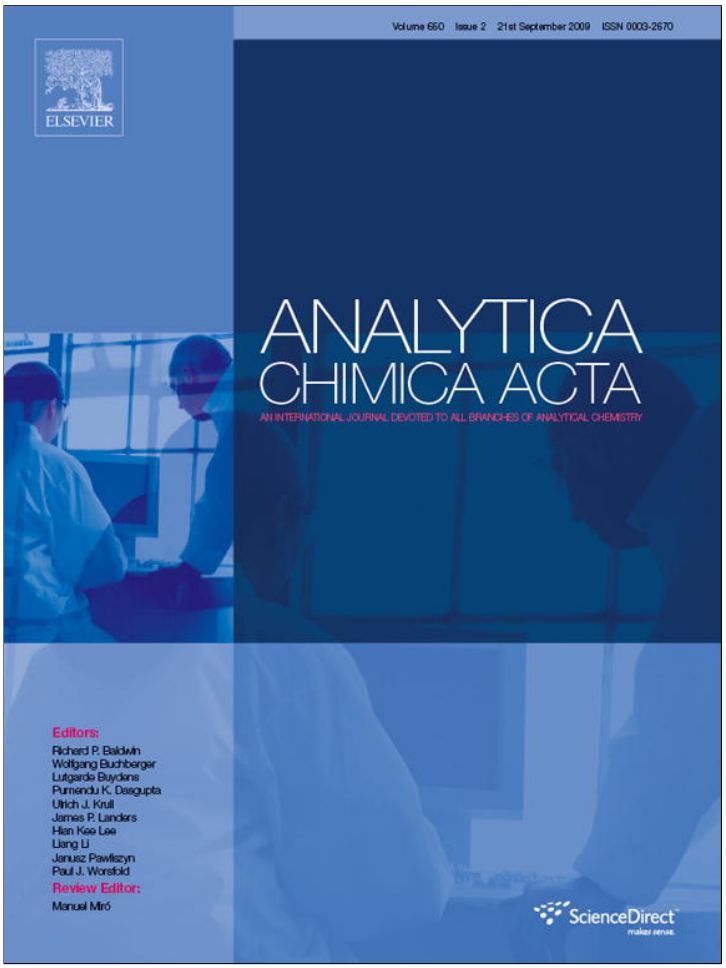

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Novel chemometric strategy based on the application of artificial neural networks to crossed mixture design for the improvement of recombinant protein production in continuous culture

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ABSTRACT

The optimal blends of six compounds that should be present in culture media used in recombinant protein production were determined by means of artificial neural networks (ANN) coupled with crossed mixture experimental design. This combination constitutes a novel approach to develop a medium for cultivating genetically engineered mammalian cells. The compounds were collected in two mixtures of three elements each, and the experimental space was determined by a crossed mixture design. Empirical data from 51 experimental units were used in a multiresponse analysis to train artificial neural networks which satisfy different requirements, in order to define two new culture media (Medium 1 and Medium 2) to be used in a continuous biopharmaceutical production process. These media were tested in a bioreactor to produce a recombinant protein in CHO cells. Remarkably, for both predicted media all responses satisfied the predefined goals pursued during the analysis, except in the case of the specific growth rate (μ) observed for Medium 1. ANN analysis proved to be a suitable methodology to be used when dealing with complex experimental designs, as frequently occurs in the optimization of production processes in the biotechnology area. The present work is a new example of the use of ANN for the resolution of a complex, real life system, successfully employed in the context of a biopharmaceutical production process.

1. Introduction

One of the aspects of bioprocesses that have attracted much interest in the last years is the design of culture media that fulfils complex requirements, such as those imposed by mammalian cells used for producing recombinant proteins. The different strategies are generally directed to reduce the total process costs and to decrease the protein content in order to minimize contamination risks and burden in downstream processing [1–3]. In addition, the main component which is desirable to be reduced or even eliminated from mammalian culture media is bovine serum [4].

Many techniques are available for developing culture media, either for prokaryotic or eukaryotic cells, varying from traditional one-variable-at-a-time method [5–8] to more complex statistical and mathematical techniques involving experimental design (for example full and partial factorials, Plackett–Burman, Hadamark matrix, central composite designs) [9–12] followed by optimization techniques such as response surface methodology (RSM), artificial neural networks (ANNs), fuzzy logic and genetic algorithms [13–18]. For an extensive review of the strategies used in the optimization of fermentation media see [19]. Unfortunately, there is no "multipurpose technique" that applies to all situations, and sometimes it becomes necessary to screen several approaches to find out the one that provides the best result in a particular case.

The aim of this work was to define the composition of two different serum-free culture media, by testing two groups of compounds that are added to a basal formulation, through a crossed mixture design. The goals pursued were to maximize the quantity of active secreting cells, the productivity and the quality of the secreted molecule (in terms of glycosylation), while minimizing toxic accumulation of catabolites during the culture of recombinant mammalian cells, using both batch and continuous processes. The responses considered in the optimization process

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were the specific growth rate (μ), the specific production rates of the inhibitory metabolites lactate (q_{lac}) and ammonium (q_{amm}) (which accumulate during the culture of cells and have been proved to affect growth and productivity as well as glycosylation [20–23]), the specific production rate of the recombinant protein (q_{prot}) and its estimated biological activity (BA).

Six components were selected to evaluate their effect on the responses: one set of compounds (hexoses referred to as H1, H2 and H3) was tested at each experimental mixture of a second group of components (named E1, E2 and E3). The concentration of the components must fulfill certain requirements, which are discussed below.

Experimental data obtained from the crossed mixture design were used to train artificial neural networks for each response. Two ANN were selected for each response, and used to predict the responses for 800 new combinations of H1, H2, H3, E1, E2 and E3. These predicted responses were combined to calculate a *Global Desirability Function* (D). The combinations of the six components which originated the highest values of function D were chosen to be tested in a continuous process in a perfused stirred tank bioreactor.

In a previous study, experimental data were used to obtain an equation for each response by means of RSM, according to a product model [13]. In such a model, the response depended on the product of both the function $f(x)$ and the function $g(z)$. The function $f(x)$ took into account the hexose composition, while the function $g(z)$ depended on the mixture of E compounds. This product model rendered a surface for each response, and was used to obtain the partial desirability functions d_i, which were then combined to generate the function D. The maximum value of the surface built for this latter function D corresponded to the mixture of components that best fulfill the predefined requirements. The new approach presented here is less complex and, in contrast to the previous one, it does not require to be performed by a highly trained technician, since it can be treated as a *black box* analysis. ANN methodology also proved to be as robust as RSM concerning the solution of a major concern of a bioprocess, such as the formulation of complex culture media for recombinant mammalian cells.

2. Theory

2.1. Crossed mixture design

Among experimental designs, a *mixture design* is a special case where a group of factors is considered as components of a mixture, and the analyzed response is a function of the proportion of each factor in the mixture. Besides, the sum of all component proportions in a mixture design is equal to 1 [24].

In a *crossed mixture design*, the combined effects of two different groups of factors on a particular response are studied, each group being analyzed as a mixture design itself.

2.2. Artificial neural networks

The ANN methodology is an information-processing, chemometric technique especially created to model non-linear information, which simulates some properties of the human brain. The so-called multilayer feed-forward networks [25,26] are often used for prediction as well as for classification. In the present work we used ANNs consisting of three layers of neurons or nodes, which are the basic computing units: the input layer, with six active neurons, corresponding to the predictor variables in regression, one hidden layer with a variable number of active neurons, and an output layer which has a single unit (each response). The neurons are connected in a hierarchical manner, i.e., the outputs of one layer of nodes are used as inputs for the next layer and so on. In the hidden layer, the sigmoid function $f(x) = 1/(1+e^{-x})$ was used, and the output of the hidden neuron j, O_j, was calculated as:

$$O_j = f\left[\sum_{i=1}^{m}(s_i w_{ij} + w_{bj})\right] \quad (1)$$

In Eq. (1), s_i is the input from neuron i in the layer above, to neuron j in the hidden layer, w_{ij} are the connection weights between neurons i and j, w_{bj} is the bias to neuron j and m is the total number of neurons in the layer above.

Linear functions are used both in the input and output layers. In the present work, learning is carried out through the back-propagation rule, for up to 11,000 epochs [25]. The number of hidden layers and neurons in each hidden layer must be selected to achieve a satisfactory fitting ability of the network, associated to a satisfactory predictive ability [27]. If the number of hidden layers or neurons in the hidden layer(s) is too high, the network, although reaching a great modelling ability, will lose the capacity for generalizing and predicting. According to ANN theory, however, most functions can be approximated using a single hidden layer [28]. The use of a subset of experiments to monitor the training is a suitable strategy, for instance, to check the predictive capability of the ANN.

It is important to stress that ANN trained with this rule have a remarkable advantage, as there is no need to know the exact form of the analytical function on which the model should be built. Furthermore, neither the functional type nor the number of model parameters need to be given [25].

2.3. Desirability function

The responses predicted with the ANN analysis were used to generate a function that assigns a value ranging from 0 to 1 to each mixture of compounds. This function, referred to as *partial desirability function for the response i* (d_i) [29], would be equal to 0 when the predicted response does not fulfill the predefined requirements, and 1 when the predicted value completely satisfies them. Details about the construction of the different desirability functions are described elsewhere [29,30].

The d_i functions are then combined to obtain a *global desirability function D* (Eq. (2)) that should be maximized choosing the best conditions of the designed variables.

$$D = \left(\prod_{i=1}^{n} d_i^{\,r_i}\right)^{1/\sum r_i} \qquad r_i = 1, 2, 3, 4, 5 \quad (2)$$

In Eq. (2), D is the value of the global desirability function, d_i is the partial desirability function for each response i and r_i is the relative importance assigned to this response. The relative importance r_i is a comparative scale for allotting emphasis to each d_i in the expression of the function D.

Finally, the mixture of the six compounds that predicts the highest value of D is selected as the best blend of components to be present in the culture medium being developed.

3. Materials and methods

3.1. Recombinant cell line and culture medium

A recombinant clone derived from a CHO.K1 cell line (ATCC CCL-61) and provided by Laboratorio de Cultivos Celulares (Santa Fe, Argentina) was used to perform the experiments. For the inoculum, cells were harvested from a suspension culture grown in a commercial serum-free medium at the middle exponential cell-growing phase, and were resuspended in 60 mL of the different media to be tested, at an initial cell density of 2×10^5 viable cells mL^{-1} (viability higher than 95%). Cells were then cultured in 100 mL spinner flasks (Bellco Glass Inc., USA) agitated at 45 rpm, and cultures were

maintained in an incubator at 37 °C and 5% CO_2 (Nuaire, USA). Seven days after the seeding, batch cultures were harvested.

The basal medium was serum-free, and consisted in a mixture of essential and non-essential amino acids, vitamins, inorganic salts and organic compounds. Different mixtures of carbon and energy sources were tested, but the exact identities of the compounds cannot be revealed due to ongoing patent registration of the developed culture media.

3.2. Cell culture in a perfused stirred tank bioreactor

A perfused stirred tank bioreactor (ALF Model, Bioengineering AG, Switzerland) was used to cultivate cells in continuous mode. Maximal working volume of the reactor was 5 L. The initial cell density was 3.5×10^5 viable cells mL^{-1}, and the perfusion of fresh medium was started on day 6 when cells reached 1.0×10^6 viable cells mL^{-1} at a rate of 0.25 working volume per day, and progressively increased up to 1 working volume per day (day 12).

3.3. Acquisition of experimental data

Daily samples were taken to perform cell counting in a Neubauer haemocytometer to determine the concentration of viable and non-viable cells, using the trypan blue exclusion method [31]. After that, samples were centrifuged 2 min at $500 \times g$ and the supernatant was frozen at −20 °C until the determination of metabolites was carried out.

The concentration of lactate in culture supernatants was determined using an autoanalyzer YSI Mod. 2700 (Yellow Spring Instruments, USA). Ammonium ion was quantitated by a colorimetric reaction [32]. The concentration of the secreted recombinant protein was measured using a specific sandwich ELISA developed in our laboratory. Supernatants collected at day 7 were concentrated by diafiltration using an Ultrafree-MC™ filter (Amicon-Millipore, USA), in successive cycles of 25 min at $2500 \times g$ at 4 °C. The biological activity of these samples was estimated by isoelectricfocusing and blotting on a nitrocellulose membrane (MSI-OSMONIC, USA), both processes were performed in a Multiphor II™ device (GE Healthcare, USA). The software ImageMaster™TotalLab (GE Healthcare, USA) was used to perform the densitometry of the bands corresponding to different glycoforms.

3.4. Crossed mixture design

A *crossed mixture design* [13,33] was selected to test different blends of the three hexoses H1, H2 and H3, and five blends of the energy-provider compounds E1, E2 and E3, simultaneously [13]. In the *mixture design* used for the hexoses, the concentrations of H1, H2 and H3 varied from 0 to 25 mmol L^{-1}, but the sum of all mixture components must be equal to 25 mmol L^{-1}. On the other hand, the concentration of E1 varies from 0 to 4 mmol L^{-1}, while E2 and E3 range from 0 to 5.1 mmol L^{-1}. The sum of E1, E2 and E3 must be equal to 5.1 mmol L^{-1}, and due to the restriction imposed to E1 this design is referred to as a *constrained mixture*.

The hexoses were arranged in a *simplex* {3,2} design augmented with the overall centroid and three axial points (S1-design), while the E-compounds were evaluated in a constrained *simplex* {3,2} with the overall centroid (S2-design), rendering a total of 65 experimental units (including the replicates). Each blend of the hexoses in the S1-design was evaluated at each point of the S2-design, as shown in Fig. 1. Hexoses are known to affect protein glycosylation in a direct manner, and this is the reason for testing several of their mixtures in this work. Compounds E2 and E3 were tested as an alternative to the use of compound E1, which is a main cellular energy source and produces a toxic metabolite by spontaneous decomposition in the culture medium.

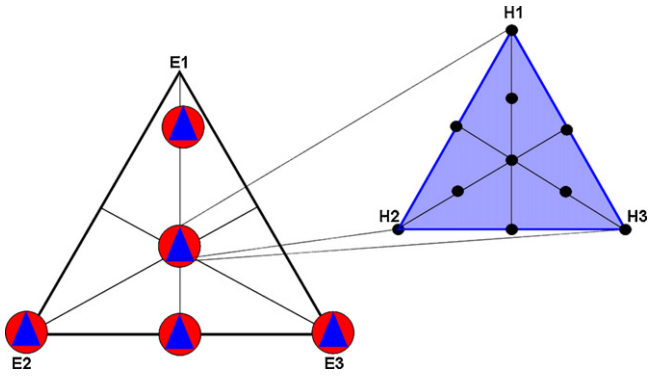

Fig. 1. Crossed mixture design S1 × S2.

3.5. Definition of the responses

A continuous culture can be considered as a biphasic process: the first phase ranges from the moment when cells are inoculated into the bioreactor to the moment when perfusion of fresh medium is initiated. During this period, the culture is similar to a batch process, since no elements are added to or removed from the bioreactor (with the exception of the gas exchange). When cells grow up to a certain concentration, perfusion begins in order to replenish nutrients and to remove catabolites that are potentially toxic.

During the first phase, a high specific growth rate (μ) is desirable to minimize process time, as well as high specific protein secretion rate (q_{prot}) and biological activity (BA). On the other hand, since catabolites accumulate during the cultivation, the specific rates of lactate production (q_{lac}) and ammonium ions (q_{amm}) should be minimal. The culture medium designed to accomplish these five goals was named *Medium 1*.

During the second phase, instead, μ is expected to be lower, in order to avoid cellular overgrowth. Since catabolites are removed because of perfusion, they are no longer of interest as responses, and consequently only μ, q_{prot} and BA were taken into account for designing the medium to be used during the perfusion phase of the culture, which was named *Medium 2*.

The parameters μ, q_{lac}, q_{amm} and q_{prot} were obtained as follows: the concentration of the variable of interest is plotted against the time integral of viable cells IVC [34], and a linear regression is performed. The slope is the value of the specific rate of production associated with the variable [2–4,13,34].

On the other hand, the biological activity of the samples was calculated according to Eq. (3):

$$BA = \sum_{i=1}^{n} BA_i \times P_i \quad (3)$$

In Eq. (3), BA_i is the biological activity associated to each isoform i of the glycoprotein, and P_i is the proportion of isoform i in the set of n isoforms present in the sample and determined by densitometry.

3.6. Training of the artificial neural networks

Experimental results for 41 experimental units were used to calibrate the ANN and 10, to monitor the training (Tables 1 and 2, respectively), using the back-propagation error method [25]. Some of the original data from the 65 experimental units were missing, and therefore these experimental units were not considered when training the ANNs. The full list of experimental results (including experiments discarded in this analysis) was presented in a previous work [13].

Table 1
Set of experimental points used for calibrating the ANN.

Experiment number	Hexoses and energy-provider compounds concentrations					
	H1	H2	H3	E1	E2	E3
	(mmol L^{-1})					
1	0.00	12.50	12.50	0.00	0.00	5.10
2	0.00	12.50	12.50	0.00	5.10	0.00
3	0.00	12.50	12.50	4.00	0.40	0.70
4	0.00	12.50	12.50	1.70	1.70	1.70
5	0.00	12.50	12.50	0.00	2.55	2.55
6	12.50	12.50	0.00	0.00	5.10	0.00
7	12.50	12.50	0.00	4.00	0.40	0.70
8	12.50	12.50	0.00	1.70	1.70	1.70
9	12.50	12.50	0.00	0.00	2.55	2.55
10	4.17	4.17	16.67	0.00	0.00	5.10
11	4.17	4.17	16.67	0.00	5.10	0.00
12	4.17	4.17	16.67	4.00	0.40	0.70
13	4.17	4.17	16.67	1.70	1.70	1.70
14	4.17	4.17	16.67	0.00	2.55	2.55
15	4.17	16.67	4.17	0.00	0.00	5.10
16	4.17	16.67	4.17	0.00	5.10	0.00
17	4.17	16.67	4.17	4.00	0.40	0.70
18	4.17	16.67	4.17	0.00	2.55	2.55
19	16.67	4.17	4.17	0.00	0.00	5.10
20	16.67	4.17	4.17	0.00	5.10	0.00
21	16.67	4.17	4.17	4.00	0.40	0.70
22	16.67	4.17	4.17	1.70	1.70	1.70
23	16.67	4.17	4.17	0.00	2.55	2.55
24	8.33	8.33	8.33	0.00	0.00	5.10
25	8.33	8.33	8.33	0.00	5.10	0.00
26	8.33	8.33	8.33	4.00	0.40	0.70
27	8.33	8.33	8.33	0.00	2.55	2.55
28	12.50	0.00	12.50	0.00	5.10	0.00
29	12.50	0.00	12.50	4.00	0.40	0.70
30	12.50	0.00	12.50	1.70	1.70	1.70
31	12.50	0.00	12.50	0.00	2.55	2.55
32	0.00	0.00	25.00	0.00	0.00	5.10
33	0.00	0.00	25.00	0.00	5.10	0.00
34	0.00	0.00	25.00	4.00	0.40	0.70
35	0.00	0.00	25.00	1.70	1.70	1.70
36	0.00	0.00	25.00	0.00	2.55	2.55
37	25.00	0.00	0.00	0.00	0.00	5.10
38	25.00	0.00	0.00	0.00	5.10	0.00
39	25.00	0.00	0.00	4.00	0.40	0.70
40	25.00	0.00	0.00	1.70	1.70	1.70
41	25.00	0.00	0.00	0.00	2.55	2.55

Table 2
Set of experimental points used for monitoring the ANN.

Experiment number	Hexoses and energy-provider compounds concentrations					
	H1	H2	H3	E1	E2	E3
	(mmol L^{-1})					
1	12.50	0.00	12.50	0.00	0.00	5.10
2	0.00	0.00	25.00	0.00	5.10	0.00
3	0.00	0.00	25.00	4.00	0.40	0.70
4	0.00	0.00	25.00	1.70	1.70	1.70
5	0.00	0.00	25.00	0.00	2.55	2.55
6	25.00	0.00	0.00	0.00	0.00	5.10
7	25.00	0.00	0.00	0.00	5.10	0.00
8	25.00	0.00	0.00	4.00	0.40	0.70
9	25.00	0.00	0.00	1.70	1.70	1.70
10	25.00	0.00	0.00	0.00	2.55	2.55

3.7. Data processing

3.7.1. Partial desirability function (d_i) calculated for Medium 1

The d_i function corresponding to each one of the responses μ, q_{lac}, q_{amm}, q_{prot} and BA was calculated as indicated in Eqs. (4)–(8). In these equations, R_p represents the response predicted by the ANN for that particular mixture of compounds.

(a) Partial desirability function for the response μ (d_μ):

$$d_\mu = \begin{cases} d_\mu = \dfrac{R_p - 0.014}{0.020 - 0.014} & 0.014 \le R_p \le 0.020 \\ d_\mu = 1 & R_p > 0.020 \\ d_\mu = 0 & R_p < 0.014 \end{cases} \quad (4)$$

In Eq. (4) R_p is expressed in [h^{-1}] units.

(b) Partial desirability function for the response q_{lac} ($d_{q_{lac}}$):

$$d_{q_{lac}} = \begin{cases} d_{q_{lac}} = 1 - \dfrac{R_p}{719} & 0 \le R_p \le 719 \\ d_{q_{lac}} = 1 & R_p < 0 \\ d_{q_{lac}} = 0 & R_p > 719 \end{cases} \quad (5)$$

In Eq. (5) R_p is expressed in [nmol 10^{-6} $cell^{-1}$ h^{-1}] units.

(c) Partial desirability function for the response q_{amm} ($d_{q_{amm}}$):

$$d_{q_{amm}} = \begin{cases} d_{q_{amm}} = \dfrac{388 - R_p}{388 - 5.8} & 5.8 \le R_p \le 388 \\ d_{q_{amm}} = 1 & R_p < 5.8 \\ d_{q_{amm}} = 0 & R_p > 388 \end{cases} \quad (6)$$

In Eq. (6) R_p is expressed in [nmol 10^{-6} $cell^{-1}$ h^{-1}] units.

(d) Partial desirability function for the response q_{prot} ($d_{q_{prot}}$):

$$d_{q_{prot}} = \begin{cases} d_{q_{prot}} = \dfrac{R_p - 4.5 \times 10^{-7}}{1.473 \times 10^{-6} - 4.5 \times 10^{-7}} & 4.5 \times 10^{-7} \le R_p \le 1.473 \times 10^{-6} \\ d_{q_{prot}} = 1 & R_p > 1.473 \times 10^{-6} \\ d_{q_{prot}} = 0 & R_p < 4.5 \times 10^{-7} \end{cases} \quad (7)$$

In Eq. (7) R_p is expressed in [% $cell^{-1}$ h^{-1}] units.

(e) Partial desirability function for the response BA (d_{BA}):

$$d_{BA} = \begin{cases} d_{BA} = \dfrac{R_p - 40,000}{73,000 - 40,000} & 40,000 \le R_p \le 73,000 \\ d_{BA} = 1 & R_p > 73,000 \\ d_{BA} = 0 & R_p < 40,000 \end{cases} \quad (8)$$

In Eq. (8) R_p is expressed in [IU mg^{-1}] units.

In Eqs. (4)–(6) the pairs of values (0.014, 0.020), (0, 719) and (5.8, 388) correspond to the minimum and the maximum empirical values obtained for the responses μ, q_{lac}, and q_{amm}, respectively. In Eqs. (7) and (8), 4.5×10^{-7} and 40,000 correspond to the lowest empirical data that include 70% of the total data of q_{prot}, and BA, respectively. The values 1.473×10^{-6} and 73,000 are the maximum empirical values achieved for these responses.

When generating the D function for Medium 1, d_i for responses μ, q_{prot} and BA were assigned a r_i = 5, while q_{lac} and q_{amm} were assigned a r_i = 1.

Table 3
Architecture and statistical parameters of the selected ANNs.

Response	ANN	Architecture	No. epochs	Best epoch	RMSEC	RMSEP
μ	ANN 1	6/6/1	10,000	3,226	0.0555	0.0545
	ANN 2	6/7/1	10,000	8,674	0.0437	0.0541
q_{lac}	ANN 3	6/7/1	10,000	10,000	0.0389	0.0541
	ANN 4	6/8/1	10,000	7,290	0.0443	0.0478
q_{amm}	ANN 5	6/3/1	5,000	5,000	0.0652	0.0455
	ANN 6	6/5/1	10,000	10,000	0.0570	0.0250
q_{prot}	ANN 7	6/6/1	10,000	10,000	0.0300	0.0490
	ANN 8	6/7/1	5,000	5,000	0.0400	0.0556
BA	ANN 9	6/5/1	10,000	10,000	0.0385	0.0504
	ANN 10	6/7/1	11,000	11,000	0.0392	0.0558

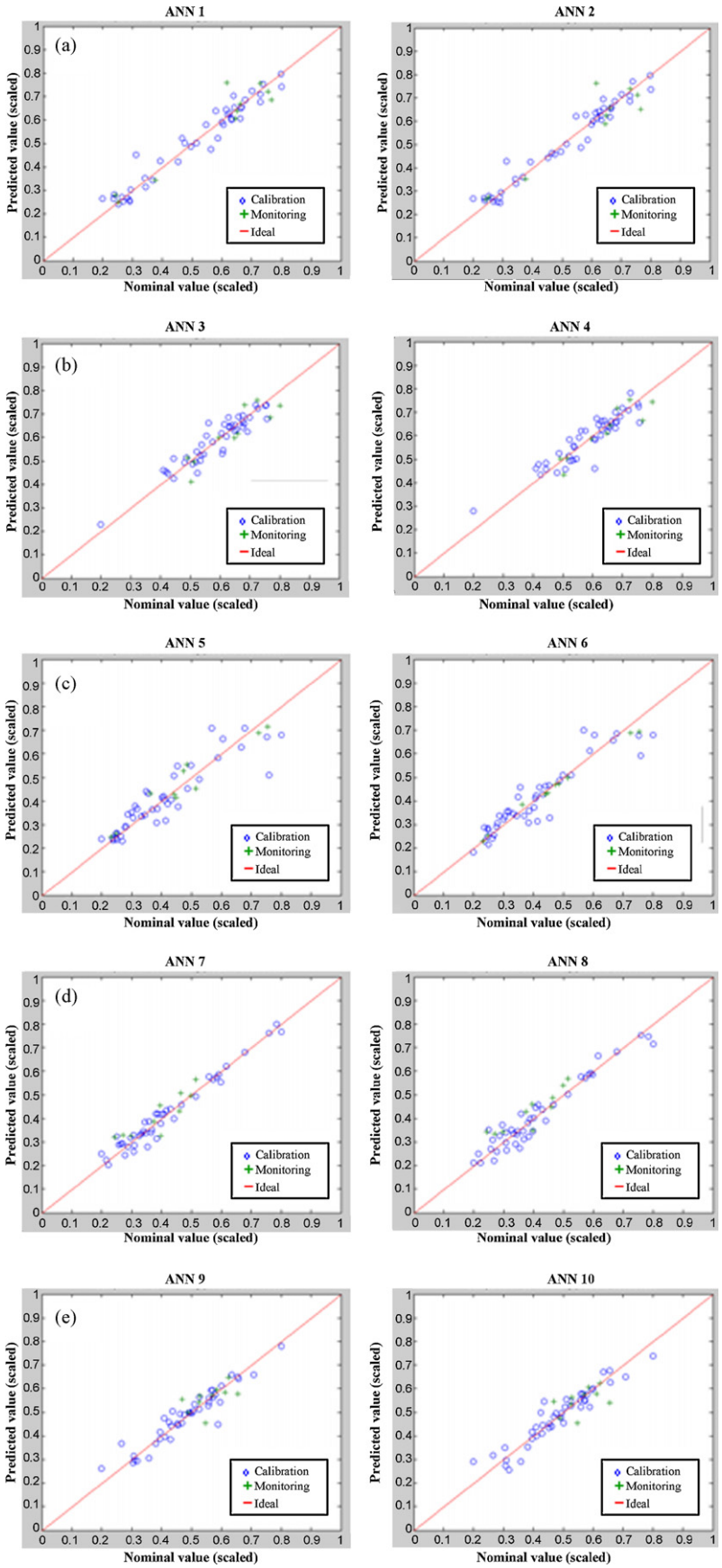

Fig. 2. Correlation between the real values used in the training of the ANN and their predictions for: (a) μ, (b) q_{lac}, (c) q_{amm}, (d) q_{prot} and (e) BA.

3.7.2. Partial desirability function (d_i) calculated for Medium 2

The d_i functions corresponding to the responses q_{prot} and BA were calculated as indicated in Eqs. (7) and (8). As μ was required to satisfy different requirements in *Medium 2*, function d_i for this response was calculated according to Eq. (9):

Partial desirability function for the response μ (d_μ):

$$d_\mu = \begin{cases} d_\mu = 1 & 0.004 \le R_p \le 0.020 \\ d_\mu = 0 & R_p < 0.004, \ R_p > 0.020 \end{cases} \quad (9)$$

In Eq. (9) R_p is expressed in [h^{-1}] units. The values 0.004 and 0.020 correspond to the lowest and the highest experimental values obtained. Likewise for *Medium 1*, μ, q_{prot} and BA were assigned a $r_i = 5$ in the expression of function *D*.

4. Results and discussion

4.1. Analysis by artificial neural networks

The general architecture of the ANNs used in this work consisted in three layers of neurons: an input layer of 6 neurons (the concentrations of H1, H2, H3, E1, E2 and E3 in the medium), a hidden layer of neurons and an output layer of 1 neuron (one for each response). A unique ANN with an output layer of 5 neurons (considering all responses) was also tested, but the training time was excessively long and the obtained errors were considered too high, and consequently the single output neuron was considered more convenient.

Several different architectures for each response were randomly tested, varying the number of the neurons of the hidden layer (data not shown). Finally, two ANNs with different architectures were selected for modelling every response. This was performed to get an insight of the robustness of the models. The criterion was to select those networks with the less number of hidden neurons (simplest architecture), presenting the lowest errors of calibration and monitoring (less than 5%). Table 3 summarizes both the architecture and the statistical parameters of the selected networks, including the best epoch in which the errors of training (RMSEC) and monitoring (RMSEP) were minimal. These errors were calculated according to Eq. (10):

$$\mathrm{RMSE} = \left[\frac{1}{I} \sum_{1}^{I} (c_{act} - c_{pred})^2 \right]^{1/2} \quad (10)$$

In Eq. (10), c_{act} and c_{pred} are the actual and the predicted concentrations in either the training or the monitoring steps, and *I* is the number of samples used in each procedure.

Fig. 2 shows the correlation between the real values used in the calibration and the predicted ones for the selected networks.

In order to analyze the robustness of the ANN approach, all possible combinations between these networks ($2^5 = 32$) were tested to predict the responses of 800 new different mixtures of the six variables, which were considered to cover the entire experimental space (Fig. 3). The predicted responses were then used to calculate their associated d_i values according to Eqs. (4)–(8) for *Medium 1*, and Eqs. (7)–(9) for *Medium 2*. These d_i values where then used to calculate the *D* value corresponding to that particular combination of H1, H2, H3, E1, E2 and E3.

In order to establish the general trend of the results, the three mixtures of compounds that rendered the highest values of *D* corresponding to each of the 32 combinations of ANN (i. e. 32×3=96 mixtures) were listed for both *Medium 1* and *Medium 2*. Tables 4 and 5 present the composition of the five mixtures for which the highest values of *D* were obtained, ordered by their relative frequency of appearance in the pool of 96 data. According to the preceding results, mixture #303

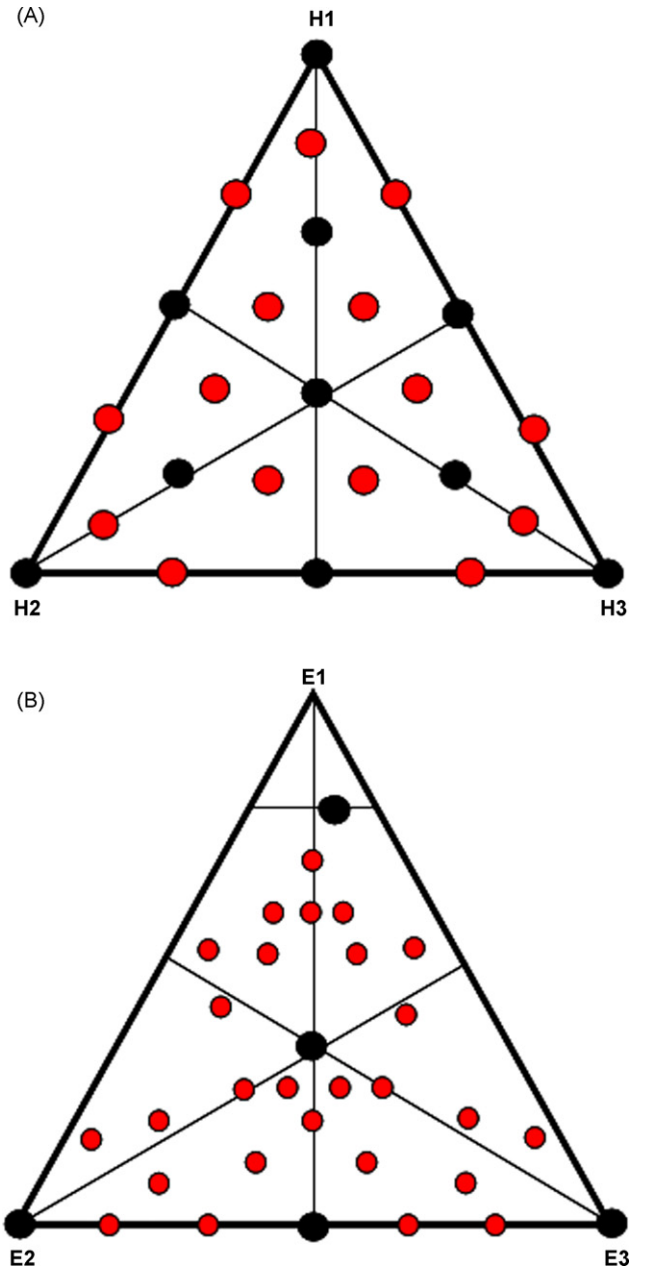

Fig. 3. Set of 25 mixtures of H1, H2, H3 (a), and 32 mixtures of E1, E2, E3 (b), which in all rendered 800 theoretical mixtures for which ANN methodology predicted the responses.

(H1=25.00 mmol L^{-1}, H2=0.00 mmol L^{-1}, H3=0.00 mmol L^{-1}, E1=2.67 mmol L^{-1}, E2=2.07 mmol L^{-1}, E3=0.36 mmol L^{-1}) was selected as *Medium 1*, while mixture #538 (H1=0.00 mmol L^{-1}, H2=6.25 mmol L^{-1}, H3=18.75 mmol L^{-1}, E1=1.417 mmol L^{-1}, E2=1.133 mmol L^{-1}, E3=2.550 mmol L^{-1}) was selected as *Medium 2*. Both media were used to cultivate cells in the bioreactor. It is remarkable that both media have a reduced content of compound E1, which was desirable to be minimized, because its ammoniagenic capability. This observation agrees with previous results obtained with the product model obtained by RSM, used in a recent optimization process [13], reinforcing the suitability of ANN analysis as an alternative to RSM.

Empirical responses obtained for this culture are presented in Table 6. It can be noticed that all responses satisfy the predefined goals during the analysis, with one exception: the specific growth rate observed for *Medium 1*. This slow growing could be due to a

Table 4
Pool of 5 points for which the highest values of the *D* function for Medium 1 was predicted.

Predictive point	Absolute frequency	Relative frequency	*D*	Composition of the mixture (mmol L^{-1})					
				H1	H2	H3	E1	E2	E3
#303	8	8.3%	0.751	25.00	0.00	0.00	2.67	2.07	0.37
#304	8	8.3%	0.715	25.00	0.00	0.00	2.00	2.33	0.78
#386	8	8.3%	0.673	2.33	2.33	20.33	2.67	0.37	2.06
#62	8	8.3%	0.549	0.00	25.00	0.00	3.40	1.03	0.67
#480	8	8.3%	0.497	6.25	18.75	0.00	2.27	1.33	1.50

Table 5
Pool of 5 points for which the highest values of the *D* function for Medium 2 was predicted.

Predictive point	Absolute frequency	Relative frequency	*D*	Composition of the mixture (mmol L^{-1})					
				H1	H2	H3	E1	E2	E3
#538	6	25%	0.801	0.00	6.25	18.75	1.42	1.13	2.55
#303	4	16.7%	0.881	25.00	0.00	0.00	2.67	2.07	0.37
#304	4	16.7%	0.865	25.00	0.00	0.00	2.00	2.33	0.78
#410	2	8.3%	0.849	2.33	2.33	20.33	1.42	1.13	2.55
#47	2	8.3%	0.846	0.00	25.00	0.00	2.67	1.2.07	0.37

Table 6
Empirical responses obtained when cells were cultured in a perfused stirred tank bioreactor.

Response	Medium 1	Medium 2
μ (h^{-1})	0.0061	0.0080
q_{lac} (nmol 10^{-6} $cell^{-1}$ h^{-1})	140.6	–
q_{amm} (nmol 10^{-6} $cell^{-1}$ h^{-1})	22	–
q_{prot} (% $cell^{-1}$ h^{-1})	4.19×10^{-6}	6.39×10^{-6}[a]
BA (IU mg^{-1})	72,900	74,300

[a] Value was calculated considering perfusion rate.

poor modelling of the response, but the analysis of cell cultures used to train the ANN proved that media with similar composition to #303 showed a high μ. The reduced growth rate observed in the bioreactor could then be due to drastic changes in cellular environment (shear stress, dissolved oxygen, for instance) which demand a period for adaptation of the cells to these new conditions.

5. Conclusions

Two different culture media were formulated according to the results obtained in this work. In particular, the combination #303 gives the maximum *D* value of 0.751 for *Medium 1*, a culture medium designed to be used during the initial phase of a continuous culture in a stirred tank bioreactor. Likewise, a value of $D = 0.801$ was calculated for mixture #538, which was selected to be used during the perfusion phase of this culture (*Medium 2*). These high values of *D* are remarkable, since five empirical responses were simultaneously optimized.

Medium 1 and *Medium 2* improved cellular metabolism, achieving the goals initially proposed. However, the response μ obtained with Medium 1 did not accomplish the predefined requirements. This could be due to an inaccurate modelling by the selected ANNs, or to a lag phase of cellular growth, usually observed when cells are subcultivated in a different culture system, as they were amplified from a spinner flask to a bioreactor.

Several ANN were trained in this work in order to confirm the predicting ability of this methodology. The results showed that the analysis can be simplified, because lesser experiments would be sufficient to train the networks, and because small differences in the architecture of the trained networks would not lead to much different results. Also, a greater number of theoretical mixtures can be tested, instead of the 800 used in this work, without increasing considerably either the time or the effort of the study. Based on these facts, it can be concluded that ANN analysis is simpler and consequently easier to perform than the product model previously developed using RSM. Besides, as the complexity of the experimental design increases (while technical capacity of carrying out experiments remains the same), the advantages of using ANN over RSM would become more evident. Still, both RSM and ANN approaches are considered suitable alternatives to be tested by biotechnologists when complex experimental designs are required, as usually happens in the production processes of biologicals.

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