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Mathematical correlations for predicting protein retention times in hydrophobic interaction chromatography ☆

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Abstract

This paper reports how a novel method for determining the surface hydrophobicity of a protein can be used to predict the dimensionless retention time (DRT) in hydrophobic interaction chromatography. The methodology has three steps. First, it is necessary to know the three-dimensional structure of the target protein. Then, it is necessary to calculate the surface hydrophobicity of the protein, considering that each amino acid has a relative contribution to the surface properties, using an appropriate equation. This will depend largely on the relative scale used to evaluate the hydrophobicity of each amino acid. Forty-two scales were investigated and two gave the best correlation, the Miyazawa–Jernigan and the Cowan–Whittaker scales. Finally, a ''quadratic model'' ($DRT = a + b\Phi_{surface} + c\Phi^2_{surface}$) is used to predict the retention time of the target protein. The methodology was able to correlate adequately the retention data for monomeric proteins not included in the generation of the model.

Keywords: Mathematical modelling; Hydrophobic interaction chromatography; Hydrophobicity; Retention time prediction; Proteins; Amino acids

1. Introduction

Hydrophobic interaction chromatography (HIC) is an important technique for protein purification; it exploits the hydrophobic nature of the solute (proteins). HIC is based on the reversible interaction between the hydrophobic patches on a protein and the hydrophobic surface of a chromatographic medium. HIC is widely used in the downstream processing of proteins as it provides an alternative basis for selectivity compared with ion-exchange and other modes of adsorption. Additionally, HIC is an ideal ''next step'' after precipitation with ammonium sulphate or elution in high salt during ion-exchange chromatography (IEC) [1]. The main variables of HIC are protein hydrophobicity, protein size [2], concentration and type of salt [3] and type of matrix [4].

There are more than 40 different scales that have been used to estimate the hydrophobicity of amino acids. There is no clear correlation or study today

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that can be used to relate any of the ''theoretical'' hydrophobicity determinations and the behavior of the proteins in a HIC column. In this paper we present a novel method for describing the surface hydrophobicity of a protein that can be used to predict the dimensionless retention time in HIC.

1.1. The retention mechanism of hydrophobic interaction chromatography

The retention mechanism of proteins in HIC has been widely studied, but none of the proposed theories has enjoyed general acceptance [5]. Melander and co-workers [6,7] proposed a thermodynamic model based on the cavity theory for the effects of neutral salt on retention in HIC. These investigators considered that the most important parameters for retention in HIC are the salt molality and the molal surface increment of the salt. Additionally, Staby and Mollerup [8] proposed a model for the solute retention behavior of a protein on HIC perfusion media, using both isocratic and gradient elution. In this case the capacity factor k' is a function of the protein activity coefficient in the mobile phase and the protein activity coefficient on the stationary phase, expressed by a simple non-linear term. Jennissen [9] suggested that the adsorption of a protein on a hydrophobic surface is a multistep reaction. Lin et al. [10] proposed a binding mechanism in HIC based on microcalorimetric studies. They considered five sequential subprocesses: (1) dehydration or deionization of the protein; (2) dehydration or deionization of the adsorbent; (3) hydrophobic interaction between proteins and hydrophobic adsorbents; (4) the structural rearrangement of the protein; (5) rearrangement of the excluded water in the bulk solution.

There are almost no studies on the relationship between retention of proteins in HIC and the physicochemical properties of proteins [such as molecular mass, surface hydrophobicity, isoelectric point (pI)]. If a quantitative relationship can be established, it will allow us to predict the retention of proteins with known properties in gradient elution. In this paper we evaluate the surface hydrophobicity of proteins to predict behavior in HIC.

2. Theory

2.1. Evaluation of the surface hydrophobicity of proteins

The surface hydrophobicity of proteins was evaluated by two methodologies proposed by Berggren et al. [11] to evaluate protein behavior in aqueous two-phase systems (ATPSs). The first assumes that each amino acid on the surface of a protein has a relative contribution to the surface properties, then:

$$\Phi_{\text{surface}} = \sum(\phi_{\text{aa}i} r_{\text{aa}i}) \tag{1}$$

where Φ_{surface} is the calculated value of the surface hydrophobicity for a given protein, i ($i = 1, \ldots, 20$) indicates the 20 different amino acids, and $\phi_{\text{aa}i}$ is the value of the hydrophobicity assigned to amino acid ''i''. $r_{\text{aa}i}$, the relative surface area exposed for each amino acid ''i'' on the surface $r_{\text{aa}i}$, is defined as:

$$r_{\text{aa}i} = \frac{s_{\text{aa}i}}{\sum s_{\text{aa}i}} \tag{2}$$

where $s_{\text{aa}i}$ is the total exposed area of the amino acid residue ''i'' in the protein and $\Sigma s_{\text{aa}i}$ is the total surface of the protein.

The second methodology considers residue accessibility on the protein surface [12]. The residue accessibility or degree of exposure (DE) is the total exposed area of each residue divided by the solvent-accessible surface area of the residue in a Gly–X_i–Gly peptide in extended conformation (where X_i represents the individual residue). Then:

$$\Phi_{\text{Residue accessibility}} = \sum(\phi_{\text{aa}i} n_{\text{aa}i}) \tag{3}$$

where $\Phi_{\text{residue accessibility}}$ is the calculated value of the hydrophobicity for a given protein considering the degree of exposure for each amino acid and $n_{\text{aa}i}$ is the equivalent number of amino acid residue ''i'' in the surface protein, defined as:

$$n_{\text{aa}i} = \frac{s_{\text{aa}i}}{s_{\text{aa}i(\text{Gly}-X_i-\text{Gly})}} \tag{4}$$

where $s_{aai(Gly-X_i-Gly)}$ or ASA_i is the solvent-accessible surface area of the residue in a Gly–X_i–Gly peptide in extended conformation, where X_i represents the individual amino acid [13].

Both methodologies were investigated for evaluating the surface hydrophobicity of a protein and its behavior in HIC.

2.2. Amino acid hydrophobicity

There are more than 40 hydrophobicity scales proposed for amino acids. For example, the Tanford scale [14], which is based on the amino acid free energy of transfer from ethanol to water; the Janin [15] and Rose et al. [16] scales, based on the amino acid's accessible surface; the Bigelow scale [17], based on the fraction of the number of different amino acids buried within proteins; the Meek [18] and Cowan–Whittaker [19] scales, based on the peptide retention times in reversed-phase HPLC (RP-HPLC), and others [21,24–46]. Therefore, it was necessary to study which scale is more useful as a design variable in HIC.

This paper describes how different amino acid hydrophobicity scales and knowledge of the three-dimensional structure of proteins can be used to determine a protein's surface hydrophobicity and thus its behavior in HIC.

3. Experimental

3.1. Materials

Eleven proteins of known three-dimensional structure were used: conalbumin (1OVT), cytochrome *c* (1HRC), ribonuclease A (1AFU), ovalbumin (1OVA), chymotrypsinogen A (2CHA), lysozyme chicken (2LYM), α-lactoalbumin (1A4V), myoglobin (1YMB), α-chymotrypsin (4CHA), and β-lactoglobulin (1CJ5) from Sigma (St. Louis, MO, USA). Thaumatin (1THV) was a gift from 4F Nutrition (Northallerton, UK). Water prepared from a Milli-Q water cleaning system (Millipore, Bedford, MA, USA) and analytical-reagent grade ammonium sulphate were used in the preparation of the eluent.

3.2. Equipment

The high-performance liquid chromatography system employed consisted of a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) equipped with a 500-μl injection loop. The chromatographic columns were 1 ml Phenyl-Sepharose Fast Flow (a gift from Amersham Pharmacia Biotech, Uppsala, Sweden). The experiments were performed at room temperature, using a flow-rate of 0.75 ml/min and 10 column volumes. Finally, retention times (t_R) were recorded.

3.3. Buffer

Elution was obtained by a decreasing gradient of ammonium sulphate. The initial eluent was 20 m*M* Bis–Tris pH 7.0 plus 2 *M* ammonium sulphate. The final eluent was 20 m*M* Bis–Tris pH 7.0 (Buffer A). The gradient used was 7.5% B/min. All buffers were filtered through 0.22 μm Millipore filters after preparation and degassed with helium for 10 min.

3.4. Sample preparation

Protein solutions were prepared to contain approximately 2.0 mg/ml dissolved in the initial eluent. All protein solutions were filtered through 0.22 μm Millipore filters.

3.5. Determination of hydrophobicity of proteins

The proteins were characterized based on their amino acid sequences. The program Graphical Representation and Analysis of Structural Properties (Grasp) [20] was used to visualize protein surfaces and to calculate the accessible surface area of single residues in a protein. The program takes as input a Protein Data Bank file (PDB, http://www.rcsb.org/pdb) and a probe radius value, the default value 1.4 Å representing a water molecule. Then, the hydrophobicity of each standard monomeric protein (Φ) was calculated using the superficial area of each

amino acid in the protein, and the different hydrophobicity scales for the amino acids.

3.6. Computation of the correlations and validation

The information obtained in the previous task, the hydrophobicity (Φ) and the dimensionless retention time (DRT) of each standard monomeric protein, was fitted. DRT was defined as:

$$\mathrm{DRT} = \frac{t_R - t_0}{t_f - t_0} \quad (5)$$

where t_R is the time corresponding to the peak of the chromatogram, t_0 is the time corresponding to the start of the salt gradient, and t_f is the time corresponding to the end of the salt gradient.

DRT is equal to 1 for an extremely hydrophobic protein (in this case the protein ankirin, a membrane protein, which showed the maximum surface hydrophobicity).

Computation of the correlations was performed using CurvExpert 1.3 Software, evaluating over 30 different mathematical models. Finally, the best amino acid hydrophobicity scale and the best method for evaluating the surface hydrophobicity to predict each protein retention time in HIC were determined.

4. Results and discussion

4.1. Classification of amino acid hydrophobicity scales

The amino acid hydrophobicity scales were classified into three groups based on the following principles: (a) direct scale based on amino acid properties such as Gibbs free energy of transfer, retention time in HPLC, polarity or aqueous two-phase partitioning coefficient; (b) indirect scales based on protein properties such as antigenic regions in proteins, accessible and buried surfaces in proteins or contact energy; and (c) mixed scales, scales based on direct and indirect scales.

All scales were classified: 59% were direct scales, 28% were indirect scales and 13% were mixed scales. The classifications are shown in Fig. 1.

4.2. Comparison of different amino acid hydrophobicity scales

In order to compare the amino acid scales, the amino acids were classified into four groups, a representative residue from each one being chosen: aspartic acid (Asp) as a charged amino acid, tryptophan (Trp) as an aromatic amino acid, isoleucine (Ile) as a long-chain aliphatic amino acid, and glycine (Gly) as a short-chain aliphatic amino acid. As can be seen in Fig. 2 the various scales differ in residue hydrophobicities. In most of them, they are ordered in a similar position: Ile and Trp, generally, present the highest level of hydrophobicity, Gly presents an intermediate level of hydrophobicity and the lowest level was that of Asp. Then, the amino acid hydrophobicities could be ranked as: long-chain aliphatic, aromatic > short-chain aliphatic > charged. This general order has been reported previously [3,4,19].

4.3. Computation of the correlations between dimensionless retention time (DRT) and protein hydrophobicity (Φ)

4.3.1. Selection scales and definition of protein hydrophobicity

Forty-two hydrophobicity scales were used for computing the protein hydrophobicities using the two methods described by Eqs. (1) and (3) to evaluate the surface hydrophobicity.

In addition, we defined a new methodology to evaluate the surface hydrophobicity based on Eq. (3). We defined a relative number for each amino acid "i" on the surface, m_{aai}, which is defined as:

$$m_{aai} = \frac{[s_{aai}/s_{aai(\mathrm{Gly}-X_i-\mathrm{Gly})}]}{\sum[s_{aai}/s_{aai(\mathrm{Gly}-X_i-\mathrm{Gly})}]} = \frac{n_{aai}}{\sum n_{aai}} \quad (6)$$

This will give a "relative" hydrophobicity value normalized according to the total protein surface, similar to Eq. (1).

Then, the surface hydrophobicity is defined as:

$$\Phi_{\text{relative number}} = \sum(\phi_{aai} m_{aai}) \quad (7)$$

The retention times of nine monomeric proteins (conalbumin, cytochrome *c*, ribonuclease A, chymo-

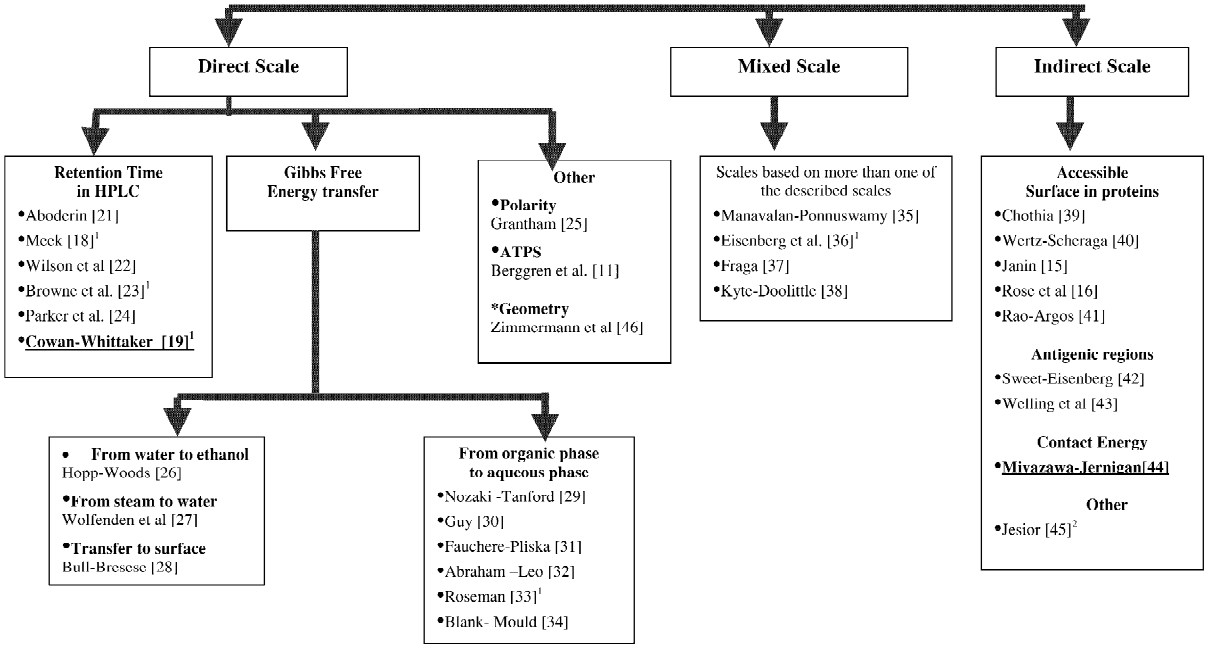

Fig. 1. Classification of amino acid hydrophobicity scales.

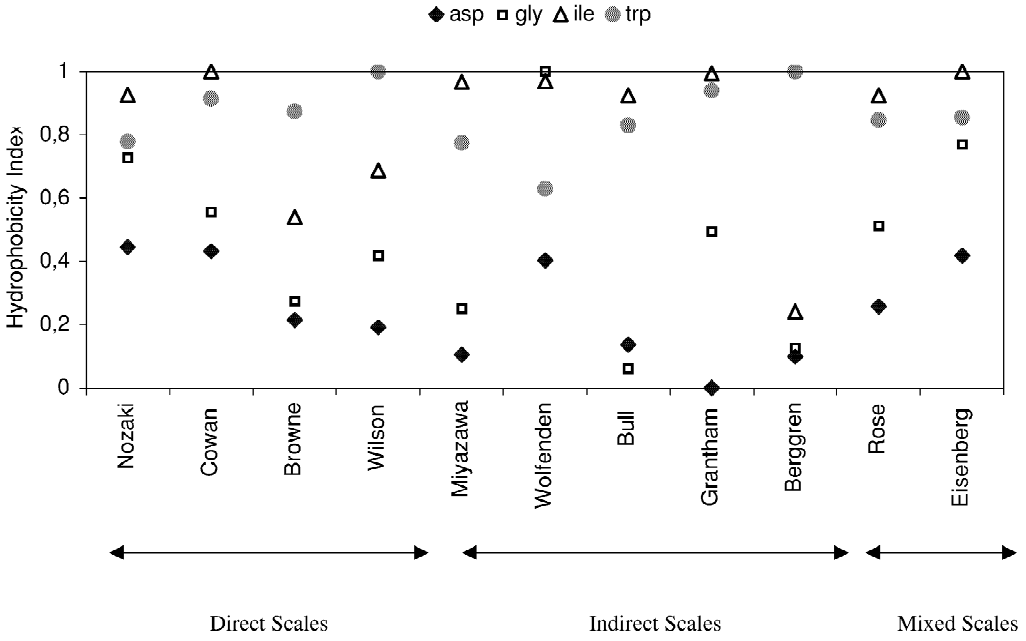

Fig. 2. Comparison of amino acid hydrophobicity using different hydrophobicity scales.

trypsinogen A, chicken lysozyme, α-lactoalbumin, myoglobin, α-chymotrypsin, thaumatin) were studied. Dimensionless retention times (DRT) and surface hydrophobicities of proteins (Φ) were correlated with a linear relation (DRT = $A\Phi + B$). The correlation coefficients (r^2) were, for Eq. (1), between 0.88 and 0.01, for Eq. (3), between 0.08 and 0.01, and for Eq. (7), between 0.87 and 0.01. Table 1 shows that the best average correlation was that obtained using Eq. (1), followed by that obtained from Eq. (7). Eq. (3) gave an extremely poor correlation coefficient ($r^2 < 0.08$).

The poor result obtained using Eq. (3) is analysed in Fig. 3, where we compare the surface hydrophobicity of 12 proteins using Eqs. (1) and (3). This same correlation [Eq. (3)] gave good results when normalized over the total possible hydrophobicity for each protein [Eq. (7)], as shown in Table 1.

In addition, Table 1 shows that the four best scales were the Miyazawa–Jernigan, Cowan–Whittaker, Brown and Wilson scales. These four scales were chosen to evaluate other mathematical models to fit the experimental data.

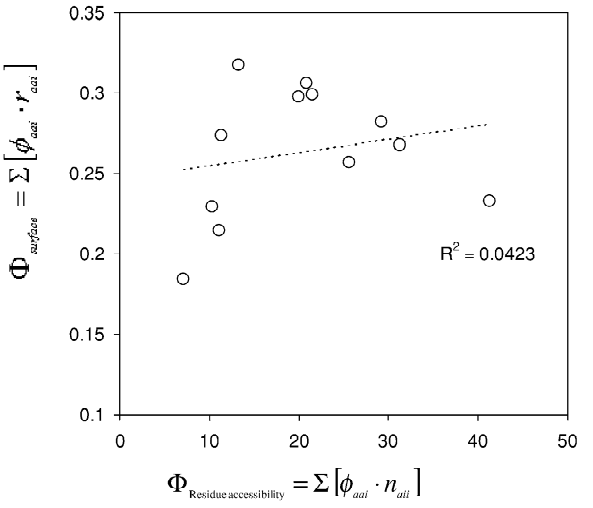

Fig. 3. Comparison of the surface hydrophobicity using Eqs. (1) and (3).

4.3.2. Best correlation

The four hydrophobicity scales (Miyazawa–Jernigan, Cowan–Whittaker, Brown and Wilson scales) were used to compute the protein hydrophobicities using the methodology given by Eq. (1). More than 30 different mathematical models were investigated, such as quadratic, polynomial, heat capacity and sinusoidal models.

The best correlations found are shown in Figs. 4

Table 1
Correlation coefficients (r^2) of the four best scales for predicting the dimensionless retention time (DRT) with a linear relation[a]

Scale	Methodology to calculate surface hydrophobicity of proteins		
	Surface[b] $\Phi = \Sigma(\phi_{aai} r_{aai})$	Residue accessibility[c] $\Phi = \Sigma(\phi_{aai} n_{aai})$	Relative number[d] $\Phi = \Sigma(\phi_{aai} m_{aai})$
Miyazawa–Jernigan [44]	0.88	0.083	0.87
Cowan–Whittaker [19]	0.88	0.038	0.88
Browne et al. [23]	0.83	0.058	0.78
Wilson et al. [22]	0.83	0.051	0.85
Average	0.855	0.058	0.845

[a] Linear relation: DRT = $A\Phi + B$.
[b] Eq. (1):

$$r_{aai} = \frac{s_{aai}}{\sum s_{aai}}.$$

[c] Eq. (3):

$$n_{aai} = \frac{s_{aai}}{s_{aai(\mathrm{Gly}-X_i-\mathrm{Gly})}}.$$

[d] Eq. (7):

$$m_{aai} = \frac{[s_{aai}/s_{aai(\mathrm{Gly}-X_i-\mathrm{Gly})}]}{\sum[s_{aai}/s_{aai(\mathrm{Gly}-X_i-\mathrm{Gly})}]} = \frac{n_{aai}}{\sum n_{aai}}.$$

and 5. They correspond to a quadratic model. An excellent correlation between the protein hydrophobicities, calculated based on the Cowan–Whittaker scale or the Miyazawa–Jernigan scale, and retention time of monomeric proteins was obtained. The Brown and Wilson scales also gave good correlations. The models were:

(i) Cowan–Whittaker scale

$$\mathrm{DRT} = -18.22\Phi^2_{\mathrm{Cowan}} + 22.07\Phi^2_{\mathrm{Cowan}} - 5.56, \quad 0 < \Phi_{\mathrm{Cowan}} < 1 \tag{8}$$

Model deviation 6.3%;

(ii) Miyazawa–Jernigan scale

$$\mathrm{DRT} = -12.14\Phi^2_{\mathrm{Miyazawa}} + 12.7\Phi_{\mathrm{Miyazawa}} - 1.74, \quad 0 < \Phi_{\mathrm{Miyazawa}} < 1 \tag{9}$$

Model deviation 7.3%.

Both scales, the Cowan–Whittaker and Miyazawa–Jernigan scales, have very good correlation coefficients (r^2) of 0.96 and 0.94, respectively. Therefore, we suggest that both scales can be used for predicting the protein dimensionless residence time in HIC. The Brown and Wilson scales had correlation coefficients of 0.89 and 0.92, respectively.

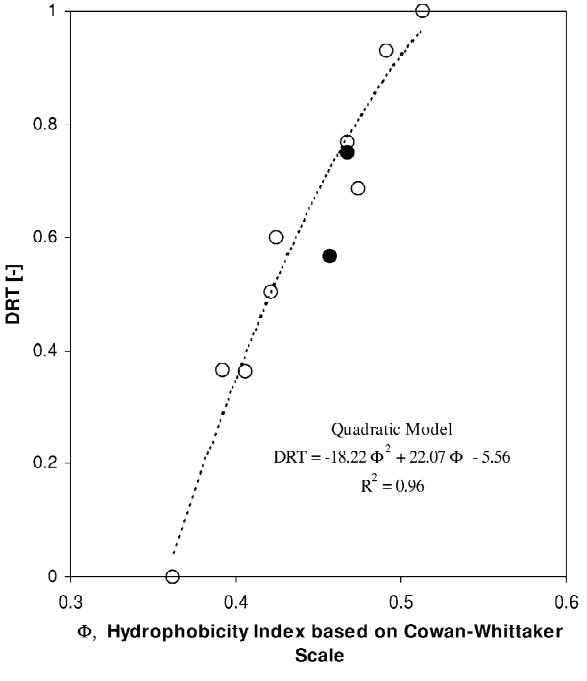

Fig. 4. Relation between dimensionless retention time (DRT) on Phenyl-Sepharose–2 *M* ammonium sulphate and the protein hydrophobicity (Φ) based on the Cowan–Whittaker hydrophobicity scale. (○) Experimental data, (———) model, (●) protein used for model validation.

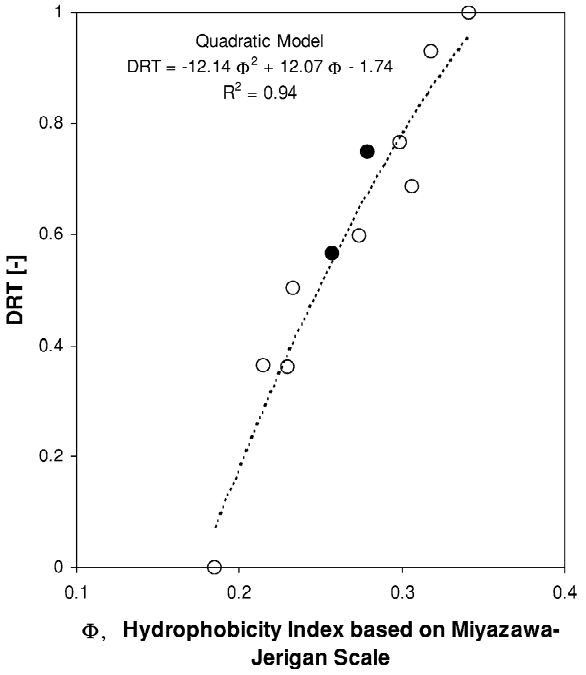

Fig. 5. Relation between dimensionless retention time (DRT) on Phenyl-Sepharose–2 *M* ammonium sulphate and the protein hydrophobicity (Φ) based on the Miyazawa–Jernigan hydrophobicity scale. (○) Experimental data, (———) model, (●) protein used for model validation.

The Cowan–Whittaker scale, shown in Table 2, is a direct scale that was obtained using hydrophobicity indices determined by the amino acid retention time in RP-HPLC at pH 3.0 [19].

The Miyazawa–Jernigan scale, shown in Table 2, is an indirect scale that corresponds to the ability of the amino acids to be hidden in the protein's core and it was obtained using the contact energy derived from three-dimensional protein data [44].

The surface hydrophobicity was evaluated using Eq. (1), which considers that each amino acid on the surface of a protein has a relative contribution to the surface properties.

Table 2
Best amino acid hydrophobicity scales used for predicting the dimensionless retention time in HIC

Amino acid	Scale			
	Cowan–Whittaker, pH 3.0		Miyazawa–Jernigen	
	Original [19]	Normalized	Original [44]	Normalized
Ala	0.42	0.660	5.33	0.391
Arg	−1.56	0.176	4.18	0.202
Asn	−1.03	0.306	3.71	0.125
Asp	−0.51	0.433	3.56	0.105
Cys	0.84	0.763	7.93	0.819
Gln	−0.96	0.323	3.87	0.151
Glu	−0.37	0.467	3.65	0.115
Gly	0.00	0.557	4.48	0.252
His	−2.28	0.000	5.10	0.354
Ile	1.81	1.000	8.83	0.967
Leu	1.80	0.998	8.47	0.908
Lys	−2.03	0.061	2.95	0.000
Met	1.18	0.846	8.95	0.987
Phe	1.74	0.983	9.03	1.000
Pro	0.86	0.768	3.87	0.151
Ser	−0.64	0.401	4.09	0.188
Thr	−0.26	0.494	4.49	0.253
Trp	1.46	0.914	7.66	0.775
Tyr	0.51	0.682	5.89	0.484
Val	1.34	0.885	7.63	0.770

4.3.3. Validation

To validate the previous correlations [Eqs. (8) and (9)], the retention times of two monomeric standard proteins (ovalbumin and α-amylase) were estimated. Figs. 4 and 5 show that the Cowan–Whittaker and Miyazawa–Jernigan correlations are valid for monomeric proteins, with a global average error of less than 10% (see Table 3). Thus, we consider that the correlations are adequate for the monomeric proteins tested. As shown in Table 3, the Miyazawa–Jernigan prediction (average error 5%) was better than the Cowan–Whittaker prediction (average error 15%) for the two proteins tested.

5. Conclusion

The methodology described in this paper is a first

Table 3
Comparison between estimated and experimental dimensionless retention time in Phenyl-Sepharose–2 *M* ammonium sulphate for monomeric proteins

Scale	Protein	DRT experimental	DRT[a] predicted	Error[b] (%)	Average error (%)	Global average error[c] (%)
Cowan[a]	α-Amylase	0.749	0.774	3.4	15.2	
	Ovalbumin	0.567	0.720	27.0		9.9
Miyazawa[d]	α-Amylase	0.749	0.681	9.2	4.7	
	Ovalbumin	0.567	0.566	0.09		

[a] $DRT = -18.22\Phi^2_{Cowan} + 22.07\Phi_{Cowan} - 5.56$.
[b] $Error = |DRT_{experimental} - DRT_{predicted}| \cdot 100/DRT_{experimental}$.
[c] Total average error: average error considering both proteins and both scales.
[d] $DRT = -12.14\Phi^2_{Miyazawa} + 12.7\Phi_{Miyazawa} - 1.74$.

approach for predicting monomeric protein retention times in HIC based on the surface properties of the proteins and the hydrophobicities of their individual amino acids.

The methodology has three steps. First, it is necessary to know the three-dimensional structure of the target protein. Then, it is necessary to calculate the surface hydrophobicity of the protein, considering that each amino acid has a relative contribution to the surface properties, using an appropriate equation. Of the more than 40 amino acid hydrophobicity scales investigated, the Miyazawa–Jernigan and the Cowan–Whittaker scales gave the best results. Finally, a ''quadratic model'' ($DRT = a + b\Phi_{surface} + c\Phi^2_{surface}$) was used to predict the retention time of the target protein with good accuracy ($r^2 = 0.94$–0.96).

This methodology was able to correlate adequately the retention data for two monomeric proteins not included in the generation of the model (deviation 5–15%).

We propose, in future work, to examine the utility of this methodology for a larger number of monomeric proteins under different operational conditions and for the separation of mixtures of monomeric proteins.

Acknowledgements

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References

[1] A.A. Shukla, K.M. Sunasara, R.G. Rupp, S.M. Cramer, Biotechnol. Bioeng. 68 (2000) 672.
[2] J.L. Fausnaugh, L.A. Kennedy, F.E. Regnier, J. Chromatogr. 317 (1984) 141.
[3] G. Sofer, L. Hagel (Eds.), Handbook of Process Chromatography: A Guide to Optimization, Scale-up, and Validation, Academic Press, San Diego, CA, 1998, p. 284.
[4] K.-O. Erikkson, in: J.-C. Janson, L. Ryden (Eds.), Protein Purification: Principles, High-resolution Methods, and Applications, 2nd ed., Wiley–Liss, New York, 1998, p. 283.
[5] J.A. Queiroz, C.T. Tomaz, J.M.S. Cabral, J. Biotechnol. 87 (2001) 143.
[6] W. Melander, Cs. Horváth, Biochem. Biophys. 183 (1977) 200.
[7] W. Melander, D. Corradini, C. Horváth, J. Chromatogr. 317 (1984) 67.
[8] A. Staby, J. Mollerup, J. Chromatogr. A 734 (1996) 205.
[9] H.P. Jennissen, J. Colloid Interface Sci. 111 (1986) 570.
[10] F.Y. Lin, W.Y. Chen, R.-C. Ruaan, H.-M. Huang, J. Chromatogr. A 872 (2000) 37.
[11] K. Berggren, A. Wolf, J.A. Asenjo, B.A. Andrews, F. Tjerneld, Biochim. Biophys. Acta, Protein Struct. M 1596 (2002) 253.
[12] B. Lee, F.M. Richards, J. Mol. Biol. 55 (1971) 379.
[13] S. Miller, J. Janin, A.M. Lesk, C. Chothia, J. Mol. Biol. 196 (1987) 641.
[14] C. Tanford, J. Am. Chem. Soc. 84 (1962) 4240.
[15] J. Janin, Nature 277 (1979) 491.
[16] G.D. Rose, A.R. Geselowitz, G.J. Lesser, R.H. Lee, M.H. Zehfus, Science 229 (1985) 834.
[17] C.C. Bigelow, Theor. Biol. 16 (1967) 187.
[18] J.L. Meek, Proc. Natl. Acad. Sci USA 77 (1980) 1632.
[19] R. Cowan, R.G. Whittaker, Peptide Res. 3 (1990) 75.
[20] A. Nicholls, K. Sharp, B. Honig, Proteins: Struct., Funct. Genet. 11 (1991) 281.
[21] A.A. Aboderin, Int. J. Biochem. 2 (1971) 537.
[22] K.J. Wilson, A. Honegger, R. Stotzel, G.J. Hughes, Biochem. J. 199 (1981) 31.
[23] C.A. Browne, H.P.J. Bennet, S. Solomon, Anal. Biochem. 124 (1982) 201.
[24] J.M. Parker, D. Gua, R.S. Hodges, Biochemistry 25 (1986) 5425.
[25] R. Grantham, Science 185 (1974) 862.
[26] T. Hopp, K. Woods, Proc. Natl. Acad. Sci. 78 (1981) 3824.
[27] R. Wolfenden, L. Andersson, P. Cullis, C. Southgate, Biochemistry 20 (1981) 849.
[28] H.B. Bull, K. Breese, Arch. Biochem. Biophys. 161 (1974) 665.
[29] Y. Nozaki, C. Tanford, J. Biol. Chem. 246 (1971) 2211.
[30] H.R. Guy, Biophys. J. 47 (1985) 61.
[31] J.L. Fauchere, V.E. Pliska, Eur. J. Med. Chem. 18 (1983) 369.
[32] D.J. Abraham, A.J. Leo, Proteins: Struct., Funct. Genet. 2 (1987) 130.
[33] M. Roseman, J. Mol. Biol. 200 (1988) 513.
[34] S.D. Black, D.R. Mould, Anal. Biochem. 193 (1991) 72.
[35] P. Manavalan, P.K. Ponnuswamy, Nature 275 (1978) 673.
[36] D. Eisenberg, E. Schwarz, M. Komarony, R. Wall, J. Mol. Biol. 179 (1984) 125.
[37] S. Fraga, Can. J. Chem. 60 (1982) 2606.
[38] J. Kyte, R. Doolittle, J. Mol. Biol. 157 (1982) 105.
[39] C. Chothia, J. Mol. Biol. 105 (1976) 1.
[40] D.H. Wertz, H.A. Scheraga, Macromolecules 11 (1978) 9.
[41] M.I.K. Rao, P. Argos, Biochim. Biophys. Acta 869 (1986) 197.
[42] R. Sweet, D. Eisenberg, J. Mol. Biol. 171 (1983) 479.
[43] G. Welling, W. Weijer, R. Zee, S. Welling, FEBS Lett. 188 (1985) 215.
[44] S. Miyazawa, R. Jernigan, Macromolecules 18 (1985) 534.
[45] J.-C. Jesior, J. Protein Chem. 19 (2000) 93.
[46] J.M. Zimmerman, N. Eliezer, R. Simha, J. Theor. Biol. 21 (1968) 170.