Determination of cysteamine in animal feeds by high performance liquid chromatography with diode-array detection

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ABSTRACT

A high performance liquid chromatography with diode-array detection (HPLC-DAD) method for the determination of cysteamine supplementation in commercial animal feeds was developed. Samples were extracted with a mixture of 0.5 % hydrochloric acid (HCl) – acetonitrile (ACN) (90:10, v/v), matrix interferences were removed with a C18 cartridge, and cysteamine was derivatized using 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) as Ellman's reagent targeting to the thiol group in the molecule. Quantification of cysteamine was performed on a C18 column with DAD at 323 nm. The developed method had a limit of detection (LOD) of 1.1 mg/l, good linearity of the calibration curve ($R^2 \geq 0.9998$), high recoveries (> 92 %), and high reproducibility (RSD < 2.0%).

Key words: Animal feeds, Cysteamine, Ellman's reagent, HPLC-DAD

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History

- Received: 21 May 2018
- Accepted: 13 August 2018
- Published: 10 September 2018

DOI :10.32508/stdj.v21i2.427

INTRODUCTION

Nowadays, food safety is the number one concern for human health, especially with regards to existing antibiotics and toxic residues in foods. Mixing of feed additives is not only critically important for reducing production costs, but also harmful for the health of humans and animals. However, the abuse and overuse of these substances may be accumulated in animal organs potentially causing toxic effects to human health. Beta-agonists, such as clenbuterol and salbutamol, have been found in cattle and poultry feeds to increase protein content and the rate of weight gain without additional feed intake, making feed efficiency greater. The illegal use of these compounds has already led to several cases of intoxication in humans after consumption of contaminated animal liver[1]. Due to the fact that beta agonists in animal meat are constantly kept under close control, animal farmers have tried to use other chemicals instead.

Cysteamine (CS) or β-mercaptoethylamine (HS-CH_2-CH_2-NH_2) is biologically derived from cysteine metabolism. It is a specific inhibitor agent due to S-S bond in animal production to affect the endocrine system and improve the growth rate of piglets and finishing pigs[2]. Although growth hormone (GH) has more direct effects in the field of animal food enhancement to improve economic returns, CS seems to be more applicable for farmed animals and for increasing serum GH concentrations as well as the growth rate of broilers, fish, sheep and pigs[3,4]. It is well-known that GH increases muscle growth and decreases fat deposition in pigs[5].

CS contains a thiol functional group, which can be derivatized with certain disulfides, *e.g.* 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 2,2'-dithiodipyridine, 2,2'-dithiobis(5-nitropyridine), 4,4'-dithiodipyridine, and other disulfides. When a thiol compound is reacted with the excess disulfide, a mixed disulfide and corresponding thiol are formed as shown in reaction Scheme 1[6].

$$RSH + R'SSR' \rightarrow RSSR' + R'SH \quad (1)$$

Thiol-disulfide interchange is the reaction of a thiol (RSH) with a disulfide (R'SSR'), with formation of a new disulfide (RSSR') and a thiol (R'SH) derived from the original disulfide. Thiol disulfide interchange of a monothiol (RSH) with a disulfide (R'SSR') involves multiple equilibria:

$$RSH \leftrightarrow RS^- + H^+$$
$$RS^- + R'SSR' \leftrightarrow RSSR' + R'S^-$$
$$H^+ + R'S- \leftrightarrow R'SH$$

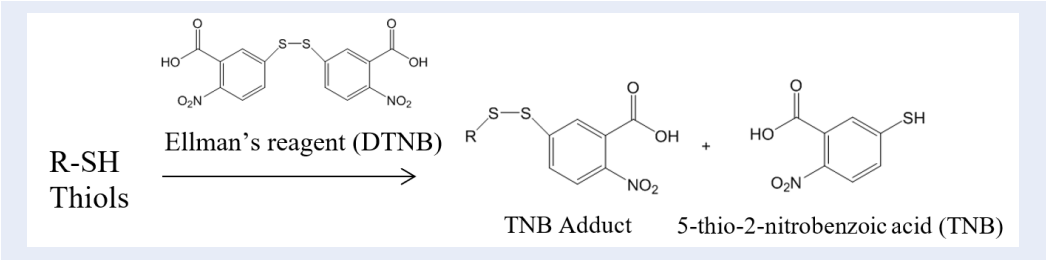
In this study, we used Ellman's reagent (DTNB) as a derivative agent to react with a thiol group of CS; the products include a 5-thio-2-nitrobenzoic acid (TNB) adduct, a concomitant release of one equivalent of 5-thiol-2-nitrobenzoic acid (TNB), and residual Ellman's reagent Scheme 1[7].

MATERIALS AND METHODS

Chemicals and apparatus

An Agilent 1200 HPLC System equipped with an InertSustain AQ-C18 column (5 μm, 250 mm x 4.6 mm

Cite this article : Nguyen Huynh P T, Nguyen P H, Nguyen M A. **Determination of cysteamine in animal feeds by high performance liquid chromatography with diode-array detection**. *Sci. Tech. Dev. J.*; 21(2):37-43.



Scheme 1: **Reaction of a thiol-containing compound with Ellman's reagent.**

id; from GL Sciences, Shinjuku, Tokyo, Japan), a DAD detector, an autosampler, and ChemStation software were employed for quantification of derivatized cysteamine. For pH adjustment, an Agilent 3200P pH meter (Agilent, Santa Clara, CA) was used.

All chemicals used in the study were of analytical grade: cysteine, cysteamine standards, and Ellman's reagent were obtained from Sigma-Aldrich (St. Louis, MO); methanol and acetonitrile (ACN) were from Fisher (USA); N, N-Dimethylformamide, formic acid, glass acetic acid, hydrochloric acid (HCl), sodium hydroxide, potassium hydroxide, ethylenediaminetetraacetic acid (EDTA), and tris(hydroxymethyl)aminomethane (Tris) were from Merck KgaA (Darmstadt, Germany); and vitamins (C, B1, B3, PP, B6, B5, B9, K3, B2) were from the Institute of Drug Quality Control (Ho Chi Minh City (HCMC), Vietnam). Bond Elute C18 cartridges (500 mg) were obtained from Agilent (Santa Clara, CA) and used for sample treatment prior to HPLC separation.

Solutions and reagents

Tris buffer (pH 8.2) was prepared by dissolving 48.44 g of tris base and 8.32 g of EDTA in 800 mL distilled water, adjusted pH to 8.2 with HCl, brought up to 1000 mL with double distilled water, and stored at room temperature. DTNB reagent solution was prepared by dissolving the compound in N, N-dimethylformamide at a concentration of 2 mg/mL and stored in the dark at 4 °C.

Calibration standards

A stock standard solution of 1000 mg/L cysteamine was prepared in methanol and the working standard solutions (0.25; 2.5; 25; 100; 125; 200; 250 mg/L) were prepared by dilution of the stock solution with tris buffer as needed.

Sample preparation and extraction

In this study, typical drug-free commercial feeds for growing-finishing pigs were collected from local markets in Vietnam, including complete feed and premix. All samples were blended then stored in zip-lock under dark and room temperature until analyzed; unused sample portions could be refrigerated for up to two months.

One g of animal feed sample and 200 mg Vitamin C (ascorbic acid) were weighed and placed into a 15-ml reaction vessel. Cysteamine was extracted with 10 ml HCl (0.5 %): ACN (90:10, v/v) with the aid of shaking for 20 min. The sample solution was cool centrifuged at 5 °C, 6000 rpm for 2 min. The supernatant was filtered through a 0.22-μm membrane, and 2 mL sample solution was passed through a C18 cartridge to remove interferences before derivatization.

Derivatization procedure

Two mL of working standard (or sample solution) and 5 mL Tris buffer (pH 8.2) were pipetted into a 10 mL volumetric flask, and pH was adjusted to 8.2-9.0 with 0.1 N HCl or 0.1 N KOH, as needed. One mL of DTNB (2 mg/mL) was added, and solution was shaken vigorously then brought up to 10 mL with Tris buffer. The solution was allowed to stand at room temperature for 60 min, followed by addition of 100 μL 37 % HCl and vigorous shaking, and then filtered through a 0.22-μm membrane and injected into the HPLC system.

Chromatographic conditions

The flow rate of the mobile phase and the injection volume were 1.0 ml/min and 20 ml for all runs, respectively. A binary solvent consisted of 0.1 % formic acid (solvent A) and ACN (solvent B) was employed. A gradient elution at room temperature was started at 90:10 = A: B (v/v) and held for 17 min, then decreased to A: B (50:50, v/v) for 1 min and held for 8 min; the detection was absorbance performed at 323 nm.

RESULTS

Effect of pH on the rate of derivatization

A basic medium was expected to stimulate the derivatization process since thiolate is a stronger nucleophile than thiol. However, the stability of DTNB decreases with increased hydroxide concentration. In this study, pH was varied from 2 to 12 (using acetic acid and adjusting to pH 2-4 with 0.1 N HCl, Tris buffer (adjusted to pH 7-12 with 0.1 N HCl or 0.1 N KOH), while the reaction time was 60 min (**Figure 1** a). It was found that the optimal pH range was between 8-9 where there was a compromise between thiolate formation and the stability of DTNB (**Figure 1** b).

Time for completion of derivatization and the effect of HCl on ceasing the reaction

The derivative reaction was studied from 10 - 120 min at room temperature (at pH 8.2). It was found that the reaction yield leveled off after 60 min and up to 90 min, then slightly increased **Figure 2**.

The yield of the reaction is not quantitative because it is reversible. Thiolate anion (RS^-) is the active nucleophile and continuously reacts with disulfide bonds at high pH. However, the reaction was effectively quenched by the addition of 100 mL 37 % HCl due to the conversion of thiolate to thiol after 60 min from the start.

Vitamin and metal ion interferences

Vitamins are essential components of animal feeds. Their levels are especially high in premix formulations. To investigate the effects of vitamins on the analysis, each vitamin and also the mixture of them were spiked into the animal feed at various levels from 100 to 1000 mg/L. As the result, all vitamins (except for vitamin K3 and B2) had insignificant effects on the recovery of the analyte (**Figure 3**). This finding is in agreement with those of Rita Gatti *et al.*[8]; in their study, menadione (vitamin K3) reacted with a thiol group in solution at room temperature and pH 8.5, therefore preventing cysteamine from reacting with DTNB (**Scheme 2**).

From the literature, it was demonstrated that ascorbic acid (vitamin C) reacts with vitamin K3, which acted as an electron transfer agent in oxidation reactions by atmospheric oxygen[10]. The use of high levels of vitamin C could prevent vitamin K3 from reacting with cysteamine. This hypothesis was verified by adding vitamin C to animal feed containing 1.0 mg/L cysteamine and spiking it with 100, 200, 500, or 1000 mg/L of each vitamin (and labeled as Mix 100 mg/L, 200 mg/L, 500 mg/L and 1000 mg/L, respectively). Indeed, 200 mg vitamin C was enough to achieve recovery greater than 90%. It should be noted that the effect of vitamin B2 was removed by passing the extracts through a C18 cartridge (**Figure 4**).

Regarding the metal ions commonly found in animal feed (*e.g.* Ca^{2+}, Mg^{2+}, Fe^{3+}, etc.), at levels as high as 100 mg/L they were completely complexed with EDTA and showed no effect on the determination of cysteamine.

Limit of detection (LOD) and limit of quantitation (LOQ)

The equation for the linear regression line and the coefficient of correlation were y=18.404 x + 26.528 and R^2 =0.9998, respectively, with the concentration range as 0.25 - 200.0 mg/L. The LOD and LOQ which correspond to the signal to noise of 3:1 and 10:1, respectively, were 1.1 mg/L for LOD and 3.3 mg/L for LOQ. Those allowed for reliable quality control of the formulations.

Precision and recovery

Spiked samples (n=9) (on animal feed matrix) at three levels (5.0, 50.0 and 75.0 mg/L) were analyzed in order to evaluate the trueness and precision of the method. The recoveries of cysteamine were over 92 % and the RSDs were less than 2 % (**Figure 5**), which complies with the international regulation set by AOAC[11].

Analysis of cysteamine contents in commercial animal feed samples

The levels of cysteamine in five different commercial animal feed samples bought from local markets are listed in **Table 1**. All five samples were prepared in triplicates and spiked at 5.0 mg/L of cysteamine from stock standards (1000 mg/L). The recoveries were determined for each sample matrix.

DISCUSSION

One of the challenges in the determination of cysteamine is the oxidation of thiol groups before and during sample treatment, especially since cysteamine is a small molecule that cannot be analyzed directly by modern analytical techniques. Furthermore, the derivatization of thiol groups with DTNB occurs much more rapidly under slightly alkaline conditions and with higher stability than neutral or acidic conditions[12]. The specific product, TNB adduct, is suitable for analysis by HPLC with UV detection at 323 nm. However, pH is one of the most important effectors for optimizing the procedure, so that it should

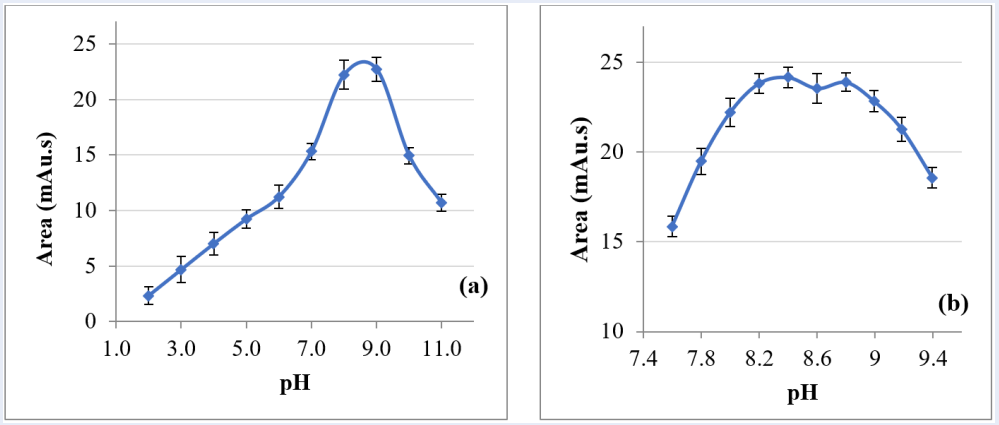


Figure 1: **Effect of pH on the formation of TNB-Adduct**. (**a**) pH from 2-12 and (**b**) pH from 7.6-9.4.

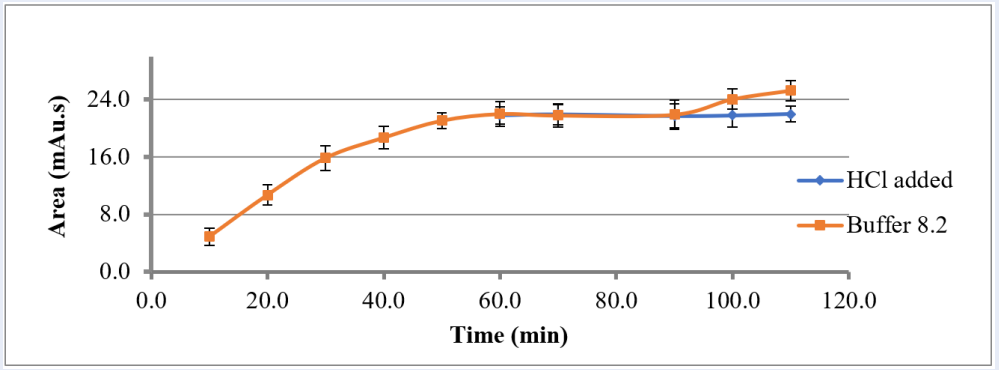
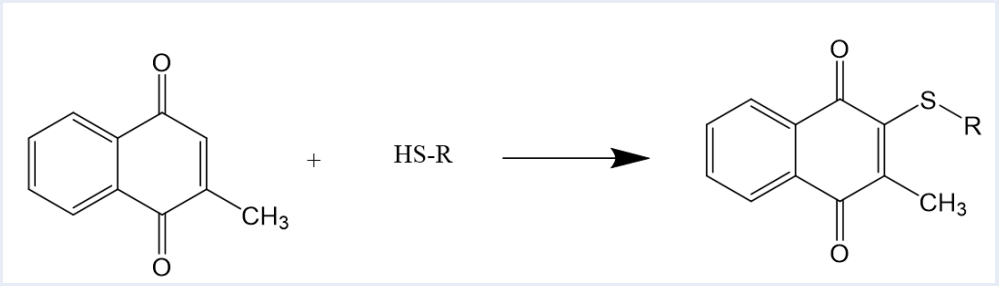


Figure 2: **Derivatization process and the effect of HCl on quenching the reaction.**



Scheme 2: **Menadione (vitamin K3) reacts with a thiol group**[9].

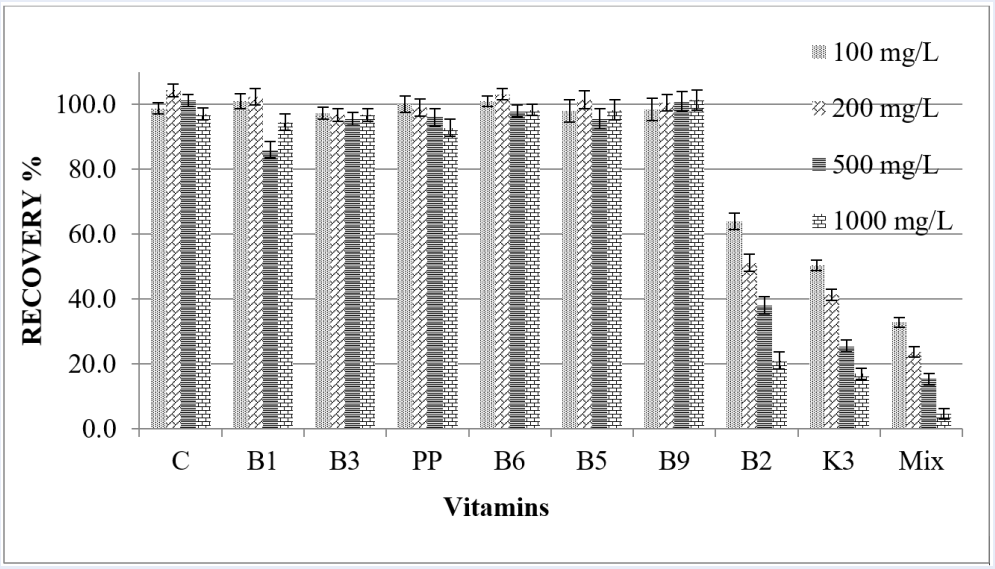


Figure 3: **Effect of vitamins on the recovery of cysteamine.**

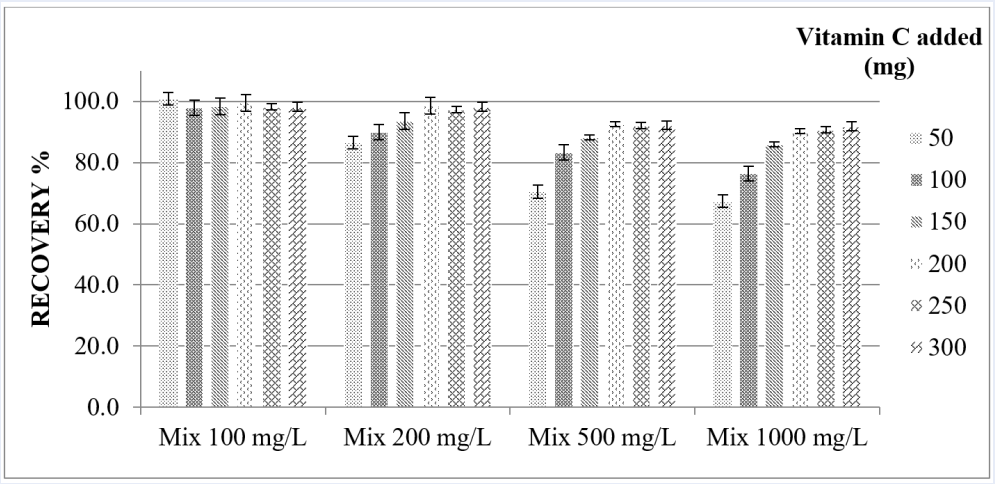


Figure 4: **Effect of vitamin C addition on the recovery of cysteamine.**

Table 1: **Cysteamine content in commercial animal feed samples (n=3, P=0.95)**

Samples	Cysteamine content (mg/kg)	RSD (%)	Recovery (%)
Premix 01	29.9	0.89	99.9
Premix 02	30.7	0.92	96.0
Complete 01	51.5	1.70	92.4
Complete 02	26.1	1.60	90.6
Complete 03	23.8	1.30	91.4

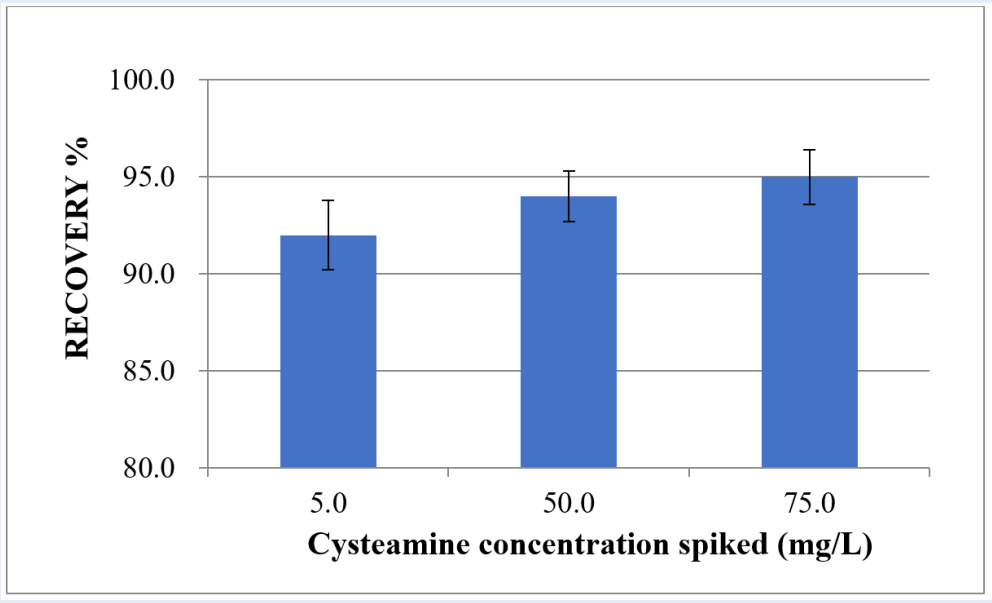


Figure 5: Recoveries of cysteamine spiked at three levels in animal feed samples.

be controlled during the process. The addition of Tris buffer brought the pH to a range between 8.0-9.0 for the derivatization reaction (60 min at room temperature) and then, an acidic solution (37% HCl) was used to stop the secondary reaction to obtain high reproducibility. Furthermore, the effects of metals, vitamin K3 and B2 were evaluated and resolved by adding EDTA solution, vitamin C and passing through C18 cartridge, respectively, in the sample preparation and derivatization reaction procedures.

In the US, Canada, Thailand and Malaysia, the use of cysteamine in animal feeds has been banned. Last year, the Ministry of Agriculture and Rural Development of Vietnam officially issued a circular to prohibit the use of cysteamine in feed productions. However, until now, there has been no study yet to demonstrate the detection limit of cysteamine in feed productions in order to prove the effectiveness or danger of cysteamine in breeding.

According to a previous study by Krzysztof Kus´mierek and his co-workers, cysteamine can be determined in plasma by liquid chromatography with ultraviolet detection at 355 nm after pre-column derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate[13]. The response of the detector is linear within the range of 0.1-40 μmol/L plasma and the LOQ was 0.1 nmol cysteamine in 1 ml of plasma. In another study, Joshua et al. developed a method for determining cysteamine in biological samples (brain, kidney, liver, and plasma) using N-(1-pyrenyl) maleimide as the derivatizing agent and analyzing by HPLC with a fluorescence detection method (λex = 330 nm, λem = 376 nm)[14]. The calibration curve for cysteamine was found to between 50-1200 nmol and the LOQ of cysteamine in biological samples using their method was 50 nmol/L.

In the present study, we focus on optimizing the derivatization reaction with 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB), *i.e.* Ellman's reagent, and analyzing by HPLC with DAD detection at 323 nm. The method we developed herein has a LOQ of 3.3 mg/L (approximately 43 nmol/L) for animal feeds, including complete and premix animal feeds. The sensitivity of our method is similar to or better than those of HPLC method described above.

CONCLUSION

In this study, the extraction, clean-up and derivatization processes were developed for the determination of cysteamine by HPLC/DAD. The proposed method was suitable for cysteamine analysis in animal feeds with good precision and accuracy, high sensitivity, and specificity. Based on these results, we believe that there is significant contamination of animal feeds in the local markets of Vietnam. In particular, a high detection rate was observed, indicating a need for the continued surveillance of cysteamine. In addition, the scope of the method may be extended to include animal feed for chicken, beef, sheep, and other meat products.

COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

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