Talanta 85 (2011) 142–150

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A novel ion-pairing chromatographic method for the simultaneous determination of both nicarbazin components in feed additives: Chemometric tools for improving the optimization and validation

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ARTICLE INFO

Article history:
Received 4 January 2011
Received in revised form 18 February 2011
Accepted 19 March 2011
Available online 31 March 2011

Keywords:
Chemometrics
Experimental design
Desirability function
Nicarbazin
Coccidiostat
Ion-pairing chromatography

ABSTRACT

The development, optimization and validation of an ion-pairing high performance liquid chromatography method for the simultaneous determination of both nicarbazin (NIC) components: 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) in bulk materials and feed additives are described. An experimental design was used for the optimization of the chromatographic system. Four variables, including mobile phase composition and oven temperature, were analyzed through a central composite design exploring their contribution to analyte separation. Five responses: peak resolutions, HDP capacity factor, HDP tailing and analysis time, were modelled by using the response surface methodology and were optimized simultaneously by implementing the desirability function. The optimum conditions resulted in a mobile phase consisting of 10.0 mmol L^{-1} of 1-heptanesulfonate, 20.0 mmol L^{-1} of sodium acetate, pH = 3.30 buffer and acetonitrile in a gradient system at a flow rate of 1.00 mL min^{-1}. Column was an INERSTIL ODS-3 (4.6 mm × 150 mm, 5 μm particle size) at 40.0 °C. Detection was performed at 300 nm by a diode array detector. The validation results of the method indicated a high selectivity and good precision characteristics, with RSD less than 1.0% for both components, both in intra and inter-assay precision studies. Linearity was proved for a range of 32.0–50.0 μg mL^{-1} of NIC in sample solution. The recovery, studied at three different fortification levels, varied from 98.0 to 101.4 for HDP and from 99.1 to 100.2 for DNC. The applicability of the method was demonstrated by determining DNC and HDP content in raw materials and commercial formulations used for coccidiosis prevention. Assays results on real samples showed that considerable differences in molecular ratio DNC:HDP exist among them.

1. Introduction

Nowadays, the use of chemometric tools should be the standard way of operating when developing, optimizing and validating a chemical process or methodology [1]. The advantages of experimental designs are well-known by chemometricians and are increasingly recognized by the whole scientific community. Its use in the separation sciences has particularly increased in the last few years [2–8]. Through the development of mathematical models, chemometrics allows to assess the statistical significance of the independent variable effects being investigated in a system, as well as evaluate their interactions using a reduced number of experiments. Moreover, employing multivariate optimization designs in which the levels of the variables are changed simultaneously in at least three different points, a second order polynomial equation may be adjusted to describe the behavior of a particular response as a function of the studied variables and their interactions. So, the popularly called response surface methodology (RSM) enables to find the optimum experimental conditions to improve certain responses that assure the best system performance. The achievement of good results in the optimization procedures resides in a correct selection of the experimental design to be employed, apart from a suitable experimental domain, i.e., the ranges in which the variables will be tested. Likewise, it is very important to fit an adequate mathematical function and to evaluate the quality of the fitted model and its accuracy to make previsions in relation to the experimental data [9].

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doi:10.1016/j.talanta.2011.03.043

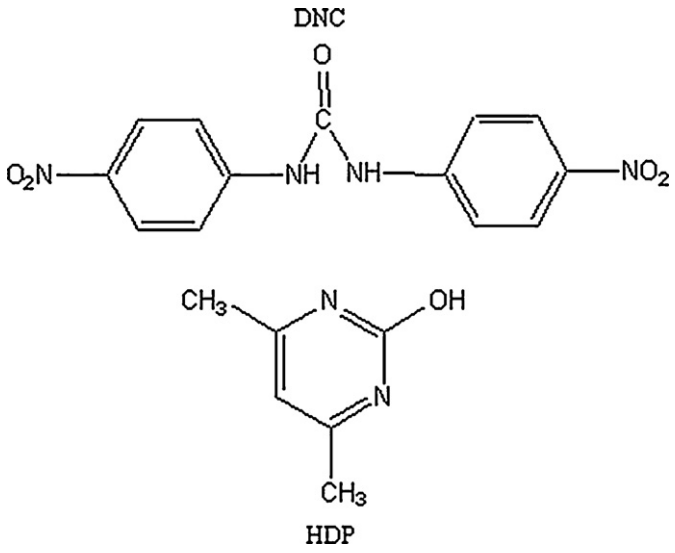

Fig. 1. Chemical structure of nicarbazin, equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP).

Otherwise, when the system involves many responses to be optimized simultaneously, the application of the procedure developed by Derringes and Suich is very useful. This method makes use of a desirability function, in which the researchers' own priorities and desires on the response values are taken into account in the optimization procedure [10,11]. This strategy becomes essential when a chromatographic method is being developed because a large number of elementary functions have to be optimized.

Nicarbazin (NIC, $C_{13}H_{10}N_4O_5{\cdot}C_6H_8N_2O$), a synthetic antibiotic that looks like a light yellow powder, is an equimolecular mixture of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) acting as an electron donor–acceptor molecular complex. The interaction sites are the electron-poor NH amide groups of the acceptor phenylurea and the electron-rich ion pairs of the nitrogen in the pyrimidone donor ring (Fig. 1). Since its development in the 1950s, it has been used for the treatment and prevention of the parasitic disease coccidiosis in the poultry industry. This is a contagious amoebic disease produced by *Eimeria sp*. that affects livestocks, particularly poultry, throughout the world. The acute manifestation of coccidiosis causes intestinal lesions which results in diarrhea and related health problems in the animals, with the consequent economic losses. Infections of coccidiosis also make chickens more susceptible to Salmonella infections [12].

NIC is approved by the U.S. Food and Drug Administration (FDA), the European Union (Regulation 1831/2003/EC) and many other countries regulations to be used as a feed additive. It may be used alone or in combination with other ionophore coccidiostat such as maduramicin ammonium, narasin or clopidol to offer a better coccidia control in coccidiosis prevention programs. These feed additives, commercialized by the veterinary pharmaceutical industry, are not intended for direct consumption by animals, but to be incorporated into mixed feeds during the production process. In order to assure a uniform mixture, it is usually recommended to prepare a concentrated premix of animal drugs with a major component of feed (corn or soya) to be incorporated later on into the ration. NIC is administrated in feed at concentrations ranging from 40 to 50 mg kg^{-1} of feed when combined with others active ingredients, and at concentrations about 125 mg kg^{-1} of feed, as single active.

Given to laying chickens, NIC produces side effects that consist in a decrease in egg hatching and, at higher doses, a decline in egg production. It seems that one mechanism by which NIC reduces the viability of eggs is by causing the disruption of the vitelline (yolk) membrane, allowing the yolk and albumin to flow together, thus creating conditions under which the embryo cannot develop. For this reason, the drug is not applied to laying hens. However, in the last few years it has been evaluated and used as a highly promising avian infertility agent controlling Canada goose populations and other overabundant wild birds [13].

In addition, it has been demonstrated that NIC absorption significantly differs among bird species and besides, that dissolution and the consequent absorption of the drug in the gut, is a function of the HDP moiety [14]. The complex DNC:HDP is 10 times more potent in the control of *Eimeria sp*. than is DNC by itself. However, HDP when used alone was observed to have no anticoccidial activity [15]. DNC is highly insoluble in aqueous media, forming hydrophobic aggregates up to 20 μm in size, being too large for absorption. Consequently, it was proposed and finally demonstrated by the scanning electron microscopy that micron-size crystals of NIC disintegrate in water to form much smaller DNC crystals [16]. A similar complex dissolution in the gut of poultry may account for the greater effectiveness of DNC when administered as a complexed rather than an uncomplexed drug.

In spite of HDP plays an important role in the bioavailability of the active DNC, literature published methods for the determination of NIC in feed additive and premixes are based in DNC assays [17–22]. This approach is very reasonable in monitoring residues since the DNC molecule is much more persistent than the HDP component in edible tissues and has therefore been established as a marker residue in liver, meat and egg [23,24]. Several methods have been developed to carry out this monitoring, employing different techniques such as high performance liquid chromatography with ultra-violet detector (HPLC-UV) [25,26], or mass detector (HPLC-electrospray MS–MS) [27], Enzyme-Linked Immuno Sorbent Assay (ELISA) [28,29] and biosensors [30]. It can be postulated that the cause of the lack of chromatographic methods for the simultaneous determination of both compounds is due to the high polarity and the basic properties of HDP, resulting in a poor retention in conventional reversed phase liquid chromatography (RP-LC).

Ion-pairing chromatography is an accessible alternative that can be used for the separation of ionic and ionizable compounds such as the ones studied in this case. The main advantages of this technique are that it does not require special stationary phases or equipment, it allows selectivity to be manipulated through changes in the mobile phase composition alone and it is suitable for the simultaneous separation of neutral and ionized compounds [31].

Interestingly, it should be considered that there are many variables that affect retention and selectivity in ion-pairing chromatography. The most important are: the type and concentration of the ion-pairing reagent, the pH and ionic strength of the mobile phase, but also the concentration and type of organic solvent. Stationary phase sorption properties and temperature are important as well. The influence of these variables is often non-linear and interdependent between them, presenting problems for the formulation of simple rules and requiring the earlier mentioned experimental design.

In this work, a novel ion-pairing high performance liquid chromatographic (IP-HPLC) method was developed, optimized and validated for its application in the simultaneous determination of DNC and HDP in bulk material and coccidiostatic formulations in combination with maduramicin ammonium. To accomplish the objective, four variables of the chromatographic system have been studied through a central composite design to optimize five responses simultaneously. Variables and design selection, model fitting and validation and the optimization criteria to reach the global desirability are discussed.

2. Experimental

2.1. Apparatus and software

All experiments were performed using an Agilent 1100 Series liquid chromatograph, equipped with a quaternary pump, membrane degasser, thermostatted column compartment, autosampler and diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). For data acquisition and processing, the Chemstation version B 0103 was used. The HPLC column was an INERSTIL ODS-3 (4.6 mm × 150 mm, 5 μm particle size) from GL Science.

Experimental design, surface response modelling and desirability function calculations were performed using the Stat-Ease Design-Expert trial Version 7.0.0.

2.2. Chemicals and reagents

NIC (CAS 330-95-0, 68.0% w/w DNC, 28.9% w/w HDP) was purchased from Sigma (Sigma–Aldrich Inc, St. Louis, USA). N,N-dimethylformamide p.a. was supplied by Anedra (San Fernando, Argentina), sodium 1-heptanesulfonate by F.J.T. Baker (Phillipsburg, NJ, USA) and sodium acetate and acetic acid by Cicarelli (San Lorenzo, Argentina). Acetonitrile and methanol HPLC-grade were obtained from Merck (Darmstadt, Germany). HPLC-grade water was obtained from a Milli-Q Biocel System (Millipore SAS, Molsheim, France).

Different buffer solutions containing sodium 1-heptanesulfonate (SHS) and sodium acetate (SA) at several concentrations and pH values were prepared in order to reach the conditions established by the experimental design. Diluents used in the optimization stage were obtained by mixing the different buffers with acetonitrile (50:50). The definitive composition of the sample diluent used for the validation studies consisted of 20.0 mmol L^{-1} of SA and 10.0 mmol L^{-1} of SHS pH = 3.30 buffer and acetonitrile (50:50).

Solutions and solvents composing the mobile phase were always filtered through 0.45 μm nylon filters. Standard and sample solutions were also filtered through a 0.20 μm syringe nylon filter before being injected in the chromatographic system.

2.3. Commercial sample

Commercial samples of the feed additive with coccidiostatic properties containing 8.0% NIC and 0.75% maduramicin ammonium were provided by a local veterinary pharmaceutical industry (FACYT S.A., Argentina). The matrix preparation consisting of a mixture of calcium carbonate and semite was also supplied by the factory.

2.4. Standard solutions

A NIC stock standard solution of 0.8 mg mL^{-1} was prepared by exactly weighing and dissolving a portion of the standard in N,N-dimethylformamide (DMF). The solution, which proved to be stable for 1 month, was conserved at 4 °C in light-resistant containers and was allowed to reach room temperature before use.

Five calibration standard solutions were prepared at the moment of use by diluting an appropriate volume of the stock standard solution in a sample diluent to reach final concentrations of 32.0, 36.0, 40.0, 44.0 and 50.0 μg mL^{-1} of NIC, covering the range from 80.0 to 120.0% of the expected concentration of the analyte in the assay solution.

Non-spiked and spiked matrix solutions were prepared as described in 2.5, adding in each case the suitable volume of stock standard solution to obtain artificial samples with 0.0, 80.0, 100.0 and 120.0% of the expected amount of the analyte.

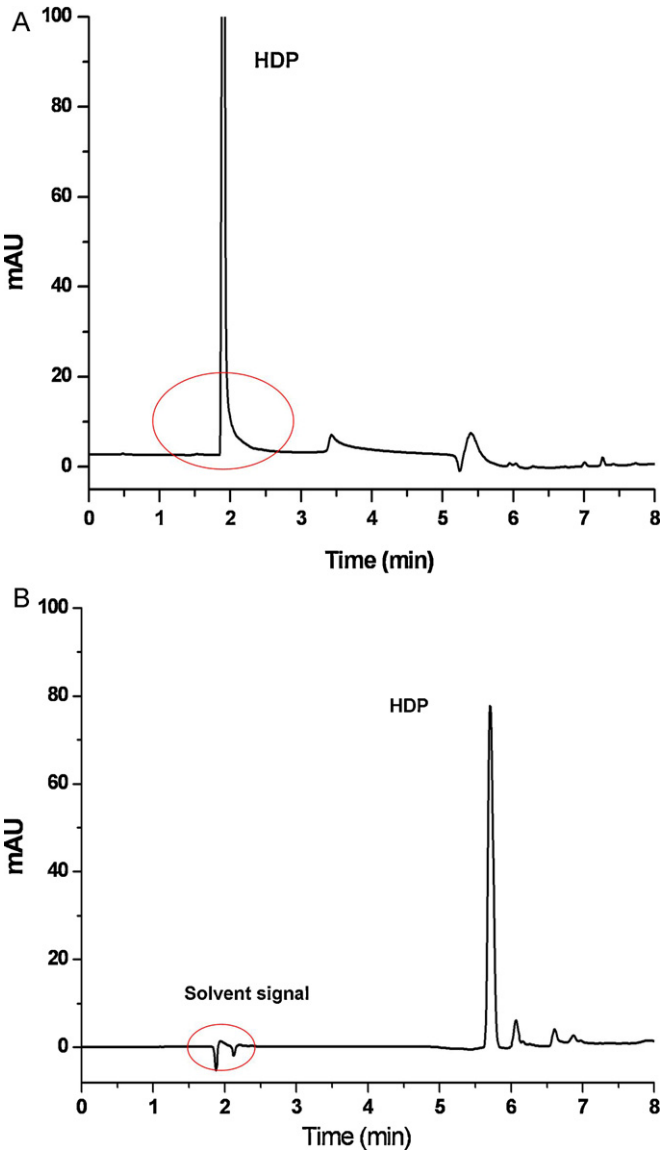

Fig. 2. HDP chromatographic signals obtained at 300 nm for a 40.0 μg mL^{-1} NIC solution. (A) Mobile phase consisting of 10.0 mmol L^{-1} of phosphate buffer pH = 3.00 and acetonitrile in a gradient system. (B) Mobile phase consisting of a buffer of 5.00 mmol L^{-1} of SHS, 10.0 mM SA at pH = 3.00 and acetonitrile in a gradient system.

2.5. Sample preparation

The commercial sample was pulverized using a grinder and it was protected against light in order to obtain a homogeneous powder. A portion of the obtained powder of about 500 mg was weighed and transferred to a 50.0 mL volumetric flask with 30 mL DMF. The mixture was sonicated for 20 min, stirring vigorously every 5 min in order to extract the active compounds and finally made up to volume with the same solvent. Then, c.a. 10 mL of the resulting suspension were centrifugated for 10 min at 2000 rpm and 1.0 mL of the supernatant was diluted to 20.0 mL with sample diluent. In this way, solutions containing c.a. 40.0 μg mL^{-1} of NIC were obtained.

2.6. Experimental design and optimization

Our challenge was to find out the optimum analytical conditions to develop a chromatographic method able to determine both components of NIC in additives with satisfactory performance and in a reasonable analysis time.

Previous experiments have shown a poor retention of HDP using a mobile phase consisting of 10.0 mmol L^{-1} of phosphate buffer pH = 3.00 and acetonitrile. Moreover, the capacity factor was extremely low and an unacceptable tailing was obtained (Fig. 2A). Using methanol or methanol:acetonitrile mixtures as organic constituents of mobile phase worse peak performance was observed.

Based on both theoretical and experimental knowledge of the issue, it was decided to test a mobile phase including SHS as an ion-pairing agent in order to change the retention mechanism of the compound. Considering this fact, different experiments were performed with a mobile phase consisting of a buffer containing 5.0 mmol L^{-1} of SHS and 10.0 mmol L^{-1} of SA at pH = 3.00 and acetonitrile, which enhanced the separation ability (Fig. 2B).

Several responses were selected for optimization purposes: (a) peak capacity factor of HDP (K'_{HDP}), (b) peak tailing of HDP (T_{HDP}), (c) peak resolution of HDP (R_{HDP}), (d) peak resolution of DNC (R_{DNC}) and (e) peak retention time of DNC (t_{DNC}) as a marker of analysis time. The factors (k): SHS and SA concentrations in the mobile phase, buffer pH and temperature of the column oven were taken into account due to the fact that these variables commonly affect the responses of systems such as the one considered herein. Concentrations of reagents were established, based on the levels usually used in mobile phases. pH was maintained within a relatively bounded acidic range to ensure the ionization of HDP without affecting the integrity of the stationary phase, while the oven temperature was tested in a relatively wide range.

2.6.1. Selection of the optimization design

Among Response-Surface designs with a symmetrical experimental domain, the following can be considered: three-level full-factorial (3-FFD), central composite (CCD), Box–Behnken (BBD), and Doehlert Matrix (DMD) designs [32].

An evaluation of these designs showed that the number of experiments (E) required for the same number of factors is different in each case. In comparison, DMD is slightly more efficient than CCD and BBD but much more efficient than 3-FFD, defining the efficiency of one experimental design as the number of coefficients in the estimated model divided by E [33,34].

Analyzing the mentioned issues, we chose a CCD because it allows us to study five levels for each variable, making the same number of experiments and, consequently, with the same efficiency than BBD which involves only three levels. It is important to remark that, although DMD would have resulted in a quite more efficient design, its application is not so simple due to the variability in the factor levels. Another advantage of the CCD is that it may be carried out in blocks. Blocking is advantageous when all of the experiments cannot be performed in the same working day.

2.6.2. Building the CCD

CCD includes: two-level factorial design points (Fp), axial or "star" points (Sp) and center points (Cp). The Sp has all of the factors set to 0, except one factor which has the value $\pm\alpha$. Levels for each factor corresponding to −1 and +1 coded value were: 10.0 and 20.0 mmol L^{-1} for SHS, 20.0 and 100.0 mmol L^{-1} for SA, 3.00 and 4.00 for pH and 25 and 40 °C for the oven temperature.

The value of α determines the location of the Sp in the CCD and usually varies from 1 to $\sqrt{k}$ (see above about k). Rotatable designs will be achieved with α = fourth root of f, where f is the number of Fp. In spherical and strictly rotatable designs the Sp will be placed farther from the center point as the number of factors increases with the risk of leaving too much of the intermediate region uninvestigated. Eventually, it could happen that the chosen α value results in some impracticable runs [32]. Thus, another option is to take a "practical" value of α: the fourth root of k. This has been shown to produce Sp that can practically be run, generating a design which still maintains adequate statistical properties (these are the so-called "near-rotatable" designs) [11]. Considering our variables and operative restriction for column care we have chosen the practical value of 1.414 for α.

Due to the large number of experiments to be performed, we decided to divide them in blocks. Thus, these experiments were carried out during three different days. The first and second blocks involved the Fp with some Cp. The remaining block consisted of the Sp with additional Cp.

Making replicates at the center point has two main objectives: to provide a measure of pure error, i.e., the error to be expected in the response if the experiment is repeated starting from scratch, and to stabilize the variance of the predicted response in the design region. In this way, it has been demonstrated that rotatable designs require at least 3–5 Cp in order to avoid severe imbalance in the prediction variance [9]. Furthermore, in order to achieve orthogonal blocks a total of 6 Cp equitably distributed in the three blocks were included.

The constructed design resulted in a CCD for $k = 4$, with $E = 30$, $Fp = 16$, $Sp = 8$, $\alpha = 1.414$ and $Cp = 6$. Experiments were divided in three blocks, with 10 runs each, which are shown in Table 1 in their actual values.

The experiments were performed in a randomized order (run order) to assure the independence of the results, minimizing the effects of uncontrolled factors. Then, the previously commented responses were evaluated.

2.7. Method validation

The experiments for method validation were done in the optimal established chromatographic conditions and following the ICH guidelines.

2.7.1. System suitability

A system suitability test was performed to confirm that the equipment and the developed method were adequate to obtain reliable results by evaluating peak performance. The test was carried out by injecting six replicates of a sample solution prepared as described in Section 2.5.

2.7.2. Selectivity

The selectivity of the method was evaluated in two stages. The first one by injecting both blank matrix and artificial samples containing maduramicin ammonium solutions, which were prepared by processing the matrix/artificial samples as previously described and by evaluating the presence of peaks at the same retention time for the analyte. The second stage consisted of forced degradation assays exposing the sample to different stress conditions. Aliquots of a sample solution were treated as follows: (a) oxidative study: with 1% H_2O_2; (b) reductive study: with elemental Zinc in 1 mol L^{-1} of HCl; (c) acidic treatment: with 1 mol L^{-1} of HCl; and (d) alkaline stress: with 1 mol L^{-1} of NaOH. In all these cases, treatments were made in a boiling water bath during 1 h under reflux and these solutions were neutralized prior to an injection in the chromatographic system. For temperature stress studies, a portion of the sample was maintained at 60 °C for 48 h, whereas for photostability another sample portion was exposed to direct sun light during 48 h and then processed as previously described to obtain sample solutions.

Table 1
Central composite design. Experiments and responses.

Std[a]	Run[a]	Factors (k)					Responses				
		Block	SHS[b]	SA[b]	pH	Temp. (°C)	K'_{HDP}	T_{HDP}	R_{HDP}[c]	R_{DNC}[c]	t_{DNC} (min)
1	12	2	10.0	20.0	3.00	25.0	1.56	1.02	6.17	0.92	9.95
2	7	1	20.0	20.0	3.00	25.0	2.04	1.19	0.98	2.14	10.44
3	8	1	10.0	20.0	4.00	25.0	2.38	1.10	1.02	8.26	10.52
4	18	2	20.0	20.0	4.00	25.0	1.84	1.26	1.00	1.47	10.15
5	1	1	10.0	20.0	3.00	40.0	4.46	1.09	8.74	2.21	10.48
6	20	2	20.0	20.0	3.00	40.0	3.88	0.95	2.48	2.00	10.45
7	13	2	10.0	20.0	4.00	40.0	1.96	1.44	1.54	6.99	10.21
8	5	1	20.0	20.0	4.00	40.0	3.29	1.03	5.09	1.12	10.17
9	9	1	10.0	100.0	3.00	25.0	1.67	1.02	16.36	1.44	10.22
10	19	2	20.0	100.0	3.00	25.0	1.84	1.51	8.11	8.23	10.27
11	14	2	10.0	100.0	4.00	25.0	2.65	1.17	1.02	9.23	10.51
12	4	1	20.0	100.0	4.00	25.0	1.75	1.15	13.23	1.54	9.94
13	15	2	10.0	100.0	3.00	40.0	4.03	0.77	6.25	1.51	10.17
14	2	1	20.0	100.0	3.00	40.0	2.49	1.51	5.79	1.09	10.28
15	3	1	10.0	100.0	4.00	40.0	2.21	1.38	1.21	2.29	10.22
16	11	2	20.0	100.0	4.00	40.0	2.48	1.39	8.78	1.76	10.27
17	10	1	15.0	60.0	3.50	32.5	1.79	1.65	8.27	7.34	10.28
18	6	1	15.0	60.0	3.50	32.5	1.69	1.18	0.97	2.24	10.49
19	17	2	15.0	60.0	3.50	32.5	2.01	1.05	12.14	1.02	10.24
20	16	2	15.0	60.0	3.50	32.5	1.85	1.13	1.01	1.90	10.41
21	22	3	7.9	60.0	3.50	32.5	1.84	1.15	12.38	1.16	10.15
22	28	3	22.1	60.0	3.50	32.5	2.16	1.40	4.73	8.18	10.29
23	21	3	15.0	60.0	2.79	32.5	2.86	1.05	3.68	1.31	10.27
24	24	3	15.0	60.0	4.21	32.5	2.84	1.07	5.93	2.13	10.49
25	30	3	15.0	60.0	3.50	21.9	1.73	1.09	9.19	1.31	9.96
26	27	3	15.0	60.0	3.50	43.1	2.55	1.22	7.60	1.63	10.29
27	23	3	15.0	3.4	3.50	32.5	1.98	1.27	1.07	3.10	10.33
28	25	3	15.0	116.6	3.50	32.5	2.31	1.37	7.58	6.92	10.10
29	26	3	15.0	60.0	3.50	32.5	4.62	1.14	1.79	1.94	10.39
30	29	3	15.0	60.0	3.50	32.5	2.11	1.20	13.61	1.02	10.24

[a] Std refers to the standard order in the design. Run refers to the experiment order.
[b] SHS and SA concentrations in mmol L^{-1}.
[c] Peaks resolutions of HDP and DNC were obtained in relation to the most proximal peaks occuring for each one, in each optimization chromatogram.

2.7.3. Linearity, precision and accuracy

Linearity was evaluated using the calibration standard solutions as described in Section 2.4 in triplicate for each level.

Precision was checked at the repeatability and intermediate precision levels. Repeatability or intra-assay variability was studied by making six replicate determinations of the commercial sample, applying the whole analytical procedure in the same day by the same analyst. Intermediate or inter-assay precision was evaluated by repeating the determination ($n = 6$) of the same sample but in another working day and conducted by another analyst.

The accuracy of the method was determined by extracting and analyzing artificial samples, prepared by fortifying matrix aliquots in levels corresponding to 80.0%, 100.0% and 120.0% of the expected amount of active compounds. Each level was studied in triplicate and recoveries of the added amounts were calculated.

3. Results and discussion

3.1. Chromatographic development

Preliminary chromatographic experiments, using a mobile phase and consisting in a buffer (5.0 mmol L^{-1} of SHS, 10.0 mmol L^{-1} of SA and pH = 3.00) with acetonitrile, allowed us to conclude about the necessity of applying a solvent gradient to reach proper retention of HDP in a first instance and the later elution of the highly retained DNC. The gradient was established varying the acetonitrile proportion as follows: 10.0% from initial time until 2.0 min, increasing to 80.0% in a linear way in 6.0 min. This proportion was maintained during 4.0 min and decreased linearly to initial conditions in 2.0 min. The total analysis time allowing column re-stabilization was set in 18.0 min. The flow rate was set at 1.00 mL min^{-1} and the injection volume was of 10 μL.

For method wavelength selection, the UV spectra of DNC and HDP in the elution conditions were evaluated by using the DAD response (Fig. 3). Due to the lower absorption properties of the HDP molecule, its maximum of absorption at 300 nm was chosen as detection wavelength because similar sensitivity for both components was attained. The other chromatographic conditions were established, based on the optimization of the results.

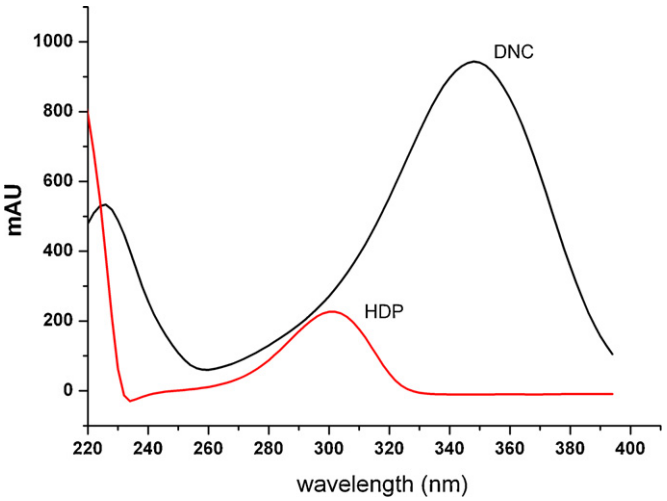

Fig. 3. Analyte spectral response recorded in the chromatographic system.

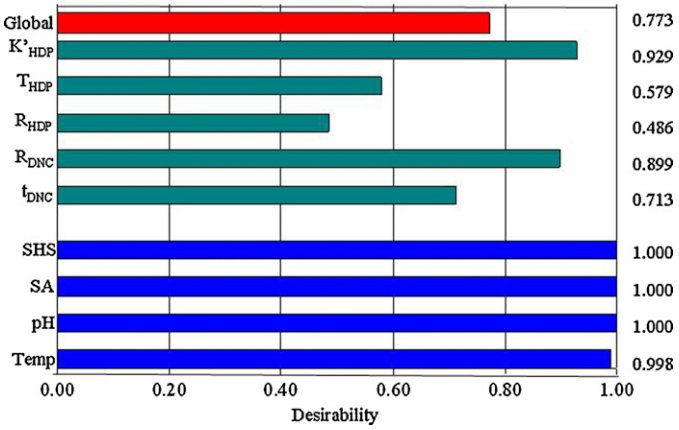

Fig. 4. Individual desirability reached by each variable in the optimized conditions: 10.0 mmol L^{-1} of SHS, 20.0 0 mmol L^{-1} of SA, pH = 3.30 and 40.0 °C oven temperature.

3.2. Optimization of the chromatographic separation

3.2.1. Models fitting and response surfaces

Data displayed in Table 1 were employed to build a model for each response fitting them to a second order polynomial function, responding to the general equation below:

$$y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii}x_i^2 + \sum_{1\le i\le j}^{k}\beta_{ij}x_i x_j + \varepsilon \quad (1)$$

where β_0 is the constant term, and β_i, β_{ii} and β_{ij} represent the coefficients of the first order terms, quadratic terms and interaction terms respectively, and ε is the residual associated to the experiments (k was mentioned before).

In each model, the terms were evaluated by ANOVA and a backward regression procedure was applied to eliminate the insignificant ones ($\alpha = 0.10$). Thus, simplified models, including only significant terms and those necessary to maintain the hierarchy were obtained and evaluated by ANOVA for model significance and lack of fit. The resulting models are shown in Table 2.

3.2.2. Desirability function and optimal conditions

As it can be seen, it is not possible by a simple visual inspection of the response surfaces obtained with the fitted models, to find out the experimental conditions (factors levels) to reach simultaneously the optimal value for all the evaluated variables. In such cases, the Derringer desirability function allows to obtain these parameters including, moreover, the researcher's priorities during the optimization procedure [9,35]. In a first step, a partial desirability function (di) for each individual response was created using the fitted models and establishing the optimization criteria for each one. Factors levels were also included in the optimization procedure, in order to prioritize the use of certain suitable conditions within the experimental region. The most desirable ranges for each design factor or response were selected, deciding if these factors or responses had to be maximized, minimized, maintained in the range or reach a target value. In addition, a weight (wi) or emphasis was given to each goal. More details about the application of this methodology can be seen in Refs. [10] and [11]. After that, the Global Desirability function (D) was obtained using the following equation:

$$D = \left(d_1^{r_1} x\, d_2^{r_2} x \cdots x\, d_n^{r_n}\right)^{1/\Sigma r_i} = \left(\prod_{i=1}^{n} d_1^{ri}\right)^{1/\Sigma r_i} \quad (2)$$

where n is the number of variables included in the optimization procedure and r_n is the importance of each factor or response relative to the others. The importance may vary from 1 for the least important variable to 5 for the most important one. Table 3 shows the criteria chosen for the optimization. Apart from optimizing the responses to reach adequate chromatographic peak parameters of the analyte, we also attempted to minimize the concentration of salts in the mobile phase to reduce costs and extend the column lifetime. Due to the fact that they are the most critical parameters, an importance of 5 was assigned to K'_{HDP} and T_{HDP}, whereas a lower importance was given to $t_{DNC;}$ having shown low variability in the experimental condition tested. The importance of the other variables was kept in an intermediate value.

Fig. 4 shows the individual desirability obtained for each variable in the optimized conditions. Note that optimal conditions were found by maintaining minimum salts concentration in the mobile phase and so a $di = 1$ was reach for these factors.

In order to evaluate the global behavior of the system, many D values were calculated using Eq. (2), where the di values were previously computed for different variable combinations. Fig. 5 shows the response surface for D, which was obtained by plotting the calculated values. In this figure most influencing factors were represented: pH and SHS concentration (for 20.0 mmol L^{-1} of SA and 40.0 °C).

Finally, values of the design variables that maximize D have been chosen as the optimal experimental conditions. They resulted in 10.0 mmol L^{-1} for SHS, 20.0 mmol L^{-1} for SA, pH = 3.30 and a temperature of 40.0 °C. Setting these experimental conditions, the following confidence interval values (95% CI) for the five responses were predicted by the fitted models: K'_{HDP} = 2.69–3.59, T_{HDP} = 0.97–1.28, R_{HDP} = 2.36–8.45, R_{DNC} = 0.82–4.08 and t_{DNC} = 10.18–10.30 min. The global desirability resulted in 0.773; which could be considered as highly acceptable taking into account the large number of variables being simultaneously optimized.

The suggested optimal conditions were then experimentally corroborated, obtaining chromatographic signals like the one presented in Fig. 6. An exhaustive inspection of the chromatographic peak parameters listed in Table 4, allows concluding about the good prediction capability of the fitted models.

Table 2
Models fitting.

Response (y)	Model	Significant terms (x_i)	Terms added to maintain hierarchy	ANOVA p value[b]	
				Model	Lack of fit
K'_{HDP}	2FI[a]	D–CD	C	0.004	0.916
T_{HDP}	2FI	AC–AB	A–B–C	0.019	0.772
R_{HDP}	2FI	B–AC	A–C	0.015	0.995
R_{DNC}	2FI	AC	A–C	0.044	0.429
t_{DNC}	2FI	AB	A–B	0.016	0.422

A = SHS concentration, B = SA concentration, C = pH, D = oven temperature.
[a] 2FI indicates a model with linear terms and interactions.
[b] p-Values less than 0.050 indicate significant parameter.

Table 3
Criteria followed for the optimization of individual factors and responses.

Variable	Goal	Limit		Weight		Importance
		Lower	Upper	Lower	Upper	
SHS	Minimize	10.0	20.0	–	1	3
SA	Minimize	20.0	100.0	–	1	3
pH	In range	3.00	4.00	–	–	3
Temperature	In range	25.0	40.0	–	–	3
K'_{HDP}	Maximize	1.60	4.62	0.5	–	5
T_{HDP}	Target = 1	0.77	1.65	0.5	0.5	5
R_{HDP}	Maximize	2.00	16.30	0.5	–	3
R_{DNC}	Maximize	2.00	9.20	0.2	–	3
t_{DNC}	Minimize	9.94	10.52	–	0.1	2

Table 4
Validation results and figures of merit.

Parameter	HDP	DNC	Acceptance criteria
System suitability ($n = 6$)			
Tailing factor (T)	1.14 (0.5)[a]	1.13 (0.2)[a]	≤ 2.0
Capacity factor (K')	2.40 (0.1)[a]	5.25 (0.5)[a]	≥ 2.0
Resolution (R)	2.56 (0.4)[a]	4.68 (0.2)[a]	≥ 2.0
Area RSD%	0.14	0.12	≤ 2.0
Linearity			
Range ($\mu g\,mL^{-1}$)	10.3–15.5	21.7–33.0	
Intercept	−2.7 (5.8)[b]	−2.1 (8.8)[b]	
Intercept	-2.7 ± 12.0	-2.1 ± 30.4	Include 0
95.0% confidence interval			
Slope	32.0 (0.4)[b]	20.5 (0.5)[b]	
Residual standard deviation	3.6	7.6	
Variance homogeneity	$F\,cal = 1.05$	$F\,cal = 2.25$	$F\,crit_{2,2\ (0.05)} = 19.00$
Lack of fit	$F\,cal = 2.25$	$F\,cal = 0.96$	$F\,crit_{13,10\ (0.05)} = 2.89$
R^2	0.997		≥0.99
Precision			
Intra-assay (RSD%)	0.38	0.46	≤2.0
Inter-assay (RSD%)	0.62	0.90	≤2.0
Variance between series	$F\,cal = 5.22$	$F\,cal = 2.25$	$F\,crit_{5,5\ (0.05)} = 7.39$
Accuracy			
Recovery (%)			
80.0% level	99.3 (0.9)[a]	101.4 (0.5)[a]	
98.0–102.0%			
100.0% level	100.2 (1.4)[a]	98.0 (0.4)[a]	
120.0% level	99.1 (0.5)[a]	100.4 (0.7)[a]	
Global recovery ($n = 9$)	99.5 (1.0)[a]	99.9 (1.6)[a]	
Difference from 100.0%	$t\,cal = 1.44$	$t\,cal = 0.19$	$t\,crit_{8\ (0.05)} = 2.31$

[a] Values between parentheses indicate RSD%.
[b] Values between parentheses indicate SD.

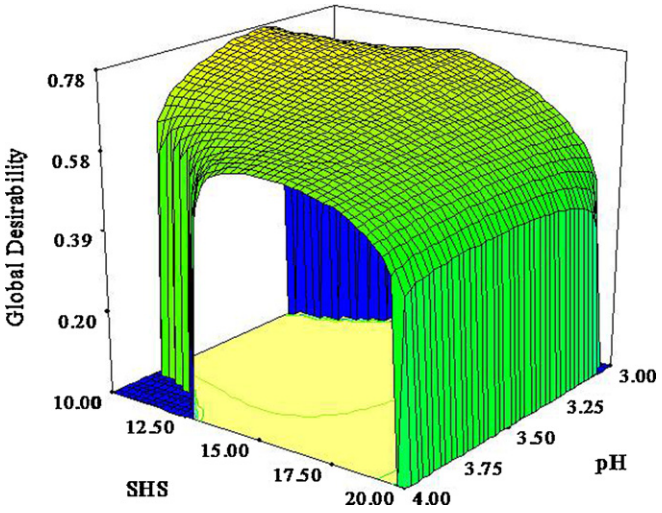

Fig. 5. Response surface of the global desirability as a function of pH and SHS concentration. The other factors are at their optimum: 20.00 mmol L^{-1} for SA and 40.0 °C for oven temperature.

3.3. Method validation and figures of merit

The evaluated system suitability parameters and the results obtained in the validation procedure are summarized in Table 4.

3.3.1. Selectivity

The sample solvent, matrix solution and artificial sample with maduramicin ammonium produced no peaks in the retention region corresponding to the analyte. The results of stress testing studies indicated in one hand, poor degradation of both components, with major degradation in reductive conditions and, on the other hand, a high selectivity of the method. This conclusion was supported on the observation that the principal peaks presented resolutions larger than 2.0 in the whole stressed samples. Finally, the photodiode array signal was also used to evaluate the homogeneity of the analyte peaks obtaining, in both cases, peak purity values larger than 0.999.

3.3.2. Linearity and range

The least squares method was applied to perform linear regressions for resulting peak areas in chromatograms, corresponding to calibration solutions vs. concentration. Calibration parameters obtained for both analytes together with the acceptance criteria

Table 5
Results obtained by analyzing NIC in commercial samples.

Sample	DNC (%, w/w)	HDP (%, w/w)	Ratio (DNC: HDP)	NIC (%, w/w)
Raw material				
1	68.0 (0.3)[a]	29.4 (0.2)	0.949:1.00	97.4 (0.3)
2	65.5 (0.4)	32.1 (0.2)	0.838:1.00	97.6 (0.4)
3	67.7 (0.4)	31.2 (0.3)	0.887:1.00	98.9 (0.4)
Feed additive				
NIC 25%	20.5 (0.5)	8.48 (0.08)	0.992:1.00	29.0 (0.5)
Feed additive				
NIC 8%	6.18 (0.06)	2.52 (0.05)	1.01:1.00	8.70 (0.05)

[a] Values between parentheses indicate SD ($n = 3$).

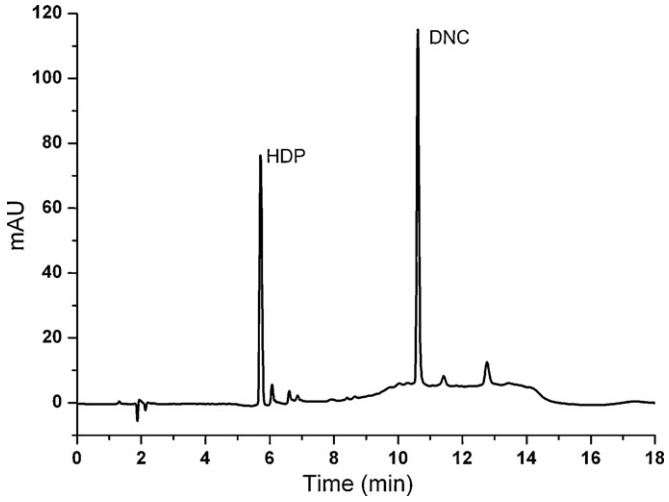

Fig. 6. Chromatogram of a real sample in the optimized conditions obtained at 300 nm for a 40.0 μg mL^{-1} NIC solution.

are listed in Table 4. The homoscedasticity of the data was evaluated trough an *F*-test of the variances at the lower and upper limit of range. The difference between the observed and the critical value of *F* was not significant ($\alpha = 0.05$). The lack of fit to linear model was also evaluated by an ANOVA test as suggested by IUPAC [36] with satisfactory results.

Since the studied range was for 80.0–120.0% of an expected concentration, as suggested by ICH guides for major active ingredients, LOD and LOQ were not calculated.

3.3.3. Precision

The relative standard deviation (RSD) of the obtained results were evaluated and an *F*-test ($\alpha = 0.05$) for comparison between series was performed showing acceptable precision parameters for the method.

3.3.4. Accuracy

Recoveries of the spiked amount of NIC in artificial samples were calculated and evaluated for each level. After that, a global recovery was computed and a *t*-test ($\alpha = 0.05$) was applied, finding that there were no statistical differences from theoretical recovery of 100.0%.

Furthermore, in order to verify the absence of the matrix effect, a linear regression employing the accuracy study data was performed for each analyte. No statistical differences were found by ANOVA ($\alpha = 0.05$) between slopes and intercepts corresponding to these regressions and those obtained for both standards prepared in solvent. These results enabled us to confirm that an external calibration procedure may be employed.

3.4. Applications

The developed method was applied to NIC components determination in several commercial samples of NIC bulk material, feed additive containing 25.0% NIC and feed additive with 8.0% NIC and 0.75% maduramicin ammonium. As it can be appreciated by examination of the results reported in Table 5, we have found considerable variations in the molecular ratio DNC:HDP among the analyzed samples.

4. Conclusions

A simple, efficient and fast method based in ion-pairing chromatography has been developed, optimized and validated for the simultaneous determination of HDP and DNC in feed additive, applicable to the routine analysis for the quality control of commercial samples. The use of chemometric tools such as the experimental design and the multiresponse optimization has shown to be of great help to achieve a fast and an efficient optimization of the chromatographic conditions.

By the application of the novel method, the molecular ratio of both compounds can also be determined in the same procedure. This is a considerable advantage of the method that allows a more complete characterization of the commercial products compared to the simple determination of DNC that has been applied so far. In addition, based on the results obtained on the specificity and stress studies, it can be concluded that the method may be used as a stability-indicating methodology.

Acknowledgments

The financial support from the "Universidad Nacional del Litoral" (Project CAI + D N° PI 12-65) and the "Consejo Nacional de Investigaciones Científicas y Técnicas" (CONICET) is gratefully acknowledged. The authors thank FACYT S.R.L for providing drugs and pharmaceuticals.

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