Mobile phases: (A) Tris buffer (pH 6); (B) Bis-Tris buffer (pH 9).	Rapid purification of a murine antibody from ascites fluid.	[71]
Ion-exchange	Poros 10 HQ Poros HQ/M	1–4	5–17	Gradient: 50–500 m*M* NaCl (constant buffer concentration of 10 m*M* Tris-HCl, pH 5.5) in 16.6 ml. Binding eluent: 100 m*M* Tris–HCl (pH 7).	Coupling the advantages of displacement chromatography with the high-rate efficiency of perfusive chromatographic supports applied to the scale-up separation of β-LG (A) and β-LG (B).	[72]
Hydrophobic interaction	Poros PE/M	8.5	3.9	Gradient: 1.5–0 *M* ammonium sulfate (pH 5) in 3.9 min.	Purification of an *E. Coli* lysate.	[66]

Table 3. Continued

Mode	Column	Flow-rate (ml/min)	Analysis time (min)	Elution. Mobile phases	Applications	Ref.
Hydrophobic interaction	Poros EP	1–10	2–16	Elution was performed under isocratic conditions using phosphate buffers at different pHs.	Rapid separation of enantiomers using BSA as immobilized stationary phase.	[73]
Affinity	Poros A	3	1.5	Loading solvent: phosphate buffer saline. Eluting solvent: 12 m*M* HCl.	Development of a fast assay to measure product purity and concentration of an *E. Coli* lysate.	[61]
Affinity	Poros A/M Poros G/M	2	<1	Loading solvent: 0.1 *M* phosphate (pH 7.5)–0.15 *M* NaCl buffer. Eluting solvent: 2% acetic acid with 0.3 *M* $MgCl_2$.	Separation of antibodies of biological interest in short analysis times.	[74]
Affinity	Poros A/M	3	3	Loading solvent: 20 m*M* Tris with 150 m*M* NaCl (pH 7.0). Eluting solvent: 12 m*M* HCl with 150 m*M* NaCl.	Rapid monitoring of the production of an antibody against fibronectin.	[75]
Affinity	Poros II A	2–2.6	3.5	Loading solvent: methanol–phosphate buffered saline (pH 7.4). Eluting solvent: citrate buffer (pH 2.5).	Development of a rapid fluorescence flow injection immunoassay using testosterone as a model analyte.	[76]
Affinity	Poros 50 OH	0.6	<1	Loading solution: 0.1 *M* $NaHCO_3$; 0.5 *M* NaCl (pH 9). Eluting solvent: 50 m*M* Tris, 100 m*M* NaCl (pH 8).	Development of a non-competitive immunoenzymatic assay of cocaine.	[77]
Affinity	Poros A	5	3	Eluting solvent: 0.1% (v/v) 12 m*M* HCl–0.15 *M* NaCl.	To develop a new chromatographic method to isolate, purificate and, quantitate the major gene involved in breast and ovarian cancers.	[78]

[a] TFA: Trifluoroacetic acid; ACN: Acetonitrile; Tris: Tris(hydroxymethyl)aminomethane; Bis-Tris: Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Bis-Tris-propane: 2-bis(hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol; BSA: Bovine serum albumin

binary gradients. Mobile phases used to be constituted mainly by water (mobile phase A) and an organic solvent (mobile phase B) which commonly is acetonitrile (ACN). In some cases, working at a determinate pH is very important and in these cases, mobile phase A usually is a buffer solution [60,62]. When perfusion RP-HPLC was applied to the separation of proteins or peptides, mobile phases usually included trifluoroacetic acid (TFA) that worked as ion-pair agent, provided a low pH, and presented protein denaturing properties. Flow-rates used varied from 1 ml/min [60] to 10 ml/min [66] when using analytical columns and from 0.025 to 0.125 ml/min in microbore and capillary columns [64,65]. Analysis times were between 0.2 and 15 min depending on which was the aim of the work to be performed: a rapid separation, a large-scale purification, etc. Of great interest is the separation of a mixture of five-proteins (ribonuclease A, cytochrome *c*, lysozyme, ovoalbumin, and β-lactoglobulin) performed by Fulton et al. [59] in only 12 s (Fig. 3).

Monitoring of perfusion RP-HPLC analysis was

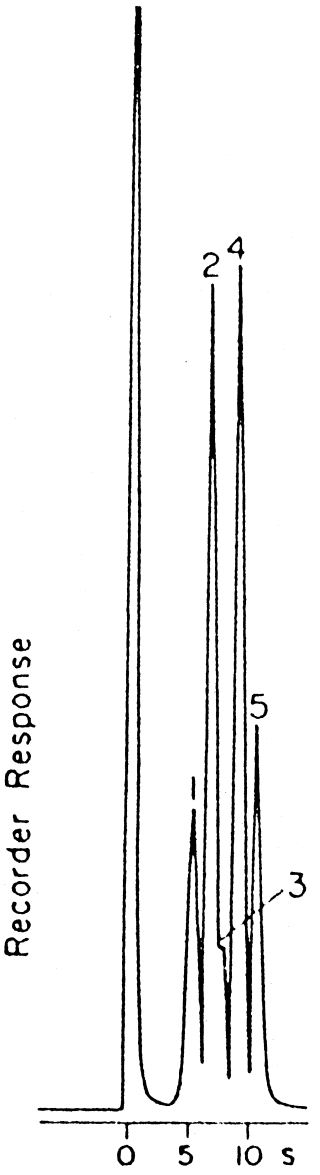

Fig. 3. High speed separation of a mixture of five proteins. Experimental conditions: Column, Poros R/M (30×2.1 mm I.D.; d_p, 20 μm); temperature, 25°C; flow-rate, 5 ml/min; UV detection, 220 nm; gradient: 20–50% B in 24 s. Mobile phases: (A) 0.1% TFA in water; (B) 0.1% TFA in ACN. Peak identification: 1, ribonuclease A; 2, cytochrome *c*; 3, lysozyme; 4, β-lactoglobulin; 5, ovoalbumin. (From Ref. [59] with permission).

achieved by ultraviolet (UV) and fluorescence detection. It is also important to highlight the coupling of electrospray ionization mass spectrometry (ESI-MS) with perfusion chromatography [64,65]. Solvents usually used for the separation of proteins (such as trifluoroacetic acid) inhibit the electrospray process and flow-rates used are higher than the optimum flow-rate of 1 μl/min required when using ESI-MS. At this respect, Kassel et al. [64] and Banks [65] explored the performance from the coupling of perfusion capillary and microbore columns with ESI-MS for the analysis of proteins with success.

Perfusion RP-HPLC has enabled the separation of biomolecules than could not be separated by conventional chromatography such as in the case of Matsumoto et al. [62] who separated phosphopeptides from their nonphosphorylated forms by working at alkaline pH and Augustijns [63] who developed a method that allowed the direct determination of chloroquine and desethylchloroquine in biological samples by working at high pH conditions.

5.2. Perfusion ion-exchange high-performance liquid-chromatography

Perfusion ion-exchange high-performance liquid chromatography (perfusion HPIEC) has also been applied to the separation of biomolecules by using linear binary gradients. Mobile phase B or eluting solvent used to be or contain a salt which normally was sodium chloride, while mobile phase A usually was a buffer solution. Separations were achieved at flow-rates ranging from 1 [67] to 15 ml/min [70] and, consequently, analysis times varied from 4 to 17 min. Detection was performed in all cases by using an UV detector.

Perfusion HPIEC has been used to perform large-scale isolations of photosynthetic complexes from bacterial and chloroplast membranes [67] and a pigment–protein complex [68] and for large-scale purifications of bioactive peptides [61], recombinant protein A from crude *E. Coli* lysate [66], an exotoxin [69], enzymes [70], and a monoclonal antibody from ascites fluid [71]. Gerstner et al. [72] enhanced mass transport in displacement chromatography by using perfusion HPIEC, which enabled displacement separations to be dramatically scale-up with respect to flow-rate.

5.3. Perfusion hydrophobic-interaction high-performance liquid chromatography

The number of examples of separations carried out with perfusion hydrophobic-interaction high-performance liquid chromatography (perfusion HPHIC) is significantly lower than the observed by other perfusion chromatographic modes. In this case, both gradient and isocratic elutions were employed and mobile phases consisted of buffer solutions. It is important to highlight the work of Hofstetter et al. [73] who used perfusion HPHIC to accelerate the separation of enantiomers (amino acid derivatives and drugs) by immobilizing bovine serum albumin on the chromatographic support which usually had been performed with silica supports.

5.4. Perfusion affinity high-performance liquid chromatography

Perfusion affinity chromatography has been used to separate proteins, antibodies, cocaine, and genes. Loading solvents normally were buffer solutions at approximately neutral pH and solvents used to elute biomolecules retained were acids (such as chloride and acetic acid) or buffer solutions at acid pH. Flow-rates usually varied from 0.6 [77] to 3 ml/min [61,75] and analysis times always were less than 3.5 min. Biomolecules were monitored by UV or fluorescence detection.

The use of perfusion affinity chromatography has constituted an important alternative to the classical time-consuming and labour-intensive immunochemical techniques. This saving process time translates in a higher reproducibility and in a saving biological activity [79]. All these facts have promoted the use of this chromatographic mode with perfusive supports. Rapid monitoring of antibodies [74,75] and fast determination of testosterone [76], cocaine [77], and a gene involved in breast and ovarian cancers [78] were some uses of perfusion affinity chromatography.

6. Applications of perfusion chromatography to the analysis of food proteins

First applications of perfusion chromatography were focussed to the separation of mixtures of standard biomolecules in surprisingly short analysis times. The capability to carry out runs in short analysis times, affects the development of chromatographic methods, being possible to study the influence of many variables that could not previously be explored in depth within normal time constraint [6]. Thus, since the answer can be obtained more quickly, the number of samples processed per unit time can also be increased. This fact is very important for routine analysis, e.g. food analysis.

Accurate assessment of composition is a prerequisite feature of the commercial and dietary evaluation of foods and food ingredients. Analysis of food is traditionally based on classical methods for its major constituents: water, protein, fat, ash, and carbohydrates. Since products have become more sophisticated, the assessment of food composition is becoming a more complex analytical task. Thus, the need for reliable, accurate, and reproducible information has become increasingly evident. On the other hand, obtaining relevant information at sufficient speed and low cost is another important problem in food research and food production, e.g. in on-line process control.

Determination of the protein content of foodstuffs has traditionally been carried out by Kjeldahl analysis which is based on the determination of the total content in protein by means of the determination of the nitrogen content. This tedious method does not allow to specify what kind of protein and what amount of a specific protein is present in a foodstuff. Chromatographic and immunochemical methods have been developed for these purposes. Nevertheless, analysis times constitute an important drawback, especially when these methods are applied to food industry.

Perfusion chromatography constitutes a promising technique for the analysis of food proteins in short analysis times. In fact, reducing analysis times has been the main goal when applying perfusion chromatography for the analysis of food proteins. Some examples of the application of perfusion chromatography for the analysis of food proteins are grouped in Table 4.

Rapid, easy, and accurate analysis of whey proteins are currently a matter of major interest for the dairy industry in the quality control of milk and

Table 4
Analysis of food proteins by perfusion chromatography[a]

Mode	Column	Flow-rate (ml/min)	Analysis time (min)	Elution. Mobile phases	Applications	Ref.
Reversed-phase	Poros 1 10R packed column	3	1.5	Gradient: 25–35% B in 1.5 min. Mobile phases: (A) ACN–water–formic acid (5:75:20, v/v/v); (B) ACN–water (93:7, v/v).	Rapid separation of bovine, ovine, and caprine whey proteins.	[80]
Reversed-phase	Poros R2/H	3	3	Gradient: 5–25% B in 1.7 min; 25–45% B in 1.3 min. Mobile phases: (A) 0.1% TFA in water; (B) 0.1% TFA in ACN.	Optimization and validation of a method for the quantitation of soybean proteins in commercial soybean products from soybean protein isolate.	[81]
Reversed-phase	Poros R2/H	3	3	Gradient: 5–25% B in 1.7 min; 25–45% B in 1.3 min. Mobile phases: (A) 0.1% TFA in water; (B) 0.1% TFA in ACN.	Validation of a method for the quantitation of soybean proteins in commercial soybean products directly prepared from whole soybeans.	[82]
Reversed-phase	Poros R2/H	3	3	Gradient: 5–25% B in 1.7 min; 25–45% B in 1.3 min. Mobile phases: (A) 0.1% TFA in water; (B) 0.1% TFA in ACN.	Characterization of commercial soybean products on the basis of their protein profile.	[83]
Reversed-phase	Poros R2/H	3	5	Gradient: 5–25% B in 1.7 min; 25–34% B in 0.3 min; 34–41% B in 3 min. Mobile phases: A, 0.1% TFA in water; B, 0.1% TFA in ACN.	Optimization of a method for the rapid separation of soybean and bovine whey proteins. Application to the detection of adulterations of whey proteins in commercial soybean milks.	[84]
Ion-exchange	Poros 20HQ	5	8	Gradient: 0–0.35 *M* NaCl in 6 min.	Separation of bovine whey proteins.	[85]
Ion-exchange	Poros PI	1	35	Gradient: 0.005–2 *M* NaCl in 50 m*M* Tris–HCl pH 7.4 and 0.02% Triton X-100.	Isolation of early light-inducible proteins from pea at preparative scale.	[86]

[a] ACN: Acetonitrile; TFA: Trifluoroacetic acid; Tris: Tris(hydroxymethyl)aminomethane.

related products. Conventional chromatographic methods for the determination of whey proteins entail long analysis times and strong interactions between proteins and stationary phases normally used. An additional limitation is the incomplete separation of the two main genetic variants (A and B) of bovine β-lactoglobulin. At this respect, Torre et al. [80] developed a perfusion RP-HPLC method for the rapid separation (about 1.5 min at a flow-rate of 4 ml/min) of bovine, ovine, and caprine whey proteins (Fig. 4). The optimized method allowed the separation of these proteins with resolution around 1.0 for the two variants of β-lactoglobulin. This method was applied to the detection of milk mixtures from different animal species being suitable to check fraudulent additions of milks from different species in the manufacture of dairy products. Perfusion HPIEC has also been used for the rapid separation of whey proteins [85]. In this work, Girardet et al. compared four rapid techniques (membrane convective chromatography, perfusion chromatography, continuous bed chromatography, and capillary electrophoresis) for the separation of these proteins. Analysis times obtained in all cases were similar and close to 8 min (Fig. 4). This analysis time obtained in the separation of bovine whey proteins by perfusion HPIEC (at a flow-rate of 5 ml/min) was much larger than the obtained by perfusion RP-HPLC by

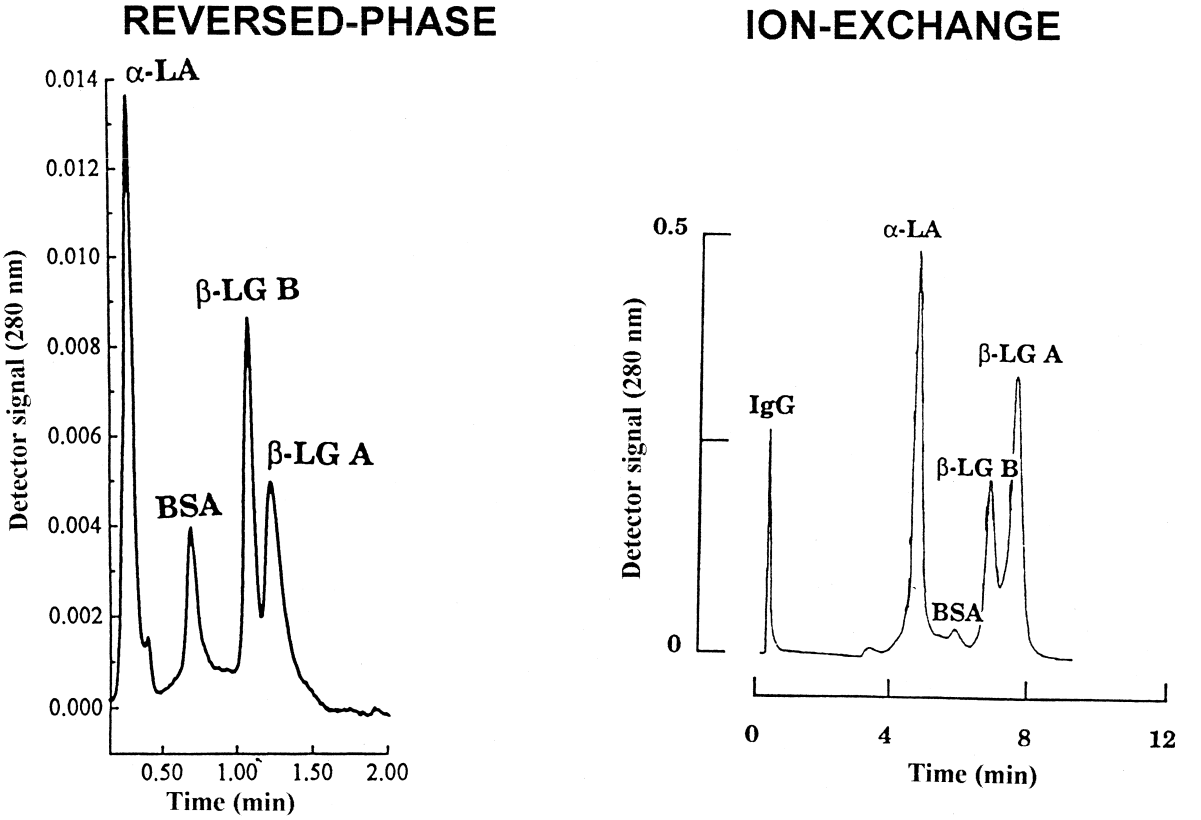

Fig. 4. Separations of bovine whey proteins by perfusion HPLC. Experimental conditions: Reversed-phase: Column, Poros1 10R support packed in a 50×2.1 mm I.D. column (d_p, 10 μm); temperature, 50°C; flow-rate, 4 ml/min; UV detection, 280 nm; gradient: 25–35% B in 1.12 min. Mobile phases: (A) ACN–water–formic acid (5:75:20, v/v/v); (B) ACN–water (93:7, v/v). Ion-exchange: Column, Poros 20HQ (10×1 cm I.D.; d_p, 20 μm); temperature, 25°C; flow-rate, 5 ml/min; UV detection, 280 nm; gradient: 0–0.035 *M* NaCl in 6 min. Peak identification: α-LA, α-lactoalbumin; BSA, bovine seroalbumin; β-LG B, β-lactoglobulin B; β-LG A, β-lactoglobulin A. (From Refs. [80] and [85] with permission).

Torre et al. [80] in which case separation took place in less than 1.5 min at a flow-rate of 4 ml/min. By other way, the order of appearance of whey proteins by using both techniques was the same: α-lactalbumin, bovine seroalbumin, β-lactoglobulin B and, finally, β-lactoglobulin A.

Soybean proteins have also been analyzed by perfusion chromatography. Soybean is an important source of good quality vegetable proteins whose use as foodstuff or as ingredient in other foodstuffs is significantly increasing. This fact has promoted the need of developing methodologies allowing the analysis of these proteins. García et al. [81] optimized a perfusion RP-HPLC method consisting of a linear binary gradient acetonitrile–water–0.1% trifluoroacetic acid for the analysis of soybean proteins in commercial soybean products. By using this method, soybean proteins from soybean protein isolate (SPI) were separated in 8 peaks in less than 3 min (at a flow-rate of 3 ml/min), which constituted an analysis time short enough to be valid in routine analysis. After validation, this optimized method was considered adequate to achieve the rapid quantitation of soybean proteins in real samples. Therefore, the method was applied to the determination of soybean proteins in commercial products derived from soybean such as soybean dairy-like products (milks and infant formulas), soybean flours, and textured soybean. With this purpose, the standard used was the SPI. From these studies it was observed that products

prepared from SPI could be quantitated by the optimized method, but when this method was applied to the quantitation of soybean proteins in products directly prepared from whole soybeans, results obtained were very different from those expected. Indeed, for these products directly prepared from whole soybeans, SPI did not seem to be a suitable standard for the determination of the protein content. For this reason the authors studied the possibility of using soybean flour as standard for the determination of the protein content in products prepared from whole soybeans [82]. Soybean flour enabled a better estimation of the protein content in these products than the obtained when using SPI as standard [82]. This optimized method was also applied to the characterization of commercial soybean products on the basis of their chromatographic profiles and results were compared with those obtained by conventional RP-HPLC with a polymeric stationary phase [83]. Perfusion chromatography enabled a further and faster characterization of commercial soybean products than conventional chromatography. In fact, perfusion chromatography allowed the discrimination among different kinds of soybean products (infant formulas, liquid milks, powdered milks, soybean flour, textured soybean, and SPI) and, within every kind of product, between those prepared from SPI and those directly prepared from whole soybeans [83].

García et al. [84] also developed a perfusion method for the rapid detection of potential adulterations resulting from the addition of bovine whey proteins to soybean dairy-like products which are an alternative for people allergic to these animal whey proteins. This method was applied to the determination of bovine whey proteins in a commercial soybean milk in whose formulation the manufacturer had added these proteins. Fig. 5 shows the chromatogram corresponding to the whey obtained by acidic precipitation from a solution of this powdered soybean milk by perfusion RP-HPLC. In Fig. 5 appears the chromatogram obtained with the same solution of the soybean milk by using a conventional column [87]. Analysis time yielded by perfusion chromatog-

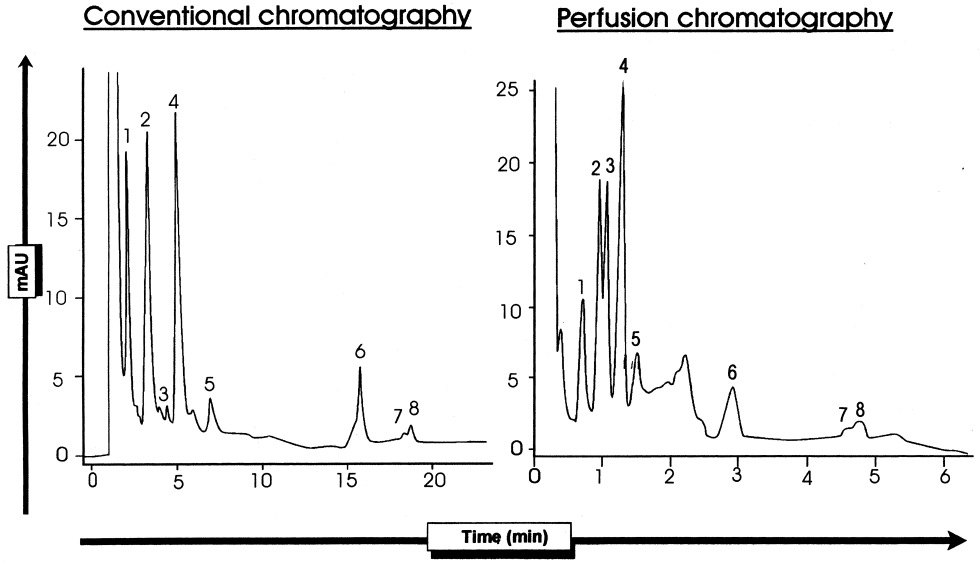

Fig. 5. Chromatograms corresponding to the whey obtained by acidic precipitation from a solution of powdered soybean milk (approximately 50 mg/ml) by conventional and perfusion RP-HPLC. Experimental conditions: Conventional RP-HPLC: Column, PLRP-S (150×4.6 mm I.D.; d_p, 8 μm); temperature, 50°C; flow-rate, 1 ml/min; UV detection, 254 nm; gradient: 20% B for 1 min, 20–42% B in 15 min, 42–46% B in 4 min, and 46–100% B in 0.5 min. Perfusion RP-HPLC: Column, Poros R2/H (50×4.6 mm I.D.; d_p, 10 μm); temperature, 60°C; flow-rate, 3 ml/min; UV detection, 254 nm; gradient: 5–25% B in 1.7 min, 25–34% B in 0.3 min, and 34–41% B in 3 min. Mobile phases: (A) 0.1% TFA in water; (B) 0.1% TFA in ACN. Peak identification: 1–5, soybean proteins; 6, α-lactoglobulin; 7–8, β-lactoglobulin (A+B) (From Refs. [84] and [87] with permission).

raphy was four times shorter than that obtained by conventional chromatography. Furthermore, with perfusion chromatography it was possible to reach lower detection limits for bovine whey proteins than those obtained by means of conventional chromatography with a polymeric stationary phase.

Finally, Adamska et al. [86] used perfusion chromatography to isolate early light-inducible proteins (ELIPs) from pea at preparative scale for investigating whether pigments were bound to these proteins. Although analysis time obtained was surprisingly high in comparison with other perfusion chromatographic methods (35 min), this was the first time that native ELIPs from higher plants were isolated enabling biochemical and biophysical analysis of these proteins [86].

7. Conclusions

The bimodal pore distribution which present perfusion supports enables intraparticle convection, making possible the separation of biomolecules with high molecular weights in shorter analysis times than with conventional chromatographic supports without compromising resolution and loading capacity. This is due to liquid flows through the particles, convectively transporting solute to the active surfaces in the interior of the sorbent and enhancing the mass transport performance of these supports. The introduction of perfusion chromatography supposes an important reduction of separation times and an increase in productivity in both analytical and preparative chromatography. This fact has significant implications for analysis, on-line monitoring, method development, scale-up, etc. which make perfusion chromatography a potential and promising tool for the very rapid analysis of proteins, especially, in the quality control of foodstuffs.

Acknowledgements

The authors thank the European Comission and the Comision Interministerial de Ciencia y Tecnología (CICYT) (Spain) for project 2FD97-1300 and also the CICYT (Spain) for project PB98-0714. M.C.G. thanks the Universidad de Alcalá for project E005/2000.

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