Accepted Manuscript

Title: Quality by Design approach in the development of hydrophilic interaction liquid chromatographic method for the analysis of iohexol and its impurities

Author: Marko Jovanović Tijana Rakić Anja Tumpa Biljana Jančić Stojanović

PII: S0731-7085(15)00151-X
DOI: http://dx.doi.org/doi:10.1016/j.jpba.2015.02.046
Reference: PBA 9988

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 3-12-2014
Revised date: 20-2-2015
Accepted date: 22-2-2015

Please cite this article as: M. Jovanović, T. Rakić, A. Tumpa, B.J. Stojanović, Quality by Design approach in the development of hydrophilic interaction liquid chromatographic method for the analysis of iohexol and its impurities, *Journal of Pharmaceutical and Biomedical Analysis* (2015), http://dx.doi.org/10.1016/j.jpba.2015.02.046

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Highlights

- QbD approach in HILIC method development is presented
- Analysis of iohexol and its related compounds is performed
- Monte Carlo simulation is applied for model uncertainty estimation
- Design Space is defined

Accepted Manuscript

ACCEPTED MANUSCRIPT

Quality by Design approach in the development of hydrophilic interaction liquid chromatographic method for the analysis of iohexol and its impurities

Marko Jovanović, Tijana Rakić*, Anja Tumpa, Biljana Jančić Stojanović

University of Belgrade – Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450, 11000 Belgrade, Serbia

*corresponding author: Tijana Rakić, University of Belgrade – Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450; Phone: +381 11 3951 333; e-mail: tijana.rakic@pharmacy.bg.ac.rs

Accepted Manuscript

ACCEPTED MANUSCRIPT

Abstract

This study presents the development of hydrophilic interaction liquid chromatographic method for the analysis of iohexol, its *endo* - isomer and three impurities following Quality by Design (QbD) approach. The main objective of the method was to identify the conditions where adequate separation quality in minimal analysis duration could be achieved within a robust region that guarantees the stability of method performance. The relationship between critical process parameters (acetonitrile content in the mobile phase, pH of the water phase and ammonium acetate concentration in the water phase) and critical quality attributes is created applying Design of Experiments methodology. The defined mathematical models and Monte Carlo simulation are used to evaluate the risk of uncertainty in models prediction and incertitude in adjusting the process parameters and to identify the design space. The borders of the design space are experimentally verified and confirmed that the quality of the method is preserved in this region. Moreover, Plackett-Burman design is applied for experimental robustness testing and method is fully validated to verify the adequacy of selected optimal conditions: the analytical column ZIC HILIC (100 mm x 4.6 mm, 5 μm particle size); mobile phase consisted of acetonitrile – water phase (72 mM ammonium acetate, pH adjusted to 6.5 with glacial acetic acid) (86.7:13.3) v/v; column temperature 25 ºC, mobile phase flow rate 1 mL min^{-1}, wavelength of detection 254 nm.

Keywords: Quality by Design, Design Space, iohexol, impurities, HILIC, Monte Carlo simulation

ACCEPTED MANUSCRIPT

1. Introduction

Modern trends in pharmaceutical industry and numerous regulatory documents in this field (Food and Drug Administration's (FDA) Good Manufacturing Practice for 21st Century [1], International Conference of Harmonization (ICH) Q8 [2]) strongly suggest the implementation of Quality by Design (QbD) concept in pharmaceutical product development and consequently, in analytical method development. QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [2].

Chemometrical tools such as Design of experiments (DoE) methodology are closely related to QbD and many basic concepts are very similar [3, 4]. Therefore DoE methodology combined with methodologies for identification of design space provides deep understanding of analytical systems and enable the identification of experimental region where the quality will be assured. Since liquid chromatography (LC) is the most commonly applied separation technique in pharmaceutical industry, the QbD concept is studied in LC systems by groups of authors such as Hubert at al. [3, 5, 6], Molnar at al. [7, 8], Orlandini, Furlanetto at al. [4, 9, 10]. However, the literature examination revealed that there are no papers dealing with risk management and design space in hydrophilic interaction liquid chromatography (HILIC).

HILIC has gained popularity in the analysis of polar and moderately polar analytes in recent years [11]. Since certain number of drugs and their impurities are polar, HILIC can represent a valuable alternative for their chromatographic separation and

ACCEPTED MANUSCRIPT

determination. In HILIC polar or moderately polar stationary phases and polar highly-organic mobile phases (representing the mixtures of > 60% of organic solvent and < 40% of water or aqueous buffer) are used. As a result, the selectivity in HILIC separation change with respect to the RP-LC separation.[12]. Bare silica columns and their polar modifications (with diol, polyethylene glycol, aminopropyl, amide, zwitterionic and other groups) are the columns of choice in this type of chromatography [11].

Zwitterionic stationary phases, which on their surface contain sulfoalkilbetaine functional groups, have relatively recently found application in HILIC [11, 12]. Such stationary phases possess both positive (strongly acidic sulfonic acid) and negative (strongly basic quaternary ammonium groups) charge separated with short alkil spacer. Since both types of charged groups are present in 1:1 molar ratio, it is reported that surface net charge is zero. Sulfoalkilbetaine phases strongly adsorb water; hence the retention of the analytes is driven mainly by polar interactions (hydrogen bonding and dipole-dipole). Nevertheless, weak electrostatic interactions can affect the retention of the analytes carrying either positive or negative charge.

Retention mechanism of separation in HILIC is rather complex and can involve three different types of processes: partitioning, surface adsorption and electrostatic interaction [13 - 16]. Consequently, retention behavior and the selectivity of the chosen analytes on the selected column are very often under strong influence of the factors related to the mobile phase composition. Therefore, this fact can be considered an advantage when the aim of the research is the optimization of chromatographic separation of the analytes' mixture.

Accepted Manuscript

ACCEPTED MANUSCRIPT

The incorporation of QbD strategy in HILIC method development is very important since it provides better understanding of this complex type of chromatography and enables dealing with optimization challenges in controlled manner. Moreover, the establishment of DS in HILIC is very important since this system is generally more vulnerable to slight experimental condition changes comparing to RP-LC. Therefore, the aim of this study was to present the QbD method development of HILIC method for the analysis of model mixture consisted of iohexol, its *endo* isomer and its three related compounds A, B and C. Chemical structures of these analytes are presented in Figure 1.

Figure 1

Regarding the structures of the analytes, they can be considered neutral in the pH range commonly applied in chromatographic systems. USP monograph of iohexol suggests gradient RP–LC method for the quantification of iohexol and its related compounds in pharmaceutical dosage forms [17]. In the literature, one paper involving the analysis of iohexol and related compounds A and B in iohexol solution for parenteral administration by LC–MS/MS method was found [18]. Our group has recently published the work suggesting the isocratic method for the simultaneous separation and quantification of iohexol and three related compounds A, B and C in iohexol solution for parenteral administration on diol stationary phase in HILIC mode [19]. However, the molecule of iohexol is present in two stereoisomeric forms: *endo* and *exo*, out of which *exo* isomer is dominant [20] and the suggested HILIC method did not provide the separation of *endo* and *exo* isomers of iohexol; both peaks eluted at the same retention time. Simultaneous analysis of iohexol and USP related compound B in canine and feline samples was also reported [20].

ACCEPTED MANUSCRIPT

Materials and methods

1.1. Chemicals and reagents

The analyzed substances iohexol and its related compounds A, B and C (obtained from *GE Healthcare Inc.*, South Central Europa) were working standards. All the reagents utilized in this study were of the analytical grade. The mobile phase and the solvents were prepared from acetonitrile (*Lab Scan*, Ireland), ammonium acetate (*Verdugt b.v.*, Netherlands), glacial acetic acid (*Zorka Pharma*, Serbia) and HPLC grade water. Omnipaque^{TM} solution of iohexole for parenteral use (*GE Healthcare Inc.*, South Central Europa) was kindly donated from local distributer.

1.2. Chromatographic conditions

The experiments were performed on chromatographic system *Finnigan Surveyor Thermo Scientific* consisting of HPLC Pump, Autosampler Plus and UV/VIS Plus Detector. *ChromQuest* software was used for data collection. The analytical column used was ZIC HILIC (100 mm x 4.6 mm, 5 μm particle size) (*Merck, KGaA,* Germany). Throughout the whole experimental procedure the following instrumental chromatographic conditions were maintained: flow rate of the mobile phase 1 mL min^{-1}, column temperature 25 °C, UV detection at 254 nm.

1.3. Mobile phase

Mobile phase consisted of acetonitrile and water phase (with added ammonium acetate and glacial acetic acid) where the amount of organic solvent, ammonium acetate concentration in the aqueous phase and pH of the aqueous phase were varied according to the experimental plan. Mobile phase under optimal chromatographic conditions was

ACCEPTED MANUSCRIPT

as follows: acetonitrile – 72 mmol L^{-1} ammonium acetate in water adjusted with acetic acid to pH 6.5 (86.7:13.3, v/v).

1.4. Standard solutions

Stock solutions for the method optimization and robustness testing contained100 μg mL^{-1} of iohexol, and 10 μg mL^{-1} of all the related compounds in the mixture of acetonitrile – 50 mmol L^{-1} ammonium acetate in water adjusted with acetic acid to pH 5.0 (85:15, v/v). Placebo mixture for selectivity estimation was prepared in a concentration ratio corresponding to the content in the pharmaceutical dosage form (solution for parenteral use). A standard solution, containing 500 μg mL^{-1} of iohexol and 0.5 μg mL^{-1} of each related compound was utilized to prove the selectivity. Seven solutions containing iohexol (250–750 μg mL^{-1}) and its related compounds A, B and C (0.25–0.75 μg mL^{-1}) were prepared in the mobile phase for linearity estimation. The accuracy estimation is performed using three series of three solutions containing placebo, iohexol in concentrations 400 μg mL^{-1}, 500 μg mL^{-1} and 600 μg mL^{-1} and its related compounds A, B and C in concentrations 0.4 μg mL^{-1}, 0.5 μg mL^{-1} and 0.6 μg mL^{-1}. The precision estimation was performed on real samples using Omnipaque™ containing 350 mg of iodine per mL (approximately 755 mg of iohexol per mL). The sample was diluted to contain 500 μg mL^{-1} of iohexol and spiked with related compounds in concentration of 0.5 μg mL^{-1}. Real samples testing was performed using Omnipaque™ diluted in the mobile phase to obtain the working solutions theoretically containing 500 μg mL^{-1} of iohexol. This procedure was repeated six times.

1.5. Software

Experimental plan and data analysis according to Box-Behenken design is created in DesignExpert 7.0.0. (Stat-Ease Inc., Minneapolis, MN, USA). The values of log P of the

Accepted Manuscript

ACCEPTED MANUSCRIPT

analyzed compounds were estimated in MarvinSketch 6.1.0 (ChemAxon Kft., Budapest, Hungary). Method optimization and Design Space definition is performed in MODDE 10.1 (UMETRICS, Umea, Sweden).

2. Results and discussion

In this paper QbD approach for development of HILIC method is presented through the following phases: 1) Analytical target profile (ATP) and critical quality attributes (CQAs); 2) Quality risk assessment (QRA) and critical process parameters (CPPs); 3) Investigation of knowledge space and Critical quality attributes modeling; 4) Optimization and design space; 5) Robustness testing and Method validation.

2.1. Analytical target profile and critical quality attributes

First objective of this work was the thorough investigation of chromatographic behavior of analyzed substances. Consequently, as first set of CQAs retention factors of investigated substances are selected. The second objective of the study was the development of method for the analysis of iohexol and its impurities where the maximal separation of substances in minimal analysis duration will be achieved. Moreover, in accordance with QbD principles, the optimal conditions should be surrounded with satisfactory design space in order to provide adequate robustness of the method. Therefore the optimal conditions are searched as experimental point where maximal selectivity factor of critical peak pair (*exo* and *endo* iohexol) in minimal analysis duration and with sufficient surrounding design space could be obtained.

2.2. Quality risk assessment and critical process parameters

Regarding the fact that mobile phases in HILIC usually represent aqueous- highly organic mixtures containing a buffer in a certain concentration, three main CPPs

ACCEPTED MANUSCRIPT

characterizing the mobile phase composition were identified: acetonitrile content in the mobile phase, concentration of ammonium acetate in the water and pH of the water phase (pH was adjusted with glacial acetic acid).

2.3. Investigation of knowledge space and CQA modeling

KS presents the part of the experimental space defined by the ranges of CPPs variation. Analyzing the preliminary retention data, intervals of CPPs were defined (Table 1). The experimental space is further on searched applying Box-Behnken design (Table 1).

Table 1

Multiple linear regression and least squares method were applied for creation of mathematical models for retention factors of the investigated substances and the results are presented in Table 2.

Table 2

Analyzing the retention behavior of investigated substances it was noted that acetonitrile content in the mobile phase strongly influenced the retention of all the analytes. The increase in acetonitrile content led to stronger retention of the analytes, which is in accordance with theoretical knowledge of HILIC. The magnitude of the influence of acetonitrile content on analytes' retention followed the order: related compound C < related compound B < related compound A < *exo* iohexol < *endo* iohexol. Taking into account the Log P values of iohexol and its related compounds A, B and C, that were estimated to be –2.59, –1.46, –1.35 and –0.64, respectively (MarvinSketch), it can be concluded that their elution order followed the pattern of the increased hydrophilicity. The fact that other mobile phase factors did not have

Accepted Manuscript

ACCEPTED MANUSCRIPT

statistically significant influence on the analytes' retention suggests the absence of direct interactions between the column surface and analytes. This can be for two reasons: (*i*) the surface of sulfoakilbetaine phases strongly adsorb water within the complete experimental range of factor combinations allowing the partitioning to take place, and (*ii*) the components of the mixture were not charged molecules, therefore electrostatic interactions were not likely to occur.

After modelling of retention factors, the mathematical model for selectivity factor of critical peak pair $\alpha_{4,5}$ was obtained in the following form:

$$\alpha_{4,5} = 1.17 + 0.061x_1 - 0.002x_2 + 0.009x_3 + 0.0009x_1x_2 + 0.004x_1x_3 - 0.0004x_2x_3 + 0.013{x_1}^2 + 0.009{x_2}^2 + 0.006{x_3}^2$$

Selectivity factor is chosen as separation criterion since it was proved that in all the experiments the peak width did not interfere with estimation of separation quality. The obtained model for $\alpha_{4,5}$ was characterized by satisfactory values of relevant statistical parameters. The second optimization target, minimal analysis duration, is measured by the model obtained for retention factor of the last eluting peak (k_5) presented in Table 2.

In order to visualize the dependence of selected optimization CQAs on investigated CPPs contour plots are constructed and presented in Supplementary files 1 and 2.

2.4. Optimization and Design space

The threshold of acceptable values were defined for $\alpha_{4,5}$ (1.15) and k_5 (10) in order to identify the set of experimental conditions with acceptable method performances. In the first phase sweet spot regions are constructed by overlay of contour plots for $\alpha_{4,5}$ and k_5 and presented in Figure 2.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Figure 2

However, sweet spots explain only the influence of CPPs variation on CQAs. On the other hand, recent advances in pharmaceutical science highlight the implementation of model uncertainty as additional source of variation of method performance [7 - 10]. In case of multiobjective optimization where several responses are described by individual functions, the resulting design space could be irregular and the mathematical function that describes it becomes very complex. Therefore, the alternative for identification of DS is the division of knowledge space into small subspaces and calculation the probability of fulfilling specification within each region. In this paper DS is created applying Monte Carlo simulations and obtained mathematical models using MODDE 10.1 software.

The robust optimization is performed expanding the design space around each potentially satisfactory point within knowledge space and choosing the one surrounded with the greatest DS. The identified point is characterized by the following CPPs combination: acetonitrile content in the mobile phase 86.7%, pH value of the water phase 6.5 and ammonium acetate concentration in the water phase 72 mmol L^{-1}. The resulting DS is presented in Figure 3.

Figure 3

The obtained boarders of DS (85.2% – 88.1% for acetonitrile content in the mobile phase, 4.7 – 7.8 for pH of the water phase and 49.1 – 94.8 mmol L^{-1} for ammonium acetate concentration in the water phase) present the region where the changes of CPPs will not disturb the quality of the method with the probability of 99%. The identified

ACCEPTED MANUSCRIPT

optimal conditions and Design space boarders are verified experimentally and the obtained results are presented in Figure 4 and Supplementary file 3.

Figure 4

2.5. Robustness testing and Method validation

Design of Experiments methodology is applied to experimentally investigate the robustness of the optimum. The factors whose variation is monitored in this phase and the experimental plan according to Plackett-Burman matrix are presented in Supplementary file 4. In the last stage of the study the developed HILIC method is validated. In Supplementary file 5 chromatograms of placebo and analyzed mixture are presented. The results of the remaining validation parameters and acceptance criteria [21] are summarized in Supplementary file 6. Finally, the developed method was applied to real sample and the obtained results were in agreement with the declared content.

3. Conclusion

This study presented the usefulness of QbD approach implementation in HILIC method development. The importance of this strategy in modern pharmaceutical analysis is emphasized and each step of QbD process is described in details. The definition of critical quality attributes and critical process parameters is explained. Special attention is devoted to DoE methodology application for creation of reliable mathematical models for knowledge space examination. The application of Monte Carlo simulation for propagation of model uncertainty and uncertainty of process parameters adjusting is used for creation of DS and robust optimization. The verification of the DS, multivariate

Accepted Manuscript

ACCEPTED MANUSCRIPT

experimental robustness testing and validation confirmed that systematic building of quality leads to the creation of highly reliable chromatographic methods.

Acknowledgements The authors thank the Ministry of Education, Science and Technological Development of the Republic of Serbia for supporting these investigations in Project 172052.

Accepted Manuscript

ACCEPTED MANUSCRIPT

References

1. US Food and Drug Administration (FDA), Department of Health and Human Services, Pharmaceutical Quality for 21st Century A Risk Based Approach Progress Report, May 2007. (http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm128080.html).

2. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Topic Q8 (R2): Pharmaceutical Development, ICH, Geneva, Switzerland, 2009.

3. E. Rozet, P. Lebrun, B. Debrus, P. Boulanger, Ph. Hubert, Design Space for analytical methods, TRAC- Trend. Anal. Chem. 42 (2013) 157-167.

4. S. Orlandini, S. Pinzauti, S. Furulanetto, Application of quality by design to the development of analytical separation methods, Anal. Bioanal. Chem. 405 (2013) 443-450.

5. P. Lebrun, B. Govaerts, B. Debrus, A. Ceccato, G. Caliaro, P. Hubert, B. Boulanger, Development of a new predictive modelling technique to find with confidence equivalence zone and design space of chromatographic analytical methods, Chem. Intell. Lab. 9 (2008) 4-16.

6. B. Debrus, P. Lebrun, J. Mbinze Kindenge, F. Lecomte, A. Ceccato, G. Caliaro, J. Mavar Tayey Mbay, B. Boulanger, R.D. Marini, E. Rozet, Ph. Hubert, Innovative high-performance liquid chromatography method development for the screening of 19 antimalarial drugs based on a generic approach, using design of experiments,

Accepted Manuscript

ACCEPTED MANUSCRIPT

independent component analysis and design space, J. Chromatogr. A 1218 (2011) 5205-5215.

7. I. Molnár, H.J. Rieger, K.E. Monks, Aspects of the "Design Space" in high pressure liquid chromatography method development, J. Chromatogr. A 1217 (2010) 3193-3200.

8. K.E. Monks, H.J. Rieger, I. Molnár, Expanding the term "Design Space" in high performance liquid chromatography (I), J. Pharm. Biomed. Anal. 56 (2011) 874-879.

9. S. Furlanetto, S. Orlandini, B. Pasquini, M. Del Bubba, S. Pinzauti, Quality by Design approach in the development of a solvent-modified micellar electrokinetic chromatography method: Finding the design space for the determination of amitriptyline and its impurities, Anal. Chim. Acta 802 (2013) 113-124.

10. S. Orlandini , B. Pasquini, M. Stocchero, S. Pinzauti, S. Furlanetto, An integrated quality by design and mixture-process variable approach in the development of a capillary electrophoresis method for the analysis of almotriptan and its impurities, J Chromatogr A 1339 (2014) 200-209.

11. P. Jandera, Stationary and mobile phases in hydrophilic interaction chromatography: a review, Anal. Chim. Acta. 692 (2011) 1-25.

12. P. Hemstrom, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29 (2006) 1784-1821.

13. B. Buszewski, S. Noga, Hydrophilic interaction chromatography (HILIC) – a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231-247.

ACCEPTED MANUSCRIPT

14. A. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, J. Chromatogr. 449 (1990) 177-196.

15. D.V. McCalley, Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography, J. Chromatogr. A 1217 (2010) 3408-3417.

16. S. Echeverría, P Herrero, F. Borrull, N. Fontanals, E. Pocurull, Performance of zwitterionic hydrophilic interaction LC for the determination of iodinated X-ray contrast agents, J. Sep. Sci. 36 (2013), 3688-3695.

17. U.S. Pharmacopoeia 35 (USP 35), The United States Pharmacopoeial Convention, Rockville, 2012.

18. J. Liu, M. Cai, L. Tan, H.Y. Ji, J.P. Shi, Y. Wang, Simultaneous determination of two related compounds in iohexol and its injection by LC-MS/MS, Chin. Pharm. J. 45 (2010) 468-471.

19. M. Jovanović, T. Rakić, B. Jančić-Stojanović, D. Ivanović, M. Medenica, Retention mechanisms assessment and method development for the analysis of iohexol and its related compounds in hydrophilic interaction liquid chromatography, Anal. Bioanal. Chem. 17 (2014) 4217-4232.

20. S. De Baere, P. Smets, N. Finch, R. Heiene, P. De Backer, S. Daminet, S. Croubels, Quantitative determination of exo- and endo-iohexol in canine and feline samples using high performance liquid chromatography with ultraviolet detection, J. Pharm. Biomed. Anal. 61 (2012) 50-56.

Accepted Manuscript

ACCEPTED MANUSCRIPT

21. J.B. Crowther, In: Ahuja S, Scypinski S (eds): Handbook of Modern Pharmaceutical Analysis, 2nd edn. Academic Press, New York, 2001.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Figure caption

Figure 1. The chemical structures of investigated substances

Figure 2. Sweet spot plots obtained by plotting acetonitrile amount vs. pH of the water phase at three ammonium acetate concentration in the water phase: A) 20 mM; B) 50 mM and C) 80 mM defined by requirements $\alpha_{4,5} > 1.15$ and $k_5 < 10$. Regions where only one criterion is met are colored in blue, while regions where both criteria are met are colored in green (to be reproduced in color on the Web (free of charge) and in black-and-white in print)

Figure 3. Design space from three different perspectives A) acetonitrile content vs. pH of the water phase, while ammonium acetate concentration is 72 mM; B) acetonitrile content vs. ammonium acetate concentration while pH of the water phase is 6.5; C) pH of the water phase vs. ammonium acetate concentration while acetonitrile content is 86.7% (to be reproduced in color on the Web (free of charge) and in black-and-white in print)

Figure 4. The experimentally obtained chromatogram under the optimal conditions

Figure caption for supplementary files

Supplementary 1. Contour plots for k_5 obtained by plotting acetonitrile amount vs. pH of the water phase at three ammonium acetate concentration in the water phase: A) 20 mM; B) 50 mM and C) 80 mM

Accepted Manuscript

ACCEPTED MANUSCRIPT

Supplementary 2. Contour plots for $\alpha_{4,5}$ obtained by plotting acetonitrile amount vs. pH of the water phase at three ammonium acetate concentration in the water phase: A) 20 mM; B) 50 mM and C) 80 mM

Supplementary 5. Representative chromatograms of A) placebo mixture and B) laboratory mixture for precision testing. Peak annotation: 1 - related substance C; 2 – related substance B; 3 – related substance A; 4 – *exo* iohexol; 5 – *endo* iohexol

Accepted Manuscript

ACCEPTED MANUSCRIPT

	x_1	x_2	x_3	k_1	k_2	k_3	k_4	k_5
1	80	3.0	50	0.11	0.41	0.71	1.11	1.39
2	90	3.0	50	1.45	3.28	6.16	12.04	15.31
3	80	7.0	50	0.10	0.38	0.66	1.03	1.29
4	90	7.0	50	1.30	2.86	5.41	10.31	13.13
5	80	5.0	20	0.08	0.35	0.61	0.96	1.20
6	90	5.0	20	1.26	2.80	5.20	9.95	12.51
7	80	5.0	80	0.11	0.40	0.71	1.12	1.40
8	90	5.0	80	1.35	2.90	5.62	10.66	13.73
9	85	3.0	20	0.36	0.93	1.58	2.63	3.27
10	85	7.0	20	0.37	0.89	1.53	2.55	3.17
11	85	3.0	80	0.44	1.04	1.83	3.05	3.84
12	85	7.0	80	0.42	0.96	1.70	2.81	3.53
13	85	5.0	50	0.38	0.92	1.61	2.66	3.34
14	85	5.0	50	0.39	0.91	1.61	2.67	3.35
15	85	5.0	50	0.40	0.92	1.62	2.68	3.18

Table 1. Plan of experiments and the obtained retention data

x_1 –acetonitrile content in the mobile phase (%);x_2 – pH of the water phase; x_3 – ammonium acetate concentration in the water phase (mmol L^{-1}); k_1– retention time of related compound C; k_2– retention time of related compound B; k_3– retention time of related compound A; k_4– retention time of *exo* iohexol; k_5– retention time of *endo* iohexol.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 2. Coefficients of the obtained second-order polynomial retention models in terms of coded factor values and statistical analysis

	k_1		k_2		k_3		k_4		k_5	
	coefficient	***p*–value**	**coefficient**	***p*–value**	**coefficient**	***p*–value**	**coefficient**	***p*–value**	**coefficient**	
	0.390	<0.0001*	0.920	<0.0001*	1.610	<0.0001*	2.670	<0.0001*	3.290	<
	0.620	<0.0001*	1.290	<0.0001*	2.460	<0.0001*	4.840	<0.0001*	6.180	<
	–0.020	0.1117	–0.071	0.0578	–0.120	0.0676	–0.270	0.0878	–0.340	
	0.034	0.0235*	0.043	0.1984	0.120	0.0771	0.190	0.1862	0.290	
	–0.034	0.0686	–0.094	0.0707	–0.180	0.0657	–0.410	0.0683	–0.520	
	0.016	0.3200	0.014	0.7523	0.079	0.3380	0.140	0.4669	0.260	
	–0.007	0.6531	–0.011	0.7986	–0.021	0.7907	–0.041	0.8282	–0.051	
	0.320	<0.0001*	0.740	<0.0001*	1.490	<0.0001*	3.180	<0.0001*	4.120	<
	0.024	0.1814	0.080	0.1199	0.120	0.1729	0.270	0.2056	0.370	
	–0.013	0.4317	0.042	0.3682	–0.075	0.3785	–0.180	0.3748	–0.200	
	0.9988		0.9978		0.9980		0.9972		0.997	
	0.9965		0.9938		0.9945		0.9922		0.992	
	0.9804		0.9648		0.9688		0.9557		0.955	

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

k_1– retention time of related compound C; k_2– retention time of related compound B; k_3– retention time of related compound A; k_4– retention time of *exo* iohexol; k_5– retention time of *endo* iohexol

* Coefficients significant for p-value < 0.05

ACCEPTED MANUSCRIPT

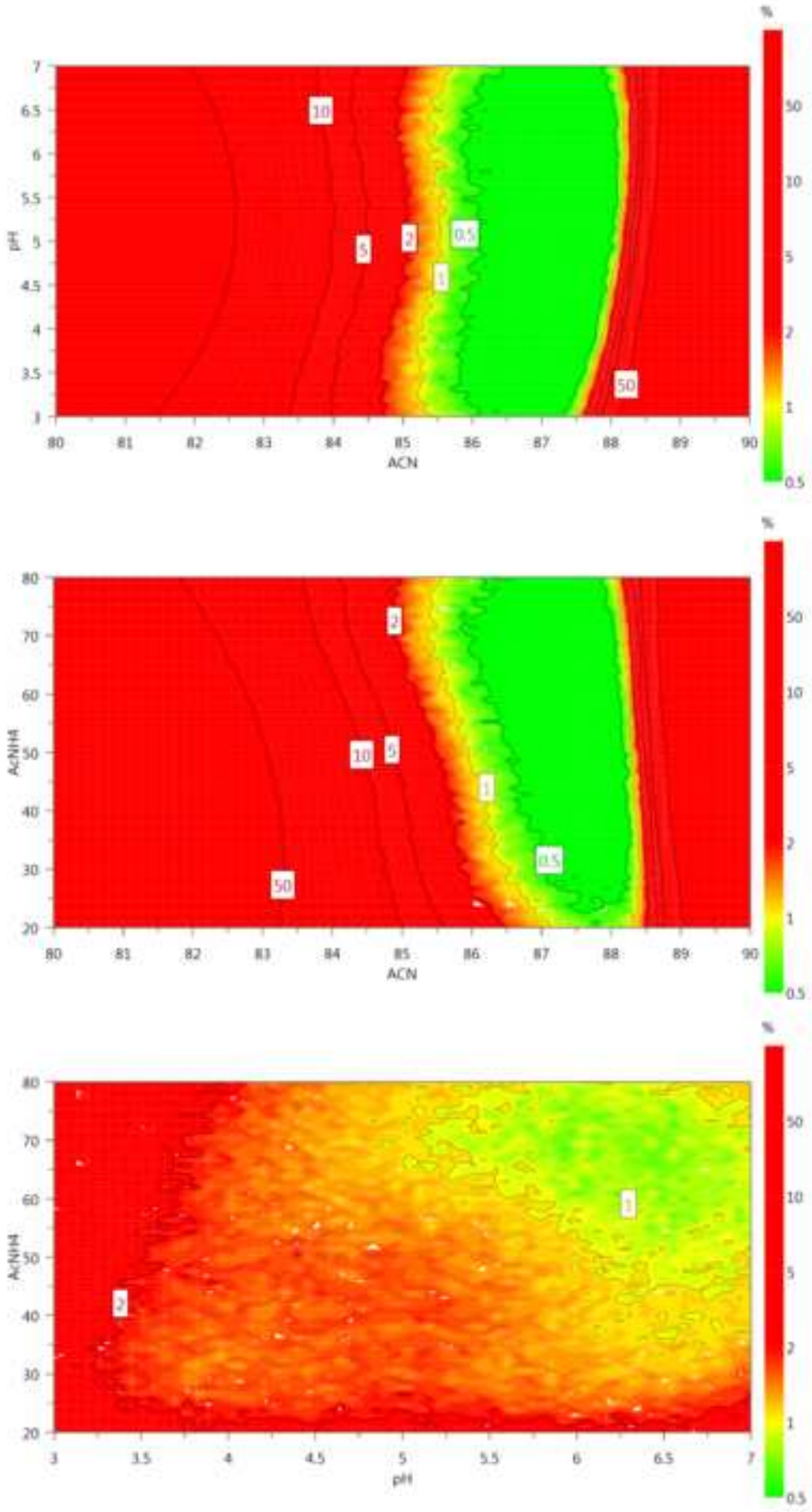
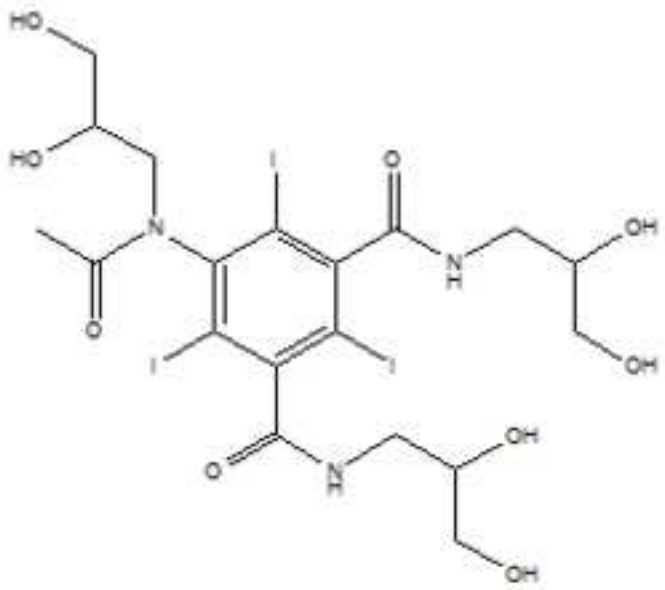
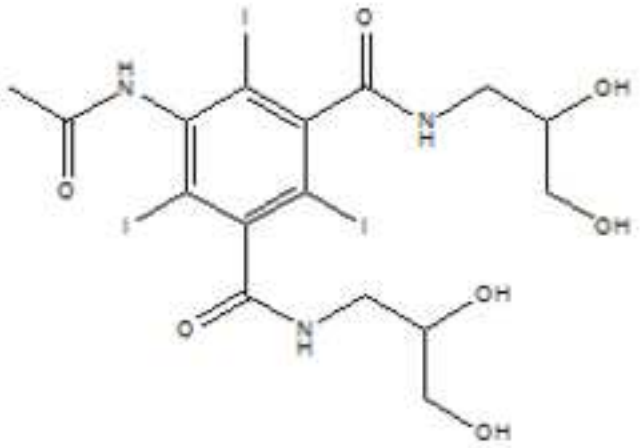


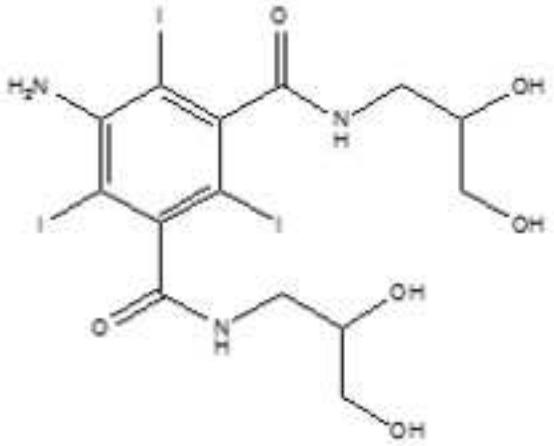
Figure 1



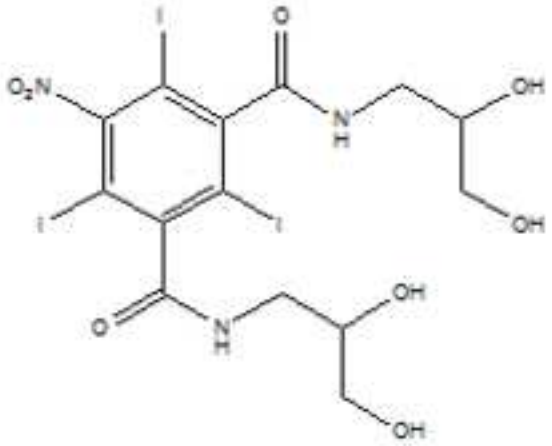
Iohexol



Related Compound A



Related Compound B



Related Compound C

Figure 2

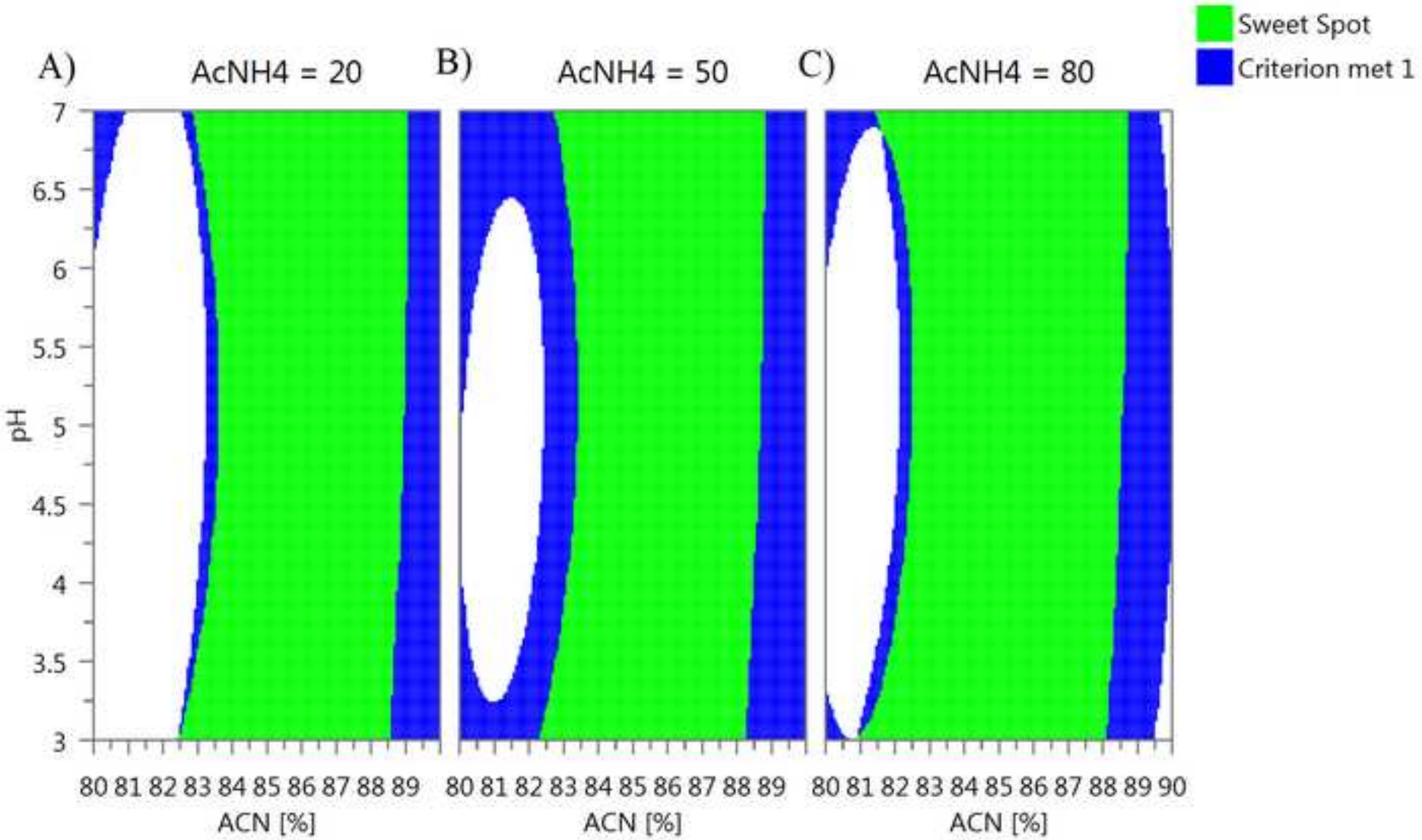
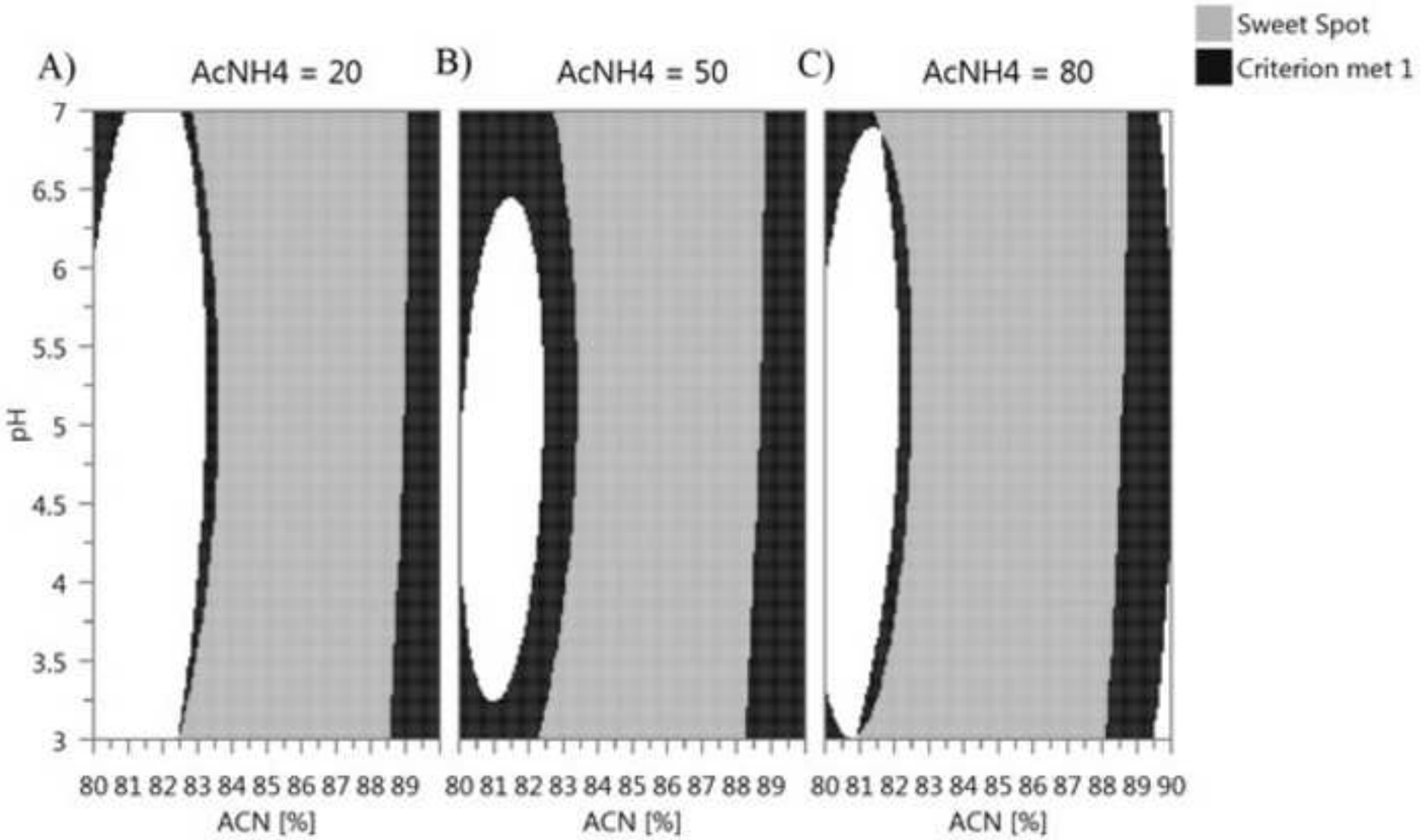
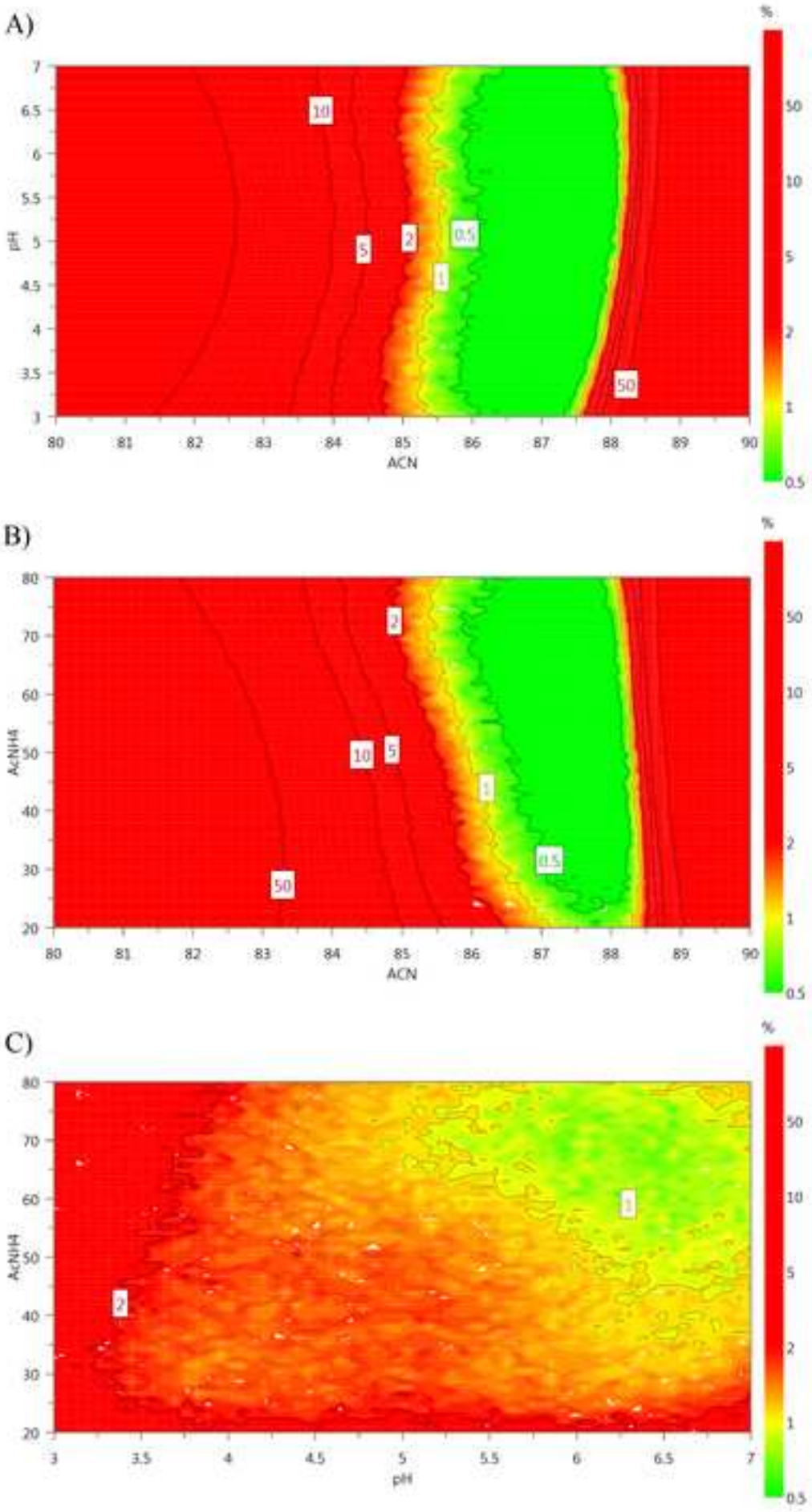

Sweet Spot
Criterion met 1
A)
AcNH4 = 20
B)
AcNH4 = 50
C)
AcNH4 = 80
pH
7
6.5
6
5.5
5
4.5
4
3.5
3
80 81 82 83 84 85 86 87 88 89
ACN [%]
80 81 82 83 84 85 86 87 88 89
ACN [%]
80 81 82 83 84 85 86 87 88 89 90
ACN [%]

Figure 2 black and white



ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT

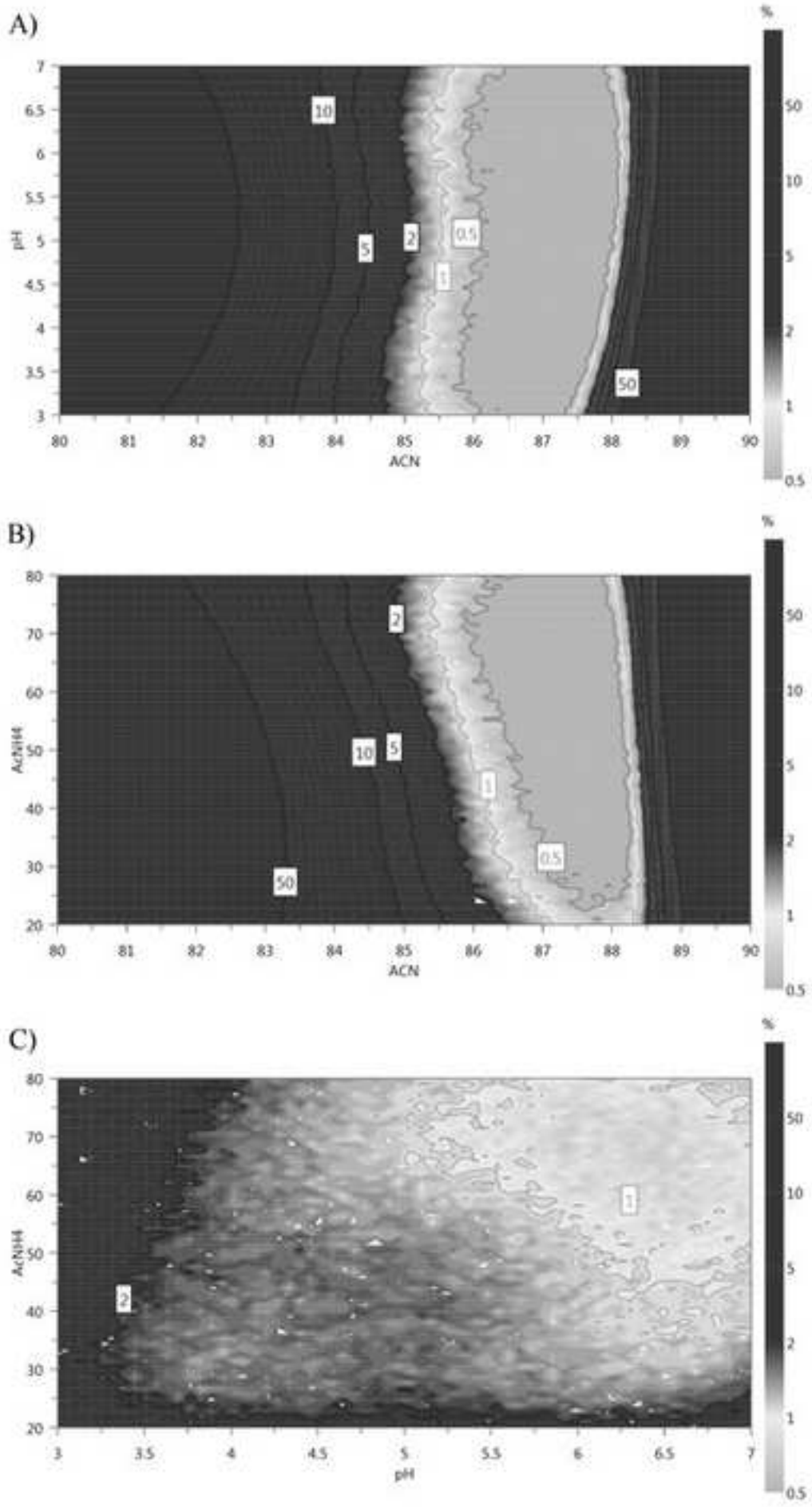
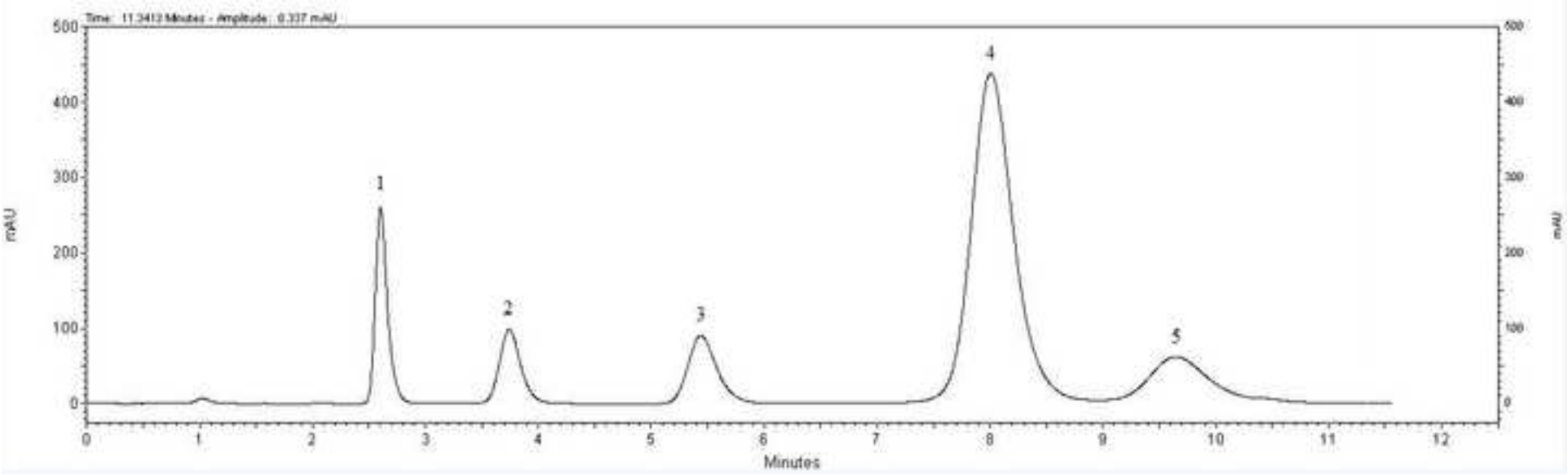


Figure 4



Manuscript