DETERMINATION OF AMMELIDE (AMD) AND DICYANDIAMIDE (DCD) IN ANIMAL FEED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A MS/MS DETECTOR (HPLC-MS/MS)

Phan Thi Thanh Dieu[1], Bui Van Tam[2], Dang Van Su[1*]

[1]*Ho Chi Minh City University of Food Industry*

[2]*National Centre for Veterinary Drugs and Bio-Products Control No.2*

*Email: *sudv@hufi.edu.vn*

Received: 12 April 2021; Accepted: 19 July 2021

ABSTRACT

The purpose of this study is to determine the content of adulterated protein enhancers, ammelide (AMD) and dicyandiamide (DCD), in animal feed according to the sample preparation process including the cleaning process by solid phase extraction (SPE) and quantification by HPLC-MS/MS. Method limit detection and quantitation values for both AMD and DCD were of 5 and 10 ppb, respectively. The good performance of the method in term of low threshold of detection, high sensitivity and reliability made it possible to be effectively applied at National Centre for Veterinary Drugs and Bio-Products Control No.2 to control the content of AMD and DCD in the basis of actual animal feed samples.

Keywords: Pseudo protein enhancers, AMD, DCD, animal feed, SPE, HPLC-MS/MS.

1. INTRODUCTION

The study of AMD ($C_3H_4N_4O_2$) and DCD ($C_2H_4N_4$) has recently become an important aspect of food chemistry. AMD is a compound used for syntheses of a large variety of materials for applications such as personal care products, cosmetics, child toys [1]. DCD is a chemical compound, widely applied as a soil fertilizer and prevent nitrogen loss in soil [2].

Figure 1. The structures of dicyandiamide (a) and ammelide (b)

They are characterized by a high content of nitrogen in the molecular formula, so mixed into milk, animal feed to artificially increase total protein content to cope with the product quality controls [3, 4]. In many studies, they can be reported as the causes of kidney problems after consuming several weeks [5-7]. Thus, on December 14, 2017, No. 10375 on "Chemicals used in the production and trading of feed" was issued by the Ministry of Agriculture and Rural Development [3], which requires the state agencies must ensure that the above substances are not used in livestock and the industrial laboratories have immediately developed methods to test these ones. The AMD and DCD contents in animal feed should not exceed 2.5 ppm [8, 9]. Various methods for determining the content of these substances in milk have recently been reported. Chen *et al*. proposed the process of determining DCD in

milk samples by LC-MS/MS using d-SPE and LLE techniques to clean the sample combined with the internal standard to quantify [10]. MacMahona *et al*. published the procedure for determining DCD, AMD and some melamine derivatives in infant food samples by LC-MS/MS method with LOQ from 18-162 ppb (depending on substances) [11]. In addition, some authors published the procedures for determining DCD and AMD by conventional methods such as UV [12], GC/MS [13], ion exchange chromatograph [14] or creating complexes and determining by UV-Vis [15]. In general, the above methods required the use of specific chemicals (using internal standards [10], derivatives [15]) and expensive sample preparation techniques. Determining the content of fake protein enhancers such as Ammelide (AMD) and Dicyandiamide (DCD) in animal feed by HPLC-DAD with quantitative limits of 0.1-1.000 ppm have recently been reported [16]. However, there has not been any study of AMD and DCD analysis methods in animal feed by HPLC-MS/MS. Animal feeds are a very complicated matrix because they are a mixture of many different components such as proteins, fats, antibiotics, minerals and some other components [17, 18]. The aim of this study is to develop a process to identify AMD and DCD according to the sample preparation process combined with the cleaning process by solid phase extraction (SPE) and quantification by HPLC-MS/MS, which is reported low threshold of detection, high sensitivity and reliability [19].

2. MATERIALS AND METHODS

2.1. Materials

Ammelide standard was purchased from Dr. Ehrenstorfer GmbH, ammonia solution and formic acid from Merck (Germany), cyanoguanidine from Sigma and acetonitrile (ACN) from Fisher (USA), super clean water and trifluoroacetic acid (TFA) from Acros (Belgium). All commercial obtained chemicals were of the highest purity available. DCD and AMD 1000 ppm stock standard solution, mobile phases, blank sample and standard spiked sample were prepared as steps in previous publish [16].

2.2. Methods

2.2.1. Analytical process

Samples of animal feed were ground and homogenized by IKA homogenizer. The homogenized sample (1-2 ± 0.5 g) was weighed into a 50 mL centrifuge tube with 25 mL of ACN:H_2O extraction solution (50:50, v:v). The tube was well shaken with 2500 rpm for 30 minutes, then centrifuged with 6000 rpm at 4 °C for 10 minutes. The resulting extract is filtered through a membrane filter (0.45 µm - 25 mm) [20, 21]. Solution after filtration (20 mL) was cleaned by solid phase extraction (SPE) with an extraction solvent of ACN:H_2O (50:50, v:v), SPE SCX (cation extraction) extraction column 500 mg/3 mL [22, 23]. The solution obtained after cleaning by SPE was concentrated with nitrogen gas and redissolved with 2 mL of dissolved solution. This solution was then injected into the HPLC system. A stationary phase with a HILIC column (Inertsil Sciences Inc., 5 µm (pore size 100 Å), 4.6 × 250 mm) was used for LC separation. A flow rate of 0.3 - 0.5 mL min^{-1} of mobile phase solution (50-70 ACN, pH 6-7) with phase A (ammonium acetate 10 mM) and phase B (ACN). An Agilent HPLC 1260 system and MS/MS Triple Quad 6420 was used to separate and measure AMD and DCD in all experiments [24-27].

2.2.2. Optimization for MS/MS detector

The parameters of MS/MS such as gas flows, ion spray voltage, source temperature and injection pressure were optimized based on the study of Kruve and AOAC [29-31]. The gas was set at 6-10 L min^{-1} (± 20%), ion spray voltage was 2500-5500V, the source temperature was 250-350 ^oC (± 10 ^oC) and injection pressure was 26-50 psi (± 5 psi / ± 1 L min^{-1}). Box-Behnken design (BBD) model was proposed with the number of experiments at the center of three to optimize the parameters affecting the ionization process by ESI ionization source. Standard solutions of AMD and DCD with concentrations of 50 ppb and 250 ppb were respectively selected to optimize the conditions of MS/MS.

2.2.3. Method validation

2.2.3.1. Specificity

The samples (animal feed) were added with standard AMD and DCD solutions (10-150 ppb), well mixed, dried at about 60^oC for 8 hours and then analyzed as a standard addition samples. According to Agilent manufacturer's instructions - Application Note 01916 and 5991-0416EN [32, 33], the fragmentation conditions of LC-MS/MS device were shown in Table 1.

Table 1. The fragmentation conditions of LC-MS/MS devices

Substances	Parent Ion	Daughter Ion (Confirmed Ion)	Daughter Ion (Quantified Ion)	Dwell Time (ms)	Fragment	Collision Energy (V)
AMD (ESI -)	127	84		200	100	8
			42	200	100	20
DCD (ESI +)	85	68		200	100	12
			43	200	100	18

2.2.3.2. Limit of detection and limit of quantitation

LOD is estimated based on the method of the National Institute for Food Control [34], LOQ was determined by the following formula: $LOQ = 3 - 10 \times C_{LOD}$ (*1*)

LOD and LOQ of the LC-MS/MS system were determined by injecting the standard solution with concentration of about 100 ppb or less into MS; diluting the concentration of the above solution until a signal of the peak that met the signal / noise requirements (S/N) ≥ 3 - 10 (for toxic substances) and S/N ≥ 3 (for non-toxic substances), according to SANCO/825/00 rev.8.1 16/11/2010 [35].

The LOD and LOQ of the method were as follows: from the LOD of the LC-MS/MS system, the amount of standard solution added to the blank sample was calculated so that 1 g of the standard spiked sample contained was equal to the LOD of the device; homogenizing the standard spiked sample according to ISO Guide 35:2017 [36] and EC 657/2002 [37]; the sample was processed and injected into the chromatographic system to determine S/N; increasing or decreasing the amount of standard solution added to the blank sample according to the results of S/N until it complied with the requirements for the determination of LOD and LOQ according to EC 657/2002 [37].

2.2.3.3. Working ranges

Standard solutions with concentration ranged from 1 - 1000 ppb were injected into HPLC system with injection procedure from low to high concentration solution. The standard solution was treated the same as the sample solution. The calibration curve was investigated on the MS/MS.

2.2.3.4. Precision and recovery

Based on the guidance of EC 657/2002 [37] and ISO Guide 35:2017 [36], standard spiked sample with known concentration of AMD and DCD standard solution created to investigate the precision and recovery. The concentrations of standard solution were added to the sample to investigate repeatability and recovery of LC-MS/MS from 10 ppm - 150 ppm.

The standard spiked sample was determined repeatability at a concentration of 150 ppb of AMD and DCD (3 times). The precision and the recovery of the method were evaluated by a comparison of the obtained results and the theoretical concentration of the analyte. The evaluation was based on the guidance of AOAC Appendix F [30, 31].

3. RESULTS AND DISSCUSION

3.1. Serveyed the parameters of MS/MS detector

3.1.1. Optimized the parameters of MS detector for AMD

3.1.1.1. Model compatibility

The optimization of MS parameters after calculating with Statgraphics Centurion XV (Version 15.1.02) were shown in Table 2.

Table 2. Variant values of AMD

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A: Ion chamber temperature (^oC)	230070.7855	1	230070.7855	7.84	0.1074
B: Gas flow rate (mL min^{-1})	965727.4273	1	965727.4273	32.90	0.0291
C: Injection pressure (psi)	498422.5122	1	498422.5122	16.98	0.0542
D: Voltage (V)	824894.365	1	824894.365	28.10	0.0338
AA	352653.7216	1	352653.7216	12.01	0.0741
AB	3114.052934	1	3114.052934	0.11	0.7756
AC	21219.44299	1	21219.44299	0.72	0.4847
AD	17492.3505	1	17492.3505	0.60	0.5209
BB	519.6822662	1	519.6822662	0.02	0.9063
BC	10483.14384	1	10483.14384	0.36	0.6107
BD	473476.5869	1	473476.5869	16.13	0.0568
CC	156679.2158	1	156679.2158	5.34	0.1471
CD	78827.32872	1	78827.32872	2.69	0.2429
DD	95506.70654	1	95506.70654	3.25	0.2130
Lack-of-fit	1296106.131	10	129610.6131	4.42	0.1987
Pure error	58703.62795	2	29351.81398		
Total (corr,)	4949978.295	26			

R-squared = 72.63%; R-squared (adjusted for d.f.) = 40.6983%; Standard Error of Est. = 336.007; Mean absolute error = 182.636; Durbin-Watson statistic = 1.23449 (P = 0.0458); Lag 1 residual autocorrelation = 0.359452.

The results in Table 2 showed that the gas flow rate (P-Value = 0.0127) and voltage (P-Value = 0.0192) are the factors that significantly affected the analysis results. Two factors

of ion chamber temperature (P-value = 0.1789) and injection pressure (P-value = 0.0574) did not significantly affect the analysis results, so they had no significant meaning to the ionization. The Lack-of-fit value with P-value = 0.1987 (> 0.05) showed the suitability of the model to the observed data at a 95.0% confidence level showing the model above perfectly suited to the experimental design.

Equation prediction by area: Area of AMD=-1753.63+6.73943*Gas Temp+59.243*Gas Flow+15.789*Nebulizer+0.126622*Capillary–0.0101273*Gas Temp^2–0.0303917*Gas Temp*Gas Flow – 0.0132223*Gas Temp*Nebulizer + 0.0000960407*Gas Temp*Capillary + 0.403891*Gas Flow^2 + 0.232342*Gas Flow*Nebulizer–0.0124917*Gas Flow*Capillary–0.110962*Nebulizer^2–0.00084949*Nebulizer*Capillary–0.00000528235*Capillary^2 *(1)*

Where: Gas Temp: Ion chamber temperature (^oC)

Gas Flow: Gas flow rate (mL min^{-1})

Nebulizer: Injection pressure (psi)

Capillary: Voltage (V)

3.1.1.2. Influence of factors on MS/MS detector

The results of factors affecting MS/MS for AMD were shown in Figures 1 and 2.

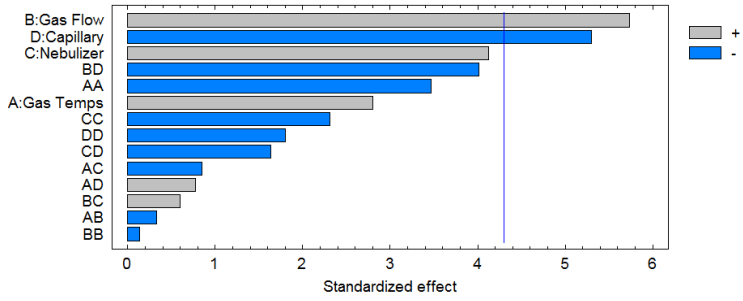

Figure 1. The standardized Pareto chart for concentration for AMD

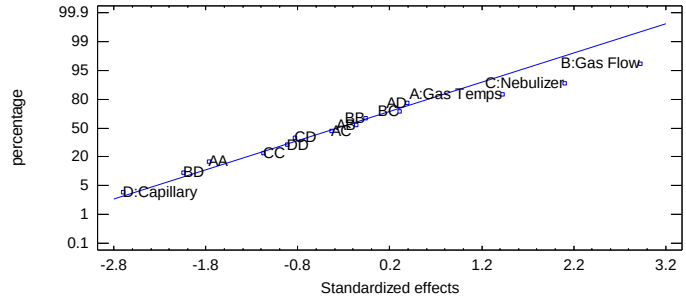

Figure 2. The main effects plot for concentration for AMD

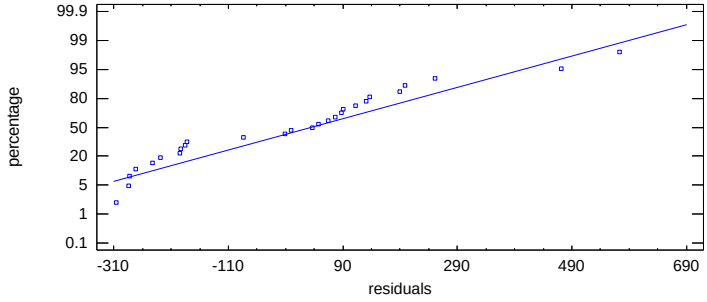

Figure 3. Normal probability plot for residuals for AMD

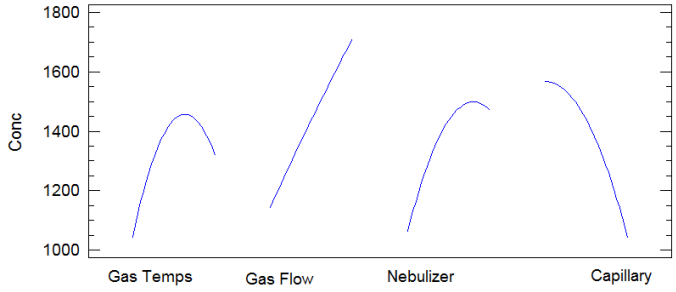

Figure 4. The normal probability plot for area

The variant values of AMD in Table 2 and the standardized Pareto chart for concentration in Figure 1 clearly showed that two main factors influenced the ionization were the gas flow rate and the voltage. The main effects plot for concentration in Figure 2 showed three survey factors that represented the extreme value were the gas flow temperature, injection pressure and voltage. Although the gas flow rate was the main influencing factor, it did not show the extreme value due to the limited specification of the survey equipment. Normal probability plot for residuals in Figure 3 and the normal probability plot for area Figure 4 showed that the obtained results were on "diagonal", proving that the experimental results were not misleaded. This confirmed that the gas flow rate and voltage were two factors that significantly affected the analysis results or the ionization of AMD.

3.1.1.3. Optimized the MS/MS conditions for AMD

Optimal MS/MS conditions were shown in Figures 5, 6 and 7.

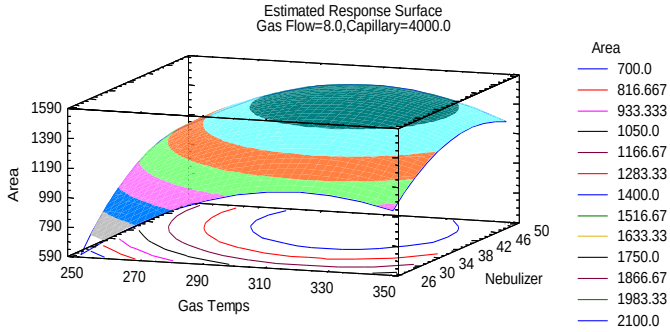

Figure 5. Estimated response surface of injection pressure and gas flow temperature for AMD

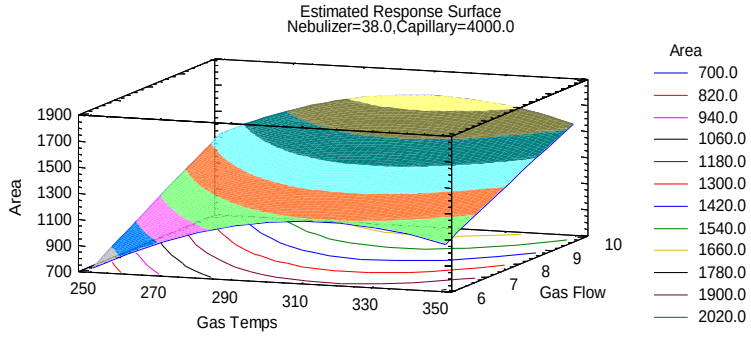

Figure 6. Estimated response surface of ion chamber temperature and gas flow rate for AMD

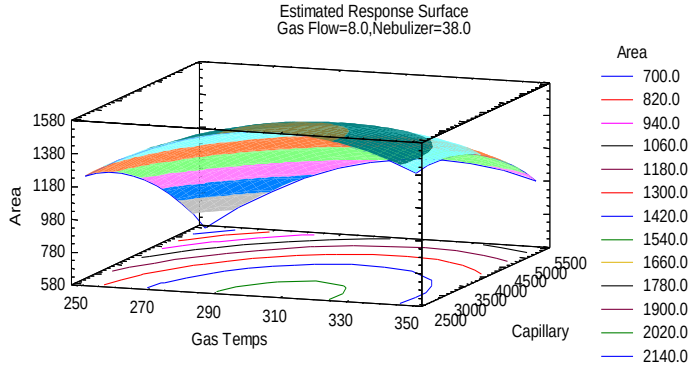

Figure 7. Estimated response surface of gas temperature and voltage for AMD

The results in Figure 6 showed that the ion chamber temperature and the gas flow rate significantly affected on the ionization of AMD. In addition, the experimental model also showed that the survey results have shown the extreme region (the top region of the model). Similarly, the injection pressure and the gas flow temperature in Figure 5, the gas temperature and the voltage in Figure 7, also showed the extreme region of the survey model. Therefore, the BBD model can be suitable in optimizing the parameters affecting the AMD ionization.

The results in Table 3 showed that the optimal conditions for MS/MS for AMD were calculated by Statgraphics Centurion XV.

Table 3. The optimal conditions for MS/MS for AMD

Parameters	Optimal conditions
Ion chamber temperature (°C)	298.61
Gas flow rate (mL min^{-1})	10.0
Injection pressure (psi)	50.0
Voltage (V)	2500.94

3.1.2. Optimized the parameters of MS detector for DCD

3.1.2.1. Model compatibility

The optimization of MS parameters for DCD after calculating with Statgraphics Centurion XV (Version 15.1.02) was shown in Table 4.

Table 4. Variant values of DCD

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A: Ion chamber temperature (°C)	223605.2641	1	223605.2641	1.67	0.3254
B: Gas flow rate (mL min^{-1})	19017501.35	1	19017501.35	142.05	0.0070
C: Injection pressure (psi)	1184324.264	1	1184324.264	8.85	0.0969
D: Voltage (V)	37663811.02	1	37663811.02	281.33	0.0035
AA	43908032.14	1	43908032.14	327.97	0.0030
AB	29166.93556	1	29166.93556	0.22	0.6866

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
AC	38.47224676	1	38.47224676	0.00	0.9880
AD	1215309.682	1	1215309.682	9.08	0.0948
BB	4943311.761	1	4943311.761	36.92	0.0260
BC	2470178.808	1	2470178.808	18.45	0.0502
BD	5195939.487	1	5195939.487	38.81	0.0248
CC	43042392.58	1	43042392.58	321.50	0.0031
CD	27003.67515	1	27003.67515	0.20	0.6973
DD	9151276.65	1	9151276.65	68.36	0.0143
Lack-of-fit	11880997.74	10	1188099.774	8.87	0.1054
Pure error	267755.7045	2	133877.8522		
Total (corr.)	145776721.7	26			

R-squared = 91.6662 %; R-squared (adjusted for d,f,) = 81.9434 %; Standard Error of Est, = 365.893; Mean absolute error = 514.358; Durbin-Watson statistic = 1.73829 (P = 0.2662); Lag 1 residual autocorrelation = 0.12426.

Equation prediction by area: Area of DCD = -141692+649.471*Gas Temps+4499.63*Gas Flow+1243.76*Nebulizer+4.13738*Capillary–1.14771*Gas Temps^2+0.853916*Gas Temps*Gas Flow+0.00516883*Gas Temps*Nebulizer+0.00734941*Gas Temps*Capillary–240.685*Gas Flow^2+32.7433*Gas Flow*Nebulizer–0.37991*GasFlow*Capillary–19.7281*Nebulizer^2 + 0.00456467*Nebulizer*Capillary – 0.000582182*Capillary^2 *(2)*

Where: Gas Temp: Ion chamber temperature (^oC); Gas Flow: Gas flow rate (mL min^{-1})

Nebulizer: Injection pressure (psi); Capillary: Voltage (V)

The results in Table 4 showed that, similarly to AMD, P-Value of the gas flow rate and voltage were 0.007 and 0.0035 respectively. These showed that gas flow rate and voltage significantly affected the ionization of DCD; Lack-of-fit value with P-Value = 0.1054 showed that the model matched the observed data at α = 95% was suitable for experimental design.

3.1.2.2. Influence of factors on MS/MS detector

The results of factors affecting MS/MS detector were shown in Figures 8 and Figures 9.

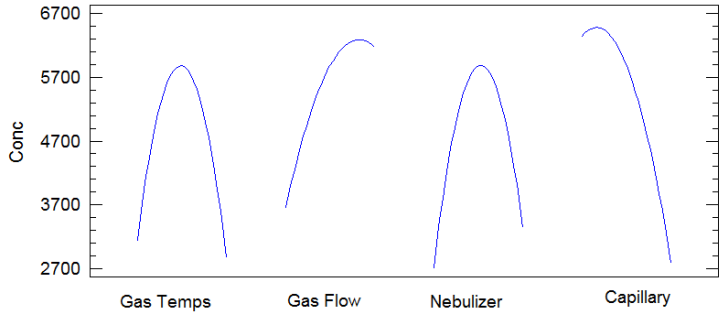

Figure 8. The main effects plot for concentration for DCD

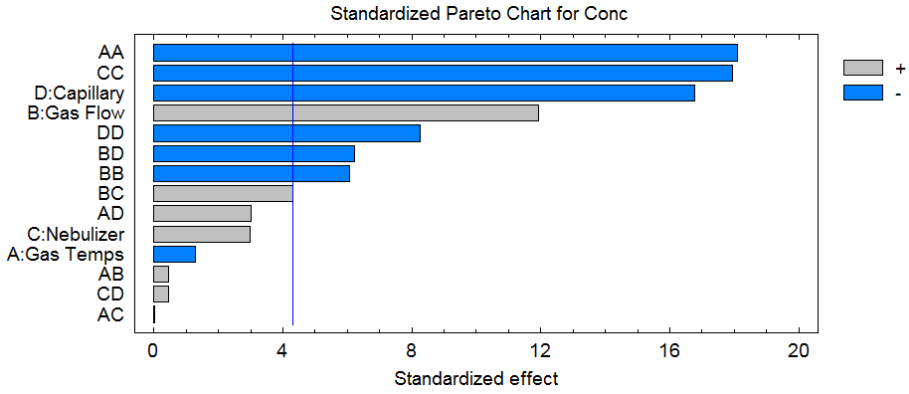

Figure 9. The standardized Pareto chart for concentration for DCD

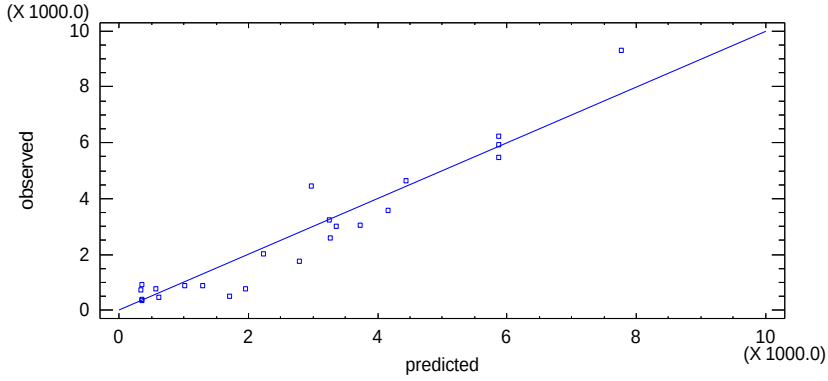

Figure 10. The normal probability plot for area for DCD

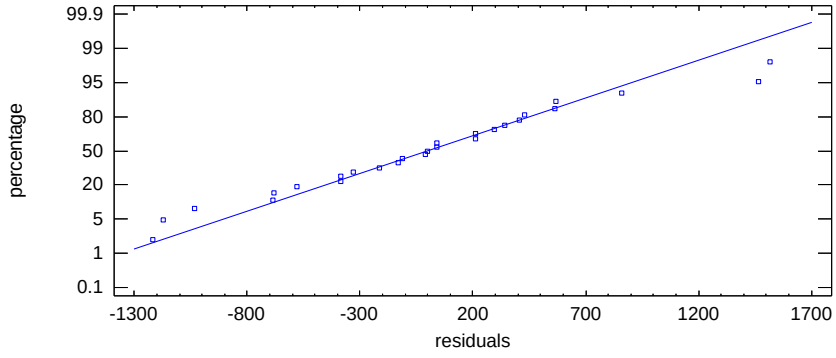

Figure 11. Normal probability plot for residuals for DCD

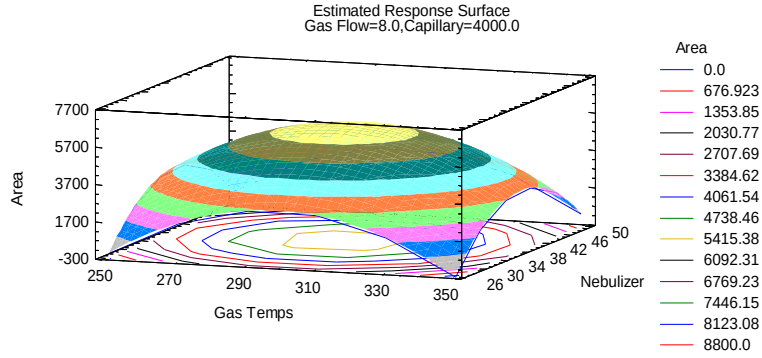

Figure 12. Estimated response surface of injection pressure and gas flow temperature for AMD

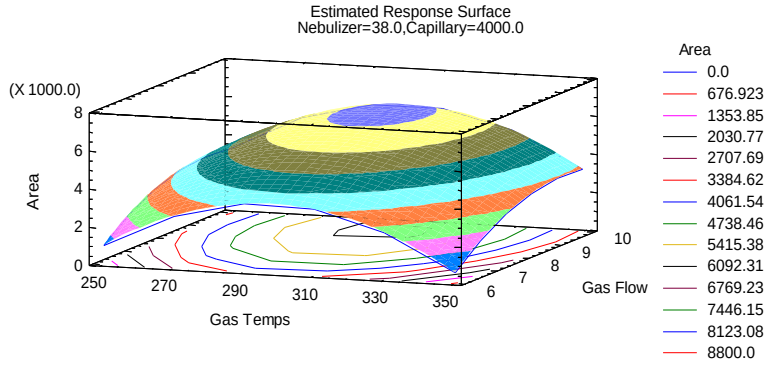

Figure 13. Estimated response surface of ion chamber temperature and gas flow rate for DCD

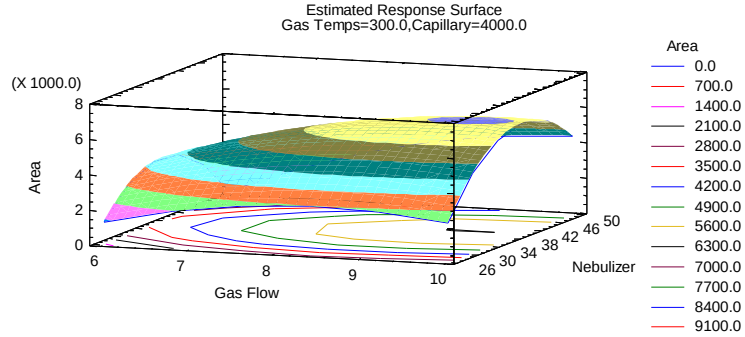

Figure 14. Estimated response surface of injection pressure and gas flow temperature for DCD

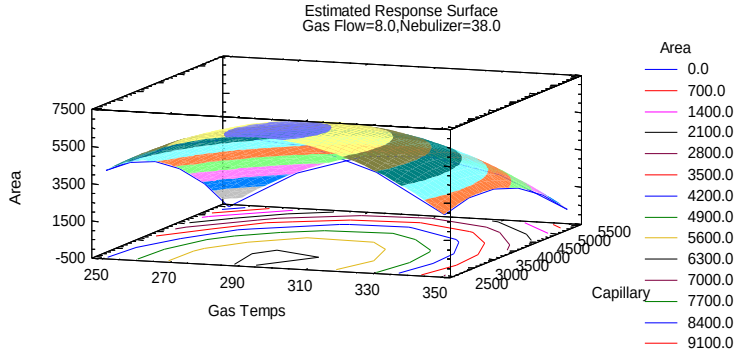

Figure 15. Estimated response surface of gas temperature and voltage for DCD

The results in Figure 8 and Figure 9 showed that gas flow rate and voltage were two factors significantly affected the analytical results. Besides, the normal probability plot for area in Figure 10 and normal probability plot for residuals for DCD in Figure11 showed that the results of the experiments were highly reliable because they were almost all located on the "diagonal line". The main effects plot for concentration for DCD in Figure 8 and estimated response surface of gas flow temperature, gas flow rate, injection pressure and voltage in Figure 12, Figure 13, Figure 14 and Figure 15 showed that the area selected for the experiments were the extreme region. Therefore, the BBD model can be suitable for investigating factors affecting the ionization of DCD. Similar to AMD, the optimal conditions for MS/MS for DCD were shown in Table 5.

Table 5. The optimal conditions for MS/MS for DCD

Parameters	Ion chamber temperature (^oC)	Gas flow rate (mL min^{-1})	Injection pressure (psi)	Voltage (V)
Optimal values	294.954	10.0	40.1465	2500.0

3.1.3. Optimized the parameters of MS detector for AMD and DCD

The results in Table 3 and Table 5 optimization of MS parameters for AMD and DCD were recommended in Table 6.

Table 6. Optimization of MS parameters for AMD and DCD

Parameters	Low	High	Optimized
Ion chamber temperature (^oC)	250.0	350.0	295.0
Gas flow rate (mL min^{-1})	6.0	10.0	10.0
Injection pressure (psi)	26.0	50.0	40.0
Voltage (V)	2500.0	5500.0	2500.0

3.1.4. Confirming for optimal conditions

Confirmed results of parameters for optimal conditions were shown in Table 7.

Table 7. Comparing the results between theoretical and experimental results

Substances	Concentration surveyed (ppb)	Theoretical optimal area	Experimented optimal area		
AMD	100	807.09	805.273	806.423	805.348
t_{theory}	4.302		$t_{experiment}$	3.792	
DCD	50	381.588	379.68	381.567	379.885
t_{theory}	4.302		$t_{experiment}$	2.025	

The results in Table 7 presented that there was no significant difference between the theoretical values and experimented ones ($t_{theory} > t_{experiment}$), so, the optimal conditions of these parameters can be applied to this research.

3.2. Validation of analytical methods

3.2.1. Specificity

The results of determination of AMD and DCD content of animal feed samples "Concentrated feed for pigs from training - finishing" from Asian company were determined that AMD and DCD content were negative. According to the guidelines of the European Analytical Society, the LC-MS/MS is believably accepted to confirm positive samples because identification points of LC-MS/MS were ≥ 4 [35].

3.2.2. The limit of detection (LOD) and the limit of quantitation (LOQ)

The results of the limit of detection (LOD) and the limit of quantitation (LOQ) for AMD and DCD were presented in Table 8.

Table 8. LOD, LOQ survey results

Substances		Concentration (ppb)	Ratio S/N (mean)	Number of injections
AMD	LOD	5	5.05	7
	LOQ	10	13.52	
DCD	LOD	5	3.26	
	LOQ	10	10.11	

LOD and LOQ of AMD and DCD of LC-MS/MS method were 5 and 10 ppb. There are many ways to determine the LOD and LOQ of the method, the simple and practical way was suggested. At quantifiable concentrations (LOQ), the RSD value met the quantitative requirements of the AOAC guide app-f (Appendix F: Guidelines for Standard Method Performance Requirements) [38].

3.2.3. Working ranges

Results of investigating linear intervals for LC-MS/MS in Figures 16 and 17 showed that AMD and DCD have linear range of 10 - 1000 ppb. Figure 18 showed the chromatogram of AMD and DCD at 500 ng/mL.

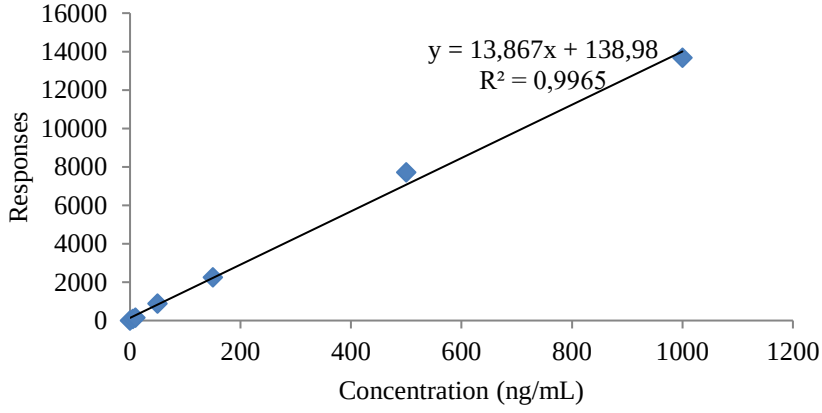

Figure 16. Investigating linear intervals of AMD for LC-MS/MS.

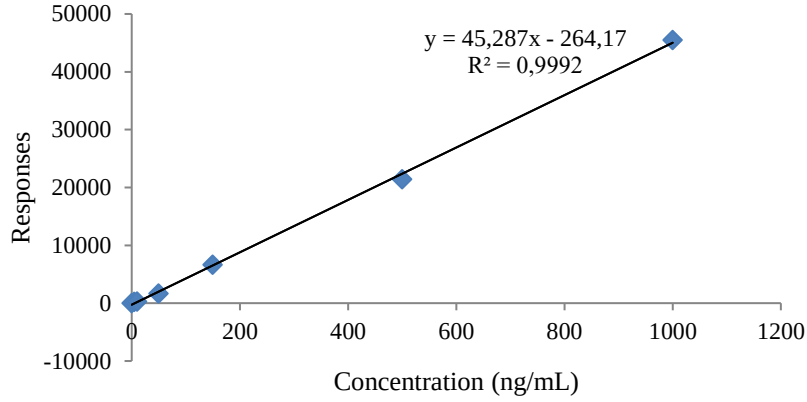

Figure 17. Investigating linear intervals of DCD for LC-MS/MS.

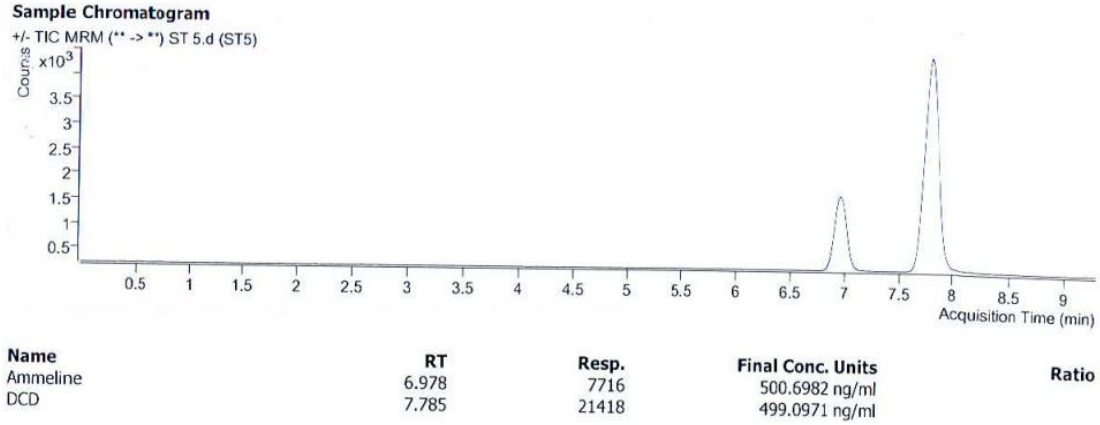

Figure 18. Chromatogram of AMD and DCD standard solutions at 500 ng/mL

3.2.4. Precision and recovery

Table 10. Precision and recovery

Substances	Level spiked (ppb)	Assay concentration (ppb)				Results		AOAC requirements	
		Sample 1	Sample 2	Sample 3	Average	Recovery %	RSD (%)	Recovery %	RSD (%)
DCD	10	8.11	8.97	8.87	8.65	86.5	5.43	60–115	< 21
	50	46.88	47.09	49.43	47.8	95.6	2.82		
	100	92.56	90.15	91.79	93.9	91.5	1.03	80–110	< 15
	150	141.8	129.4	138.5	136.6	91.1	4.70		
AMD	10	7.11	6.18	6.49	6.59	65.9	7.20	60–115	< 21
	50	48.75	49.39	48.65	48.93	97.86	0.87		
	100	98.15	97.52	99.41	98.39	98.4	1.11	80–110	< 15
	150	137.7	131.9	135.5	135.0	90.0	2.17		

Table 10 showed the results of the study on the repeatability and the recovery of the standard spiked animal feed samples. According to the AOAC (app-f) documentation of the validity of the method, the results were on completely responsive. Recoveries for the method validation ranged from 65.1 to 98.4% and the precision ranged from 0.87 to 7.2% RSD that were slightly higher than these in previous studies (84.6% - 96.8% - DCD analysis with HPLC–UV) [39], (61.4% - 117.2% - AMD analysis with GC-MS/MS) [13], compared with quantitative levels of AMD and DCD in animal feed samples by HPLC-DAD [16].

4. CONCLUSION

Based on the previous research, in this report, HPLC-MS/MS can be effectively used in the procedure for determining AMD and DCD. The method can be capable of simultaneously quantifying the AMD and DCD in the actual sample with quantitative limits of concentrations of 5 and 10 ppb, correspondingly, meeting the AOAC requirements. The method has been successfully applied at the National Centre for Veterinary Drugs and Bio-Products Control No.2 to manage the amounts of AMD and DCD in animal feeds with a relatively low threshold of detection and high sensitivity and reliability.

REFERENCES

1. National Library of Medecine. Available on: <https://pubchem.ncbi.nlm.nih.gov/compound/Ammelide>.
2. Rezaee M., Shoeibi S., Razavi-Azarkhiavi K., Ebrahim M. - Application of graphene oxide reinforced hollow fibers as a novel electromembrane extraction method for quantitative analysis of dicyandiamide in infant formula, Journal of Chemical Health Risks **8** (4) (2018) 313-321.
3. Ministry of Agriculture and Rural Development - Official Letter No. 10375 issued on chemicals used in production and trading of feed, Ha Noi (2017) (in Vietnamese).
4. Maryam J. - A review paper on melamine in milk and dairy products, Dairy & Veterinary Sciences **1** (4) (2017) 555566.
5. Dobson R.L., Motlagh S., Quijano M., Cambron R.T., Baker T.R., Pullen A.M., Regg B.T., Bigalow-Kern A.S., Vennard T., Fix A.S., Reimschuessel R., Overmann G.I., Shan Y., & Daston G.P. - Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs, Toxicological Sciences 106 (1) (2008) 251-262.
6. Scientific Opinion on Melamine in Food and Feed - EFSA Panel on Contaminants in the Food Chain (CONTAM) and EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), EFSA Journal **8** (4) (2010) 1573.
7. Cayzer J., Buckle K.N., Munday J., Ledgard S., Heiser A., Thatcher A. - Systemic granulomatous disease in dairy cattle during a dicyandiamide feeding trial, New Zealand Veterinary Journal **66** (2) (2018) 1-17.
8. Ministry of Agriculture and Rural Development - Decision No. 3762 issued on Melamine management in livestock and aquaculture, Ha Noi (2008) (in Vietnamese).
9. Ministry of Agriculture and Rural Development - QCVN 01 - 183:2016 - National technical regulation on animal feed - stipulates the maximum allowable limit for the content of mycotoxins, heavy metals and microorganisms in mixed feed for cattle and livestock hold, Ha Noi (2016) (in Vietnamese).

10. Chen X.H., Zhou L.X., Zhao Y.G., Pan S.D., Jin M.C. - Application of nanoring amino-functionalized magnetic polymer dispersive micro-solid-phase extraction and ultra fast liquid chromatography–tandem mass spectrometry in dicyandiamide residue analysis of powdered milk, Talanta **119** (2014) 187-192.
11. MacMahon S., Begley T.H., Diachenko G.W., Stromgren S.A. - A liquid chromatography-tandem mass spectrometry method for the detection of economically motivated adulteration in protein-containing foods, Journal of Chromatography A **1220** (2012) 101-107.
12. Qiu H., Sun D., Gunatilake S.R., She J., Mlsna T.E. - Analysis of trace dicyandiamide in stream water using solid phase extraction and liquid chromatography UV spectrometry, Journal of Environmental Sciences **35** (2015) 38-42.
13. Miao H., Fan S., Wu Y.N., Zhang L., Zhou P.P., Li J.G., Chen H.J., Zhao Y.F. - Simultaneous determination of melamine, ammelide, ammeline, and cyanuric acid in milk and milk products by gas chromatography-tandem mass spectrometry, Biomedical and Environmental Sciences **22** (2) (2009) 87-94.
14. Ono S., Funato T., Inoue Y., Munechika T., Yoshimura T., Morita H., Rengakuji S., Shimasaki C. - Determination of melamine derivatives, melame, meleme, ammeline and ammelide by high-performance cation-exchange chromatography, Journal of Chromatography A **815** (2) (1998) 197-204.
15. Yu H., Tang Y., Zhang G., Wang Z., Gao R. - A new chemiluminescence method for determination of dicyandiamide based on the N-bromosuccinimide-merbromin-cetyltrimethylammonium bromide system, Journal of Luminescence **157** (2015) 327-332.
16. Dang V. S., Phan T.T. D., Bui V. T., Studying to build the determination process of ammelide (AMD) and dicyandiamide (DCD) in animal feed by high-performance liquid chromatography (HPLC) with a diode-array detector (DAD), Journal of Science Technology and Food **20** (3) (2020) 67-79.
17. Duong Thanh Liem - Lecturing material on subjects: "Animal food and nutrition", Ho Chi Minh City of Agriculture publisher (2006) (in Vietnamese).
18. Le Duc Ngoan, Nguyen Thi Hoa Ly, Du Thi Thanh Hang - Lecturing material on subjects: "Animal food", Hue University of Agriculture and Forestry (2006) (in Vietnamese).
19. Application Note 01864. Available on: <https://www.agilent.com/cs/library/applications/si-01864_melamine_compounds_fda_220-ms_appn_v05.pdf>.
20. United States Department of Agriculture - CLG-MEL1.01: Determination and Confirmation of Melamine by LC/MS/MS, Effective: 12/02/2008.
21. Benvenuti M., O'Connor A. - Melamine, ammeline and cyanuric acid analysis by UPLC/MS/MS and UPLC/PDA, Waters Corporation, USA (2007).
22. TCVN 9048:2012 (ISO/TS 15495:2010) - Milk, milk products and infant formulae - Guidelines for the quantitative determination of melamine and cyanuric acid by liquid chromatography - tandem mass spectrometry (LC-MS/MS).
23. Smoker M., Krynitsky A.J. - Interim method for determination of melamine and cyanuric acid residues in foods using LC-MS/MS: Version 1.0, Laboratory Information Bulletin (LIB) 4422: Melamine and Cyanuric Acid Residues In Foods, U.S Food and Drug Administration, 2008.

24. U.S. Department of Agriculture, CLG-MEL1. 01 - Determination and confirmation of melamine by LC/MS/MS, Revision: 01, Replaces: CLG-MEL 1.00 Effective: 12/02/2008, 1-24.
25. Benvenuti M.E., O'Connor A. - Melamine, ammeline and cyanuric acid analysis by UPLC/MS/MS and UPLC/PDA, Waters Applications Note.
26. Shen Y., Han C., Zhou X., Chen X., Huang F., Zhu Z. - Microwave-assisted extraction and determination of dicyandiamide residue in infant formula samples by liquid chromatography-tandem mass spectrometry, Journal of Dairy Science **96** (11) (2003) 6877-6882.
27. Fu F., Schreiber A. - LC-MS/MS Analysis of Emerging Food Contaminants (2013).
28. Kruve A., Rebane R., Kipper K., Oldekop M-L., Evard H., Herodes K., Ravio P., Leito I. - Tutorial review on validation of liquid chromatography–mass spectrometry methods: part I, Analytica Chimica Acta **870** (2015) 29-44.
29. Kruve A., Rebane R., Kipper K., Oldekop M-L., Evard H., Herodes K., Ravio P., Leito I. - Tutorial review on validation of liquid chromatography–mass spectrometry methods: part II, Analytica Chimica Acta **870** (2015) 8-28.
30. AOAC International - Guidelines for standard method performance requirements AOAC Official Methods of Analysis, Appendix F (2016) 1-18. http://www.eoma.aoac.org/app_f.pdf.
31. AOAC Official Methods AOAC International - Standard Method Performance Requirements for Alkaloids of Mitragyna speciosa, AOAC SMPR 2015.008.
32. Application Note 01916, Simultaneous, Fast Analysis of Melamine, Cyanuric Acid, and Related Compounds in Milk and Infant Formula by LC/MS/MS. Available on: <http://www.ingenieria-analitica.com/downloads/dl/file/id/2737/product/110/simultaneous_fast_analysis_of_melamine_cyanuric_acid_and_related_compounds_in_milk_and_infant_formula_by_lc_ms_ms.pdf>.
33. 5991 0416en, Determination of Melamine and ItsAnalogues from Powdered Infant MilkUsing Polymeric Solid Phase Extraction, Available on: <https://www.scribd.com/document/420317199/5991-0416EN>.
34. National Institute for Food Control - Evaluation of methods in chemical analysis and microbiology analysis, Publishing House for Science & Technology, Ha Noi, 2010 (in Vietnamese).
35. European Commission, Directorate General Health and Consumer Protection - Guidance document on pesticide residue analytical methods, SANCO/825/00 rev.8.1 (2010).
36. ISO Guide 35:2017 - Reference materials - General and statistical principles for certification (2017).
37. Gowik P. - Criteria and requirements of commission decision 2002/657/EC, Bulletin - International Dairy Federation **383** (2003) 52-56.
38. Analysis, A.O.M.o., Appendix F: Guidelines for standard method performance requirements. 2012, AOAC International Rockville, MD, USA.
39. Qiu H., Sun D., Gunatilake S.R., She J., Mlsna T.E. - Analysis of trace dicyandiamide in stream water using solid phase extraction and liquid chromatography UV spectrometry, Journal of Environmental Sciences **35** (2015) 38-42.

TÓM TẮT

XÁC ĐỊNH HÀM LƯỢNG AMMELIDE VÀ DICYANDIAMIDE TRONG THỨC ĂN CHĂN NUÔI BẰNG SẮC KÝ LỎNG HIỆU NĂNG CAO ĐẦU DÒ MS/MS (HPLC-MS/MS)

Phan Thị Thanh Diệu[1], Bùi Văn Tâm[2], Đặng Văn Sử[1*]
[1]*Trường Đại học Công nghiệp Thực phẩm TP.HCM*
[2]*Trung tâm Kiểm nghiệm Thuốc Thú y Trung ương II*
*Email: *sudv@hufi.edu.vn*

Mục đích của nghiên cứu này là xác định hàm lượng các chất tăng cường protein giả ammelide (AMD) và dicyandiamide (DCD) trong thức ăn chăn nuôi theo quy trình chuẩn bị mẫu kết hợp với quy trình làm sạch bằng chiết pha rắn (SPE) và định lượng bằng HPLC-MS/MS. LOD và LOQ của AMD và DCD được xác định bằng LC-MS/MS với giới hạn định lượng tương ứng là 5 và 10 ppb. Phương pháp có ngưỡng phát hiện tương đối thấp, độ nhạy và độ tin cậy cao được áp dụng hiệu quả tại Trung tâm Kiểm nghiệm Thuốc thú y Trung Ương II để kiểm soát hàm lượng AMD và DCD trên cơ sở mẫu thức ăn chăn nuôi thực tế.

Từ khóa: Chất tăng đạm giả, AMD, DCD, thức ăn chăn nuôi, SPE, HPLC-MS/MS.