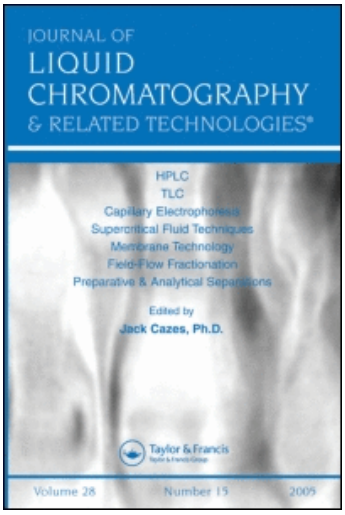

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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF EGG WHITE PROTEINS. OPTIMIZATION OF OVALBUMIN ELUTION

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ABSTRACT

This paper is an experimental contribution to the optimization of ovalbumin elution in RP-HPLC with a C4 support. The effects of TFA concentration, gradient duration, flow rate, and the nature of the solvent were studied using an experimental design. The results highlighted the major role of TFA concentration in the recovery yield of ovalbumin, with an optimum concentration range which is very narrow and much lower (0.025 %) than the levels usually applied (0.1 %). In this respect, ovalbumin seems to behave in a specific way. Among the four solvents tested, i.e. acetonitrile, isopropanol, ethanol, and a mixture of these three, the first one is definitely the most satisfactory in the area of recovery yield and efficiency. By modifying the elution conditions alone, the recovery yield of ovalbumin may range from 50 to 85 %.

Finally, this work has enabled us to define the chromatographic conditions for quantitative egg white analysis of its major proteins in only six minutes and with minimal subsequent ghosting effects.

INTRODUCTION

The increasing interest in the valorization of egg-products first found expression in the development of fractionation technologies. In this day and age, what is needed is rapid, precise, and reliable analyses methods. For egg white, this mainly concerns protein analysis. Several types of chromatography have been suggested with this objective in view.

Gel permeation chromatography has been used, but generally to prepare coarse protein fractions for subsequent analysis, or in association with other techniques. Young and Gardner[1] used this method to purify ovomucin. More recently, Awade et al.[2] have thus been able to obtain 6 major peaks and isolate ovomucin and lysozyme. But this chromatographic mode does not enable ovalbumin, ovotransferrin, and ovomucoïd to be separated; it is, however, noticeable because it is not denaturing compared with other chromatography methods, and it is the only one which currently allows for the analysis and quantification of ovomucin.

Cation exchange chromatography has been in use for a considerable time. Rhodes et al.[3] developed a method to eluate egg white proteins in 14 peaks at a high recovery rate, but with 11 successive buffers. Subsequently, this was adapted by Jolles et al.[4] and Fossum and Whitaker.[5] In the same way, anion exchange chromatography has been used by Mandeles,[6] Parkinson,[7] and Furka and Sebestyen.[8]

More recently, reversed phase chromatography, widely used for the analysis of peptides and proteins in general, has been applied to egg white analysis. This technique offers several advantages: samples are easy to prepare, separation power is high, even between different forms of a single protein, peaks are sharp, making integration, and hence, quantitative analysis easier, and it is possible to apply rapid gradients. Accordingly, Itoh et al.[9] developed a method in 48 sec, but it was only possible to distinguish 4 peaks. The real aim of these authors was, in fact, to increase the recovery yield of very hydrophobic proteins, and particularly ovalbumin, which is currently the major problem in the use of reversed phase liquid chromatography (RP-HPLC) for egg white analysis.

The question of the very high retention of ovalbumin was soon raised by Guerin and Brule[10] and Schafer et al.[11] The most satisfactory method is currently that proposed by Takeuchi et al.[12] It makes use of a relatively unhydrophobic stationary phase (C4) and wide pores (300 Å), leading to 17 peaks, 11 of which have been identified. However, these authors do not refer to the ovalbumin recovery rate, and ghost peaks may be observed with these analytical conditions. In addition to this, lysozyme is not visible.

In this work, our objective was to contribute to the development of qualitative and quantitative analytical methods for egg white proteins, using RP-HPLC, by maximizing, on one hand, ovalbumin recovery rate, and at the same time, by applying an analysis duration compatible with routine use. We chose to study the effect of 4 parameters: flow rate, concentration of ion-pairing acid, gradient slope, and solvent nature, using the statistical methodology of an experimental design.

EXPERIMENTAL

Analytical Chromatography

RP- HPLC was performed using a Thermo Separation Products chromatograph (Les Ulis, France), equipped with an inert (PEEK, poly ethyl ether ketone) pump for binary gradients, model P1000XR2/1-060, a manual Rheodyne injection valve, and an inert 20 µL injection loop, and a helium degasser system. Detection was carried out at 280 nm with a UV/visible absorbance detector, model UV 2000-126, coupled to an Spectranet SP 4500 interface; the chromatograms were processed with Winner on Windows software on a Compaq Prolinea 3-25 microcomputer.

The reversed-phase column used was a Vydac 214 TP (butyl group C4 on silica, 5 µm particle size, 300 Å pore size, 5 x 0.21 cm I.D.) (Touzart et Matignon, Vitry s/ Seine, France). The column temperature was maintained at 50°C with a Sup-RS Stabitherm column oven from Prolabo (Fontenay s/s Bois, France).

Reagents

Ovalbumin (98 % pure as determined by HPLC and SDS-PAGE) was purchased from Calbiochem (CA, USA). Ovomucoïd, ovoinhibitor, ovoglobulins, flavoprotein, avidin, lysozyme, cystatin, and ovotransferrin were obtained from Sigma (L'Isle d'Abeau Chesnes, France). These proteins were used as reference proteins for HPLC analysis.

Trifluoroacetic acid (TFA) (sequanal grade) was obtained from Pierce (IL, USA). HPLC-grade acetonitrile (ACN) was obtained from Carlo Erba (Nanterre, France). HPLC-grade absolute ethanol (EtOH) and propanol-2 (IsoP) were purchased from Prolabo (Fontenay s/s Bois, France). Water was purified by filtration using a Milli-Q system (Millipore, Molsheim, France).

Ovalbumin Solutions

A single ovalbumin mother solution (1 $g.L^{-1}$) was used for all the trials of experimental design. This solution was dispatched in 1 mL aliquots and frozen at -20°C until required for use. For every trial, an aliquot was thawed at room temperature, diluted with buffer A to obtain a solution at 0.5 $g.L^{-1}$, and stored at 5°C between each use. Beforehand, we verified that this amount of ovalbumin exceeded neither the column capacity nor the spectrophotometer capacity.

Egg White Preparation

Egg white was diluted with 19 volumes of buffer A (0.025 %-TFA) and filtered on a 0.45 µm membrane before injection.

Buffer Preparation

The composition of the buffer was different for every trial of the experimental design. TFA quantity and the nature of the organic solvent vary in buffer B. Buffer A was composed of water and X % TFA; buffer B was composed of water, X % TFA and 70 % (vol:vol) organic solvent. Elution was carried out by increasing buffer B from 10 to 100 %, for various durations. New buffers were prepared daily.

Trial Procedure

Every trial began with two blank gradient washes of the column to eliminate residual proteins. The sample (ovalbumin solution) was then injected three times onto the column; each injection was followed by two blank gradient washes. The same sample was then successively injected five times onto an inert capillary tube (PEEK).

Injection conditions for the column were different for each trial (see Results and Discussion). For injections onto the capillary tube, the flow-rate was set at 1 $mL.min^{-1}$, whatever the flow-rate of the corresponding injection on the column. We confirmed that peak area proportionally decreases with an increase in flow-rate. Comparisons between peak areas obtained with or without the column was thus possible.

For each trial, elution in the capillary tube was isocratic; the eluent buffer composition was chosen according to that observed when ovalbumin was eluted from the column.

Recovery Yield, Efficiency, and Retention Time Calculation

Recovery yield, efficiency and retention time were the three parameters we chose in order to characterize the optimization of the analysis regarding ovalbumin.

Recovery yield, R, was : $R = (a_a / A_a).100$ %, a_a denoting the peak area average of the three injections of ovalbumin solution in the column and A_a the peak area average of the five injections of the same ovalbumin solution in the capillary PEEK tube.

Efficiency was calculated using the ratio between the height and the area of the ovalbumin peak. Retention time was obtained directly from the chromatogram.

Statistical Treatment

The experiments were conducted through an experimental design strategy and treated according to a linear model described below (SPAD'N software).[13]

RESULTS AND DISCUSSION

Choice of Levels for Each Studied Factor

Preliminary trials highlighted that TFA concentration, between 0 and 0.3%, is an essential factor as regards ovalbumin retention time and recovery yield (Figure 1). To study the potential interactions between TFA concentration and other factors, we chose to work within the concentration range where recovery rate is maximum, i.e. between 0.01 and 0.025 % TFA.

Pearson and Regnier[14] and Chen and Horvath[15] showed that the recovery rate of proteins and resolution are both highly dependent on the residence time in the stationary phase. Short gradients tend to increase the recovery rate but a minimal time is necessary to separate the different proteins. We chose to study elution with gradients of 5, 12.5, and 20 min.

The most common organic solvent in RP-HPLC is acetonitrile because it allows for good resolution. However, for the most hydrophobic proteins, ethanol and isopropanol, either pure or with acetonitrile, are sometimes advised.[16] These 3 solvents and their resulting equi-volume blend were studied.

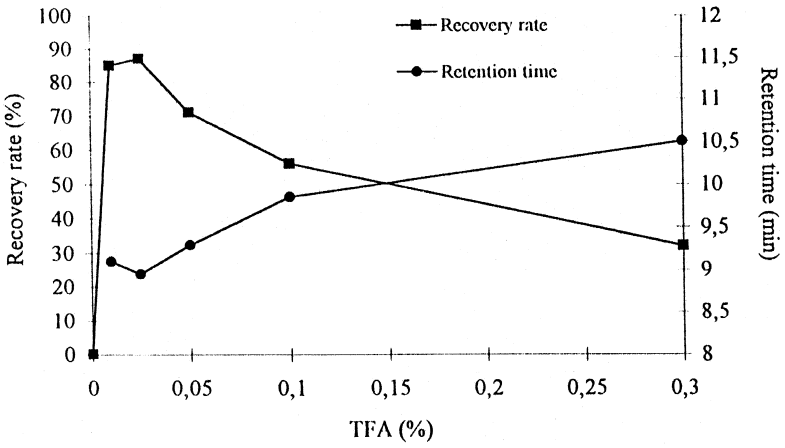

Figure 1. Recovery rate and retention time of ovalbumin vs. TFA concentration; elution with acetonitrile, gradient duration : 10 min from 10 to 100% buffer B, 1 $mL.min^{-1}$.

The other parameters were established. The spectrophotometer range was 0.1 AU (absorption unit). The quantity of ovalbumin injected was always the same since we observed, during preliminary trials, that this had no significant effect on the responses studied, within the range 12.5 - 50 µg. Temperature was maintained at 50°C, the level recommended for resolution and repeatability;[17] on the other hand, Nugent et al.[18] observed that average recovery increases as the column temperature increases from 20 to 60°C. For the column, we made our choice according to the current literature, which recommends short columns with wide pores and silica modified with a short alkyl chain.[12,14,15,19]

Strategy: Experimental Design

First, considering that for the mixture of solvents the three responses are very different from the means of the results of the pure solvents alone (e.g. for the mixture, mean efficiency is 22.15 whereas it is between 23.57 and 28.27 for the three other solvents), the mixture is considered as a fourth category of solvent.

In addition, it should be noted that the solvent mixture does not improve ovalbumin elution. It is even the least suitable solvent as regards efficiency. It therefore seems reasonable to look for an optimal solvent among pure substances.

Because we did not know the variation model of the studied factors, we chose a strategy which enabled us to test different models successively, beginning with the simplest. So, we chose a complete factorial design for the three quantitative factors (TFA concentration, gradient slope, and flow-rate), with two levels for each factor. This design is completed with two central points, necessary for introducing a quadratic term in the model and for testing the linearity of the model. The design was crossed with a standard mixture design (three pure solvents and their mixture in equal proportions) for the study of the solvent. The matrix of design points is presented in Table 1. The order of experiments was laid down by technical requirements; the first trial was repeated at the end of the experiment to test for any potential drift.

Statistical Analysis of the Results

For each response (recovery yield, efficiency, and retention time), a statistical analysis was carried out separately through an analysis of variance applied to the 40 trials of the experimental design (Table 1). Initially, we chose the most complete model, including the main effects, second order interactions and a term gathering all the quadratic effects of the quantitative factors taken together. The entire set of coefficients of the models, as well as standard deviations, are presented in Table 2. In the text, only the significant coefficients (in the sense of Student test applied to each coefficient at the level 0.01%) were taken into account in the models discussed.

Study of the Recovery Yield

The equations of the model, obtained by analysis of variance, are presented below in the usual presentation.[16] In these equations, the different factors (such (TFA), (Gradient)…) can be equal to +1, 0 or –1 according to the trial conducted (see Table1). This makes possible a direct comparison among the various coefficients.

With acetonitrile,

$$\text{Recovery Yield} = 80.7853 + 2.1113(\text{TFA}) - 1.8012(\text{Gradient}); \quad (1)$$

With ethanol,

$$\text{Recovery Yield} = 54.1273; \quad (2)$$

With isopropanol,

$$\text{Recovery Yield} = 51.6633; \quad (3)$$

Table 1

Matrix of Trials and Results of the Experimental Design*

		Matrix of Trials				Results		
	Trial Number	Factor 1 [TFA]	Factor 2 Flow Rate	Factor 3 Gradient Duration	Factor 4 Solvent Nature	Recovery Rate (%)	Efficiency (without Unity)	Retention Time (min)
1	(38)	-1	-1	-1	0	55.34	21.42	6.66
2	(35)	+1	-1	-1	0	55.49	36.86	6.50
3	(36)	-1	+1	-1	0	55.38	26.66	5.30
4	(37)	+1	+1	-1	0	56.57	42.98	5.21
5	(39)	-1	-1	+1	0	55.95	8.31	17.34
6	(34)	+1	-1	+1	0	56.34	16.87	17.00
7	(33)	-1	+1	+1	0	54.37	9.31	16.17
8	(40)	+1	+1	+1	0	56.57	14.81	15.91
9	(1)	0	0	0	0	55.64	16.96	11.16
10	(2)	0	0	0	0	57.66	17.72	11.10
11	(14)	-1	-1	-1	1	83.50	26.43	6.14
12	(11)	+1	-1	-1	1	83.38	47.57	6.04
13	(12)	-1	+1	-1	1	78.80	30.07	4.75
14	(13)	**+1**	**+1**	**-1**	**1**	**84.37**	**62.49**	**4.71**
15	(15)	-1	-1	+1	1	77.00	9.51	15.60
16	(10)	+1	-1	+1	1	78.97	20.01	15.16
17	(9)	-1	+1	+1	1	75.10	9.43	14.31
18	(16)	**+1**	**+1**	**+1**	**1**	**84.57**	**20.62**	**14.19**
19	(3)	0	0	0	1	81.22	22.01	9.91
20	(4)	0	0	0	1	83.06	22.84	9.94
21	(22)	-1	-1	-1	2	55.73	25.51	6.76
22	(19)	+1	-1	-1	2	52.30	32.01	6.67
23	(20)	-1	+1	-1	2	54.37	30.14	5.42
24	(21)	+1	+1	-1	2	55.68	44.78	5.40
25	(23)	-1	-1	+1	2	54.56	10.98	17.79
26	(18)	+1	-1	+1	2	53.31	16.31	17.47
27	(17)	-1	+1	+1	2	55.39	11.50	16.55
28	(24)	+1	+1	+1	2	55.87	17.29	16.57
29	(5)	0	0	0	2	53.09	20.85	11.39
30	(6)	0	0	0	2	53.09	21.34	11.42
31	(30)	-1	-1	-1	3	49.26	24.53	5.49
32	(27)	+1	-1	-1	3	48.69	44.96	5.22
33	(28)	-1	+1	-1	3	54.36	25.34	4.28
34	(29)	+1	+1	-1	3	55.29	52.08	4.08
35	(31)	-1	-1	+1	3	51.12	8.43	13.17
36	(26)	+1	-1	+1	3	51.72	22.68	12.46
37	(25)	-1	+1	+1	3	54.56	8.94	12.04
38	(32)	+1	+1	+1	3	52.92	20.09	11.40
39	(7)	0	0	0	3	51.60	25.56	8.10
40	(8)	0	0	0	3	49.23	24.96	8.11

Table 1 (continued)

Level	Factor 1	Factor 2	Factor 3	Solvent Nature
-1	0.01%	0.5 $mL.min^{-1}$	5 min	0:blend
0	0.0175%	0.75 $mL.min^{-1}$	12.5 min	1:acetonitrile
+1	0.25%	1 $mL.min^{-1}$	20 min	2:ethanol
				3:isopropanol

* Numbers in brackets indicate experimental order of trials.

Table 2

Coefficients Matrix of the Equations Derived by Variance Analysis[a]

Effect	Recovery Rate	Efficiency	Retention Time
Intercept	60.5738	21.5300	10.1412
ACN	20.2115**	2.8190**	-.2473**
	.450	.648	.023
EtOH	-6.4465**	-1.2080	1.2218**
	.450	.648	.023
Isop	-8.9105**	1.4780	-1.8872**
	.450	.648	.023
Blend	-4.8545**	-3.0890**	.9127**
	.450	.648	.023
TFA	.5391	7.0594**	-.1181
	.291	.418	.015
Flow rate	.6722	1.6919**	-.5994**
	.291	.418	.015
Gradient	-.3184	-10.8981**	4.8281**
duration	.291	.418	.015
$(\ )^2$	.2647	3.4363*	.2262**
	.650	.936	.034
TFA	.6803	.6750	.0338
x Flow	.291	.418	.015
TFA	.2247	-2.5425**	-.0575*
x Gradient	.291	.418	.015
Flow	-0.234	-1.7612	.0462
x Gradient	.291	.418	.015
TFA	1.5722*	2.3469*	.0306
x ACN	.504	.725	.026

(continued)

Table 2 (continued)

Coefficients Matrix of the Equations Derived by Variance Analysis[a]

Effect	Recovery Rate	Efficiency	Retention Time
TFA	-.9003	-3.0269**	.0669
x EtOH	.504	.725	.026
TFA	-.6241	2.0119	-.1094**
x Isop	.504	.725	.026
TFA	-.0478	-1.3319	.0119
x Blend	.504	.725	.026
Flow	-.6734	.6944	-.0231
x ACN	.504	.725	.026
Flow	.0041	.6706	.0056
x EtOH	.504	.725	.026
Flow	1.3703	-.9606	.0319
x Isop	.504	.725	.026
Flow	-.7009	-.4044	-.0144
x Blend	.504	.725	.026
Gradient	-1.4828*	-2.4756*	-.1256**
x ACN	.504	.725	.026
Gradient	.4497	1.3531	.6881**
x EtOH	.504	.725	.026
Gradient	.6584	.0519	-1.0781**
x Isop	.504	.725	.026
Gradient	.3747	1.0706	.5156**
x Blend	.504	.725	.026

[a] The standard deviation is indicated under each coefficient, as usual presentation.[16]

Student test significant at level 0.01* or 0.001**

$( )^2$ means $((TFA)^2 + (Gradient)^2 + (Flow)^2)$

With solvent mixture,

$$\text{Recovery Yield} = 55.7193. \quad (4)$$

Thus the most important factor is the nature of the solvent. To obtain a high recovery yield, the use of acetonitrile is preferable. It is noteworthy that the parameters of chromatography are essential only when using acetonitrile. In decreasing order of interest, as regards the solvents are: the mixture, followed by ethanol and, lastly, isopropanol. This order corresponds to the decreasing polarity of the 3 solvents, whereas one would expect a better recovery rate with

the least polar solvent, i.e. isopropanol. One suggestion concerns conformational changes of ovalbumin, produced by the solvent. Clark and Smith[20] highlighted that the secondary structure of most proteins, especially that of ovalbumin, is altered in the presence of alcohol-containing spreading solvents. These conformational changes are characterized by an increase in the helical fraction at the expense of the ß-sheet fraction of the protein structure and are considered as playing a role in the elution process itself in RP-HPLC.[21] The alcohol-induced reordered helical form possesses apolar aromatic side chains that are more exposed than in the native protein. Such residues might be expected to interact more closely with the hydrophobic ligate. Nugent et al.[18] mention that, during on-column denaturation of proteins, in the highly hydrophobic environment within the pores of the packing, the hydrophobic groups, initially concentrated within the interior of the protein molecule become free to rearrange themselves and to interact with corresponding groups on adjacent protein molecules, thus leading to aggregation and precipitation of the protein. Precipitated aggregates, highly insoluble and probably irreversibly bound to the column-packing surface, would be implicated in low protein recoveries in RP-HPLC.

In other respects, these authors suggest that the partial dissociation of these aggregates into more soluble compounds would explain ghosting effects often observed in RP-HPLC, and especially with ovalbumin. Lastly, it is also conceivable that these aggregates lead to some size retention, even without strong hydrophobic interactions.

Following these assumptions, we suggest the use of a solvent that is more polar than acetonitrile to limit protein denaturation and aggregation. The use of stationary phases with wider pores could likewise make the elution of ovalbumin easier. Another possible solution would be to treat the samples in order to leave proteins in their reordered conformation before injection onto the column. Such a treatment could consist of solubilization by the addition of detergents or chaotropic agents (urea), as suggested by Nugent et al.[18] However, in order to develop an analytical method for whole egg white, we would have to confirm that these different solutions have no deleterious effects on the elution of other proteins.

Finally, the highest recovery yield we obtained for ovalbumin is about 85 %, which is very close to that obtained by Itoh et al.[9] under optimal conditions (86 %). However, these authors used a very rapid gradient (48 sec) which did not allow for good separation of the various proteins during the whole egg white analysis.

If TFA concentration does not appear to be essential for recovery yield in these results, it is probably because we chose two levels close to the optimum. In reality, it is certainly the most important factor and we can distinguish two

regions where an increase in TFA concentration has opposite effects (Figure 1). First, TFA minimizes the interaction between cationic residues and the support by balancing the ionization of charged surface silanols; to be sure, in the absence of TFA, ovalbumin is totally retained on the column, and the use of organic solvent does not allow for any elution as interactions between the protein and the support are of an ionic type. Between 0 and 0.025 %, TFA also acts as a hydrophilic ion-pairing reagent, meaning that the hydrophobic group of TFA ($-CF_3$) interacts with the hydrophobic regions of the protein, thus conferring to ovalbumin a hydrophilic characteristic and, therefore, limiting the number of hydrophobic interactions with the support.

This phenomenon leads to the decrease in k' coefficient (capacity factor) described by Geng and Regnier[22] and consequently, the simultaneous decrease in retention time and the increase in recovery yield. On the other hand, above 0.025 % TFA, recovery yield of ovalbumin decreases and retention time increases, indicating that stronger interactions between the protein and the stationary phase appear. It is true that this phenomenon was not observed by these authors with any protein, including bovine serum albumin, but these authors used isopropanol, not acetonitrile.

Furthermore, within the experimental area studied, a "positive" interaction exists between TFA and the solvent; in the acetonitrile buffer, the highest recovery yield is obtained when the concentration of TFA is the highest.

The other two factors studied, namely gradient duration and flow rate, do not have any significant effect. Nevertheless, our observations are in accordance with documents consulted on the subject:[14,15,18] flow rate should be high and gradient duration should be short. This is generally explained by the conformational changes that have to be minimized by a residence time on the column as short as possible. However, we can observe that the theoretical recovery is always higher than 80 % with acetonitrile and 0.025 % TFA, whatever the flow rate and gradient duration.

Study of the Efficiency

As for recovery rate, the equations of the model are :

With acetonitrile,

$$\text{Efficiency} = 24.349 + 9.4063(\text{TFA}) + 1.6919(\text{Flow Rate}) - 13.3737(\text{Gradient}) + 3.4363([\text{TFA}]^2 + [\text{Flow Rate}]^2 + [\text{Gradient}]^2) - 2.5425\ (\text{TFA} \times \text{Gradient}); \quad (5)$$

With ethanol,

$$\text{Efficiency} = 21.53 + 4.0325(\text{TFA}) + 1.6919(\text{Flow Rate}) - 10.8981(\text{Gradient}) + 3.4363([\text{TFA}]^2 + [\text{Flow Rate}]^2 + [\text{Gradient}]^2) - 2.5425\ (\text{TFA x Gradient}); \quad (6)$$

With isopropanol,

$$\text{Efficiency} = 21.53 + 7.0594(\text{TFA}) + 1.6919(\text{Flow Rate}) - 10.8981(\text{Gradient}) + 3.4363([\text{TFA}]^2 + [\text{Flow Rate}]^2 + [\text{Gradient}]^2) - 2.5425\ (\text{TFA x Gradient}); \quad (7)$$

With solvent mixture,

$$\text{Efficiency} = 18.441 + 7.0594(\text{TFA}) + 1.6919(\text{Flow Rate}) - 10.8981(\text{Gradient}) + 3.4363([\text{TFA}]^2 + [\text{Flow Rate}]^2 + [\text{Gradient}]^2) - 2.5425\ (\text{TFA x Gradient}). \quad (8)$$

Gradient duration is thus the most important factor: the shorter the gradient duration, the better the efficiency, and this according to several authors.[15,18,23] This may arise from the possible existence of different protein conformers, reversed-phase being inherently denaturing. In the simplest case, it may be the native protein and the completely denatured molecule. Nonetheless, conformations of an intermediate structure are also possible. Protein molecules are thus bound in different forms, and each form requires a particular solvent concentration in order to be eluted. Then, the longer the gradient duration, the wider the gap between the first and the last eluted proteins. Similarly, a high flow rate is preferable.

TFA concentration is clearly a noteworthy factor, the highest ones being the most effective. The ion-pairing reagent produces effects in several ways. First, it minimizes the residual ionic interactions with the support and is said to stabilize protein conformation thereby limiting the number of possible conformational states.

The nature of the solvent is also important. Acetonitrile allows for the highest efficiency, a result earlier obtained by Nugent et al.[18] One explanation could be that the lower the viscosity, the easier and faster the penetration of the solvent into the pores and, in turn, the lower the time difference necessary to eluate proteins adsorbed on the surface of the support as well as those adsorbed in the pores.

Above all, this model exhibits a quadratic term and many interactions. First, the significant coefficient of the term ($[\text{TFA}]^2$ +$[\text{Flow Rate}]^2$+$[\text{Gradient Duration}]^2$) indicates that efficiency is not linearly dependent on these three

factors, and the positive sign of this coefficient indicates a minimum in the field of experimentation. As a first approximation, it is thus reasonable to think that the optimum is on the borderline of the experimental area. This observation therefore indicates that it is not possible to distinguish the three factors.

TFA also interacts with the other factors; on the one hand with solvents (acetonitrile buffer, TFA concentration should be high which is fortunately in accordance with the optimal for recovery rate), and on the other hand with the gradient duration (efficiency increases when TFA concentration increases and gradient duration decreases, which reinforces the choice of main effect levels).

Finally, gradient duration interacts with the solvent: with acetonitrile, short gradients lead to a higher efficiency. Once more, the optimal conditions concerning gradient duration are confirmed.

Study of the Retention Time

As previously indicated, the equations of the model are :

With acetonitrile,

$$\text{Retention Time} = 9.8939 - 0.1181(\text{TFA}) - 0.5994(\text{Flow Rate}) + 4.7025(\text{Gradient}) + 0.2262([\text{TFA}]^2+[\text{Flow Rate}]^2+[\text{Gradient}]^2) - 0.0575\ (\text{TFA x Gradient}) \quad (9)$$

With ethanol,

$$\text{Retention Time} = 11.363 - 0.1181(\text{TFA}) - 0.5994(\text{Flow Rate}) + 5.5162(\text{Gradient}) + 0.2262([\text{TFA}]^2+[\text{Flow Rate}]^2+[\text{Gradient}]^2) - 0.0575\ (\text{TFA x Gradient}) \quad (10)$$

With isopropanol,

$$\text{Retention Time} = 8.254 - 0.2275(\text{TFA}) - 0.5994(\text{Flow Rate}) + 3.75(\text{Gradient}) + 0.2262([\text{TFA}]^2+[\text{Flow Rate}]^2+[\text{Gradient}]^2) - 0.0575\ (\text{TFA x Gradient}) \quad (11)$$

With solvent mixture,

$$\text{Retention Time} = 11.0539 - 0.1181(\text{TFA}) - 0.5994(\text{Flow Rate}) + 5.3437(\text{Gradient}) + 0.2262([\text{TFA}]^2+[\text{Flow Rate}]^2+[\text{Gradient}]^2) - 0.0575\ (\text{TFA x Gradient}) \quad (12)$$

As expected, gradient duration is the most important factor for retention time. The longer the gradient , the longer the time required to reach the solvent concentration necessary for elution, and thus, the longer the retention time.

Solvents also play an important role in this context, retention time increasing from shortest to longest with use of isopropanol, then acetonitrile, then the solvent mixture, and lastly ethanol. This order does not match the polarity order of the solvents; it simply emphasizes that polarity value does not integrate the hydrophobic character. Polarity value accounts for the ionic interactions whereas, in the present case, the phenomenon involved is that of hydrophobic interactions, i.e., the specific organization between protein molecules and the stationary phase. Flow rate likewise influences retention time: the higher the rate, the shorter the retention time.

Again, there exists a quadratic term with many interactions. First of all, as for efficiency, one can see that retention time is not linearly dependent on the three factors studied (TFA concentration, gradient duration and flow rate), and in the experimental field, a minimum is clearly required.

TFA interacts with the solvent: as indicated earlier, with isopropanol, high TFA concentration increases retention time. This is a simple observation seeing that this solvent is not suitable because of the consequently low recovery rate. TFA also interacts with gradient duration, retention time increasing when TFA and gradient duration evolve in opposite directions.

Lastly, gradient duration interacts with the solvents. Concerning acetonitrile, the solvent chosen because of its good recovery yield and efficiency, the interaction is negative, i.e. with this solvent, low gradient duration leads to a lower retention time.

From these results the observation emerges that the optimal conditions for ovalbumin elution (recovery yield, high efficiency, and short retention time) are:

- for recovery yield: use of acetonitrile; for second order interactions, high concentration of TFA (0.025 %), and short gradient (5 min) are preferable;
- for efficiency: use of acetonitrile, with TFA 0.025 %, at 1 $mL.min^{-1}$, with a gradient duration of 5 min;
- for retention time: same conditions as above, with the exception of the solvent: isopropanol would be preferable.

Since the optimal conditions are very similar for the three parameters studied, only the nature of the solvent need be determined. Recovery yield is clearly of greater interest than retention time. Consequently, acetonitrile is the solvent of choice.

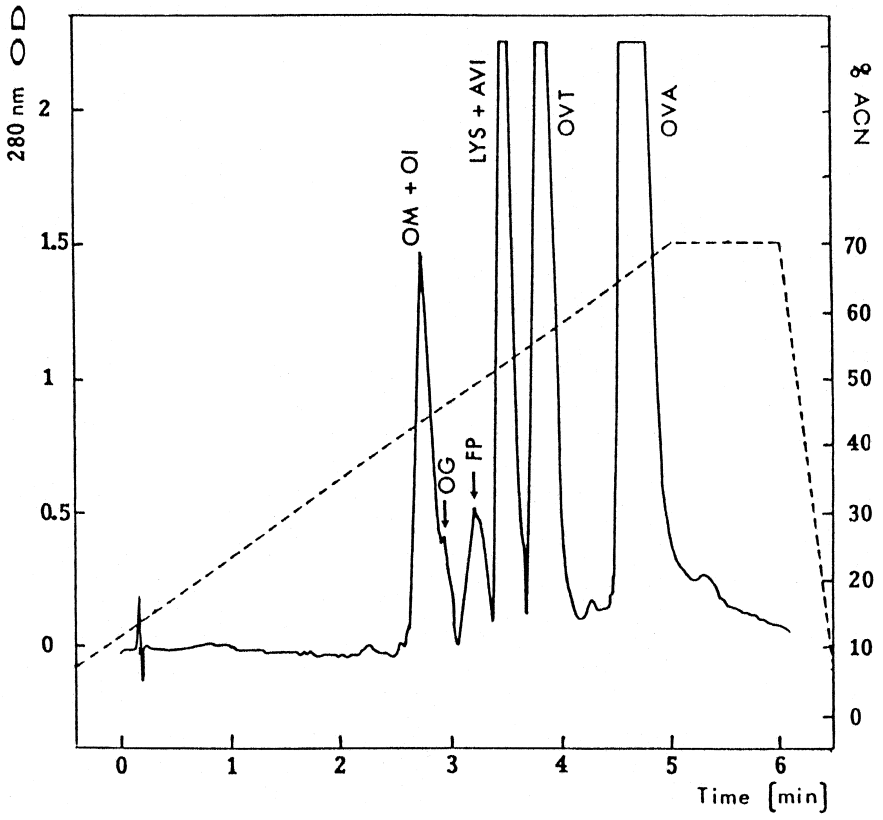

Figure 2. RP-HPLC chromatogram of egg white proteins. Elution with water-acetonitrile, 0.025% TFA, 1 mL.min^{-1}. Dashed lines indicate the concentration of acetonitrile in the phase. Peaks identified are as follows : ovomucoid (OM), ovoinhibitor (OI), ovoglobulins (OG), flavoprotein (FP), lysozyme (LYS), avidin (AVI), ovotransferrin (OT), ovalbumin (OVA).

With these previously defined conditions, one can calculate the optimal values of the three studied parameters (recovery yield, efficiency and retention time) by applying the three model equations:

equation (1) gives: recovery yield at the optimum = 84.70 % ± 2.5,
equation (5) gives: efficiency at the optimum = 61.67 ± 3.6,
equation (9) gives: retention time at the optimum = 5.21 min ± 0.13.

Standard deviations correspond to the estimation of expectation. These calculated values are very close to those experimentally obtained (trial no. 14).

Application to Egg White Analysis

The aim of this study was to suggest a method for egg white protein analysis which is quick, easy to apply, quantitative, and which minimizes the problem of low recovery of ovalbumin encountered by many authors. By applying to egg white the conditions defined in the first part of this study, we succeeded in meeting these different demands (Figure 2).

Indeed, the quantitative analysis of major egg white proteins is effective in six minutes, a very short time compared with the usual methods, and with a good recovery of ovalbumin without ghosting effects.

We can clearly observe a peak of lysozyme, whereas Takeuchi et al.[12] did not; this failure was probably caused by the dilution of the sample with distilled water in conditions where ovomucin carries away lysozyme during its precipitation.[24]

It is also noteworthy that, with the solvents defined in this study, an increase of the gradient duration up to 20 minutes allows for an improvement in resolution, without a significant decrease in ovalbumin elution (trial no. 18).

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