



Determination of N-Carbamoylglutamate in Rumen Fluid

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Abstract

N-carbamoylglutamate (NCG) is a feed additives obtained synthetically from sodium glutamate, which is a structural analogue of N-acetylglutamate. Current study is aimed at developing ion chromatographic method for the determination of NCG in rumen fluid. Successful determination was performed on cation-exchange column and non-suppressed conductivity detection with total run time of 11 minutes. Simple one-step cleanup using anion exchange cartridge was used to remove interfering compounds. Method was applied to determine NCG in rumen fluid, which was obtained from three rumen fistulated dairy cows. Incubated NCG sample in rumen fluid was assessed at various time intervals (0, 2, 4, 6, 12 and 24 h at 39 °C). Results showed that NCG was disappeared at the rate of 7.8, 12.8, 13.3, 14.2, and 17.8 % at 2, 4, 6, 12 and 24 h, respectively. It is concluded from the results that the procedure is quick and uncomplicated. NCG can be detected in rumen fluid without lengthy separation using cation-exchange chromatography. This technique can be applied for determination of NCG in rumen fluid as well as for practical use in animals feed.

Keywords: N-Carbamoylglutamate, N-Acetylglutamate, Rumen fluid, Ion chromatograph Determination, Method

Introduction

Feed additives have been used in ruminant ration for obtaining the desired results related to production and health or due to environmental concern. N-carbamoylglutamate (NCG) is a feed additives obtained synthetically from sodium glutamate, which is a structural analogue of N-acetylglutamate [1], that is an essential factor of carbamoyl phosphate synthetase-1, a rate-limiting enzyme that augments urea cycle and the arginine (AGR) production process [2] and have favorable position of lower rumen breakdown compared to ARG [3].

The NCG was studied as a source of nutritional non-protein nitrogen for ruminants [4-6] and was found to produce ammonia in the rumen,

which is utilized by microflora for manufacturing of microbial crude protein [6,7]. Thus, NCG likewise supplement a portion of a front stomach animal's protein requirements [8].

Oral administration of NCG increase the amalgamation of ARG, plasma ARG fixation, muscle protein combination, and affects entire body in development of piglets [2,9,10]. In spite of the fact that these findings that show critical role of NCG in neonatal nutrition and development, but negligible work has been done on the ruminants. Recent research on ruminant showed that NCG can improve urea cycle in gut tissue [11] and increased plasma ARG concentration, balanced amino acid profile; thereby efficiently improve dairy

production and protein when used in high producing cows [12]. Feeding NCG to mid lactating dairy cows increased the milk protein and fat contents, thereby improved the milk quality. [13].

Research demonstrated that rumen protected ARG and NCG supplementation has beneficial effect on mammalian reproduction, which are associated with complex metabolic networks [14] and improve the maternal–fetal–placental anti-oxidation capability, and promote fetal and placental development during early-to-late gestation when offered to underfed ewes [15]. Pregnancy outcomes and fetal growth improved under conditions of maternal under nutrition in ewes gestating twin fetus [16]. The NCG and ARG may act directly on granulosa cell and regulate ovarian function by slowing follicular differentiation via inhibiting IGF1 action, and steroid synthesis while stimulating granulosa cells proliferation in cattle [17]. Dietary supplementation with NCG reduced mean pulmonary hypertension, peripheral systolic blood pressure, through the restoration of endothelial nitric oxide synthase and endogenous NO synthesis in holstein heifers with high altitude pulmonary hypertension [18]. Thus, NCG is considered as potential ARG replacer feed additives in ruminant's. When ruminant are fed with feed containing protein, the first stage is microbial degradation which make it complex to quantify the utilized product in rumen. No method is available for NCG determination in rumen fluid. Therefore, establishment of easy, convenient method of NCG analysis in rumen fluid was of prime importance to understand the rumen metabolism, when animal are fed a NCG product. Thus, study was conducted to develop simple, convenient and accurate method for determination NCG in rumen fluid and to carry out the research on rumen metabolism affected by NCG.

Material and Method

Chemicals

All reagents used in current study were of analytical-reagent grade excluding NCG (Feed grade). Purified water of Milli-Q system

(Millipore, Bedford, MA, USA) was used. N-carbamyl L-Glutamate NCG (Hangzhou King Techina Technology Co. Ltd. China) purity 97%, with molecular weight 190.2 was used. The NaOH was purchased from Sino Pharma Shangahi China). NCG samples were dissolved in eluent solution and passed through a 0.45 µm filter before injecting into chromatograph.

Instrumentation for NCG analysis

For chromatographic examination an ICS-2000 (Dionex, Sunnyvale, CA, USA) liquid chromatograph furnished with a double-piston pump, a column heater, a advance conductivity detector and an injector with a 20 µL sample loop was used [19]. The conductivity detector with 6DS6 cell heater was position to 25°C and temperature adjustment to 1.7% per °C was positioned after the IC column in the non suppressed mode. The software Dionex Chromeleon 6.2 was used for data collection and handling.

Chromatographic conditions

The levels of NCG in rumen fluid were all around distance by methods of cation-exchange chromatography using NaOH as eluent and an IC column Cleanert IC-RP with Spec: 1 mL (250mm×4mm i.d.) as the partition column. The ion chromatography framework was made out of a Dionex IonPac SCSG1 guard column (50mm×4mm i.d.) and a Dionex Ion Pac SCS1 logical section (250mm×4mm i.d.). The SCS1 section was selected due to its improved analysis in general inorganic cations as well as various amines with non-suppressed conductivity finding. The optimized eluent solution contained NaOH. For partitioning a stable temperature of 25 °C and a flow-rate of 1 mL/min were completed in a isocratic state.

Rumen fluid collection and processing

In vitro glass syringe system was used for incubating the NCG samples in rumen fluid [20]. Rumen fluid were obtained equally from three ruminally fistulated Chinese Holstein lactating

cows before morning feeding (Hay and concentrate 60:40 ratio, respectively) and filtered through four layers of surgical gauze into a separating funnel (flushed with a mixture of 95% N₂ and 5% CO₂). Before incubation, the glass syringes were cleaned through washing and drying. About 200 mg substrate containing Hay (Chinese wild grass) and Corn (maize) with ratio of 50:50 were weighed into 100 mL calibrated glass syringes in triplicate. To prevent the escape of gas from syringe, Vaseline oil was applied to the pistons. NCG solution with the concentration of 1mmolL⁻¹ (5.7 µg/syringe) and 0 mmol (control) were added into syringe before the incubation [3]. The syringes were pre-warmed at 39 °C before adding 30 ± 1 mL of rumen liquor-buffer mixture (ratio 1:2) into each syringe. Blank test syringe containing equivalent capacity of buffered ruminal fluid without substrate was used to confirm the differences in the composition and activity of rumen fluid. Syringes containing NCG samples were incubated in water bath with automatic temperature, shaker and grid for supporting syringes at 39°C for 2, 4, 6, 12 and 24 h. In each point time, three syringes from each treatment and one blank were terminated and rumen fluid with the help of pipette were collected into 2 mL eppendorf tubes for determination of the NCG. Immediately after termination of incubation 1 mL rumen sample centrifuged at 4000 RCF for 10 minutes at room temperature and supernant were stored at -4°C until analysis [3].

Preparation of rumen sample for NCG determination

Procedure

Before injecting, all rumen incubated NCG samples were dissolved by the eluent solution and passed through 0.45mm filters. The samples were clear from free the organic acid thorough anion separation column with NaOH mobile phase elution and conductive detector. Prior to analysis 1 mL centrifuged rumen sample was kept at room temperature to easy get the sample into syringe. 20 µL sample were added into 10 mL Milli Q water for dilution, after that 25 µL sample were taken by 1 mL syringe with filter of 0.22 µm size porosity then injected into Ion chromatography Dionex ICS

2000 RFIC USA). The rumen incubated NCG sample required little or no pretreatment without filter the sample 0.22 µm size before injection IC column Cleanert IC-RP with Spec: 1 mL (Agela Technologies).

Preparation of standard curve for NCG determination

For the establishment of standard curve, the NCG stock solution was prepared by dissolving 25mg of NCG into 100 mL Milli-Q Water HPLC grade and final dilute solution was made by adding of 1 mL NCG stock solution with 10 mL deionized water with concentration of 0.025 mg/mL equivalent to 25ppm. The NCG concentration of 0.046, 0.092, 0.183, 0.458 and 0.578 µg/mL.

Results and Discussion

The concentration of NCG in the rumen fluid were determined by calculating the peak area developed by NCG on Ion Chromatography as shown in (Fig. 1. C & D) and multiplied by equation developed by standard line, while blank and control sample (Fig. 1. A & B) produce 0 peak area. A clear peak at retention time for NCG is 9.35 minutes whereas no signal was seen in corresponding controls at the same retention time. It indicates that no any other molecule interfere with method for NCG determination in rumen fluid. Samples incubated for 2 and 24 hours showed clearly developed peak areas. Under optimized conditions, concentration levels mentioned in experimental section of NCG indicated good linearity's between the concentrations of NCG and detector response ($R^2=1$) (Fig. 2). Accuracy was obtained by assessing standard solutions at minimum and maximum in triplicate where relative standard deviation was observed for 1.5 and 4.71 µgmL⁻¹ for 12 and 6 h incubated rumen fluid samples. NCG concentration in rumen fluid during different incubation period were obtained by calculating the peak area developed on chromatograph for 2 and 24 h of incubation were 175.3 ± 1.63 (SD), and 156.4 ± 4.27 (SD) µgmL⁻¹ (Table 1). It was inferred that cation- exchange chromatographic

technique with a non-suppressed conductivity detection is a reliable technique for measurement of NCG in rumen fluid. Moreover, the method was rapid and uncomplicated. NCG can be detected in rumen fluid without lengthy separation process using one step clean-up stage. The method can be used for routine analysis of NCG in feed additive.

Table-1 shows NCG content in rumen fluid at various time intervals. It was disappeared at 2, 4, 6, 12 and 24 h as 7.8, 12.8, 13.3, 14.2, and 17.8 %, respectively. It is inferred that rumen micro flora has low effect on degradation rate on

NCG break-down as compared to front stomach for urea [6]. Our work confirms that of Mcallam et al [4] in an in vitro framework, while breakdown of NCG in the rumen was a natural procedure instead of a basic inversion of the response. Previously, conducted experiments on synthesis of NCG by microbes of rumen and recommended that urea is the basic nitrogenous element produced in the breakdown of NCG; also indicate as a source of non-protein nitrogen for ruminant feed [21]. We obtained the degradation rate at maximum possible levels within specific time period. This is first study on NCG degradation in rumen fluid in vitro.

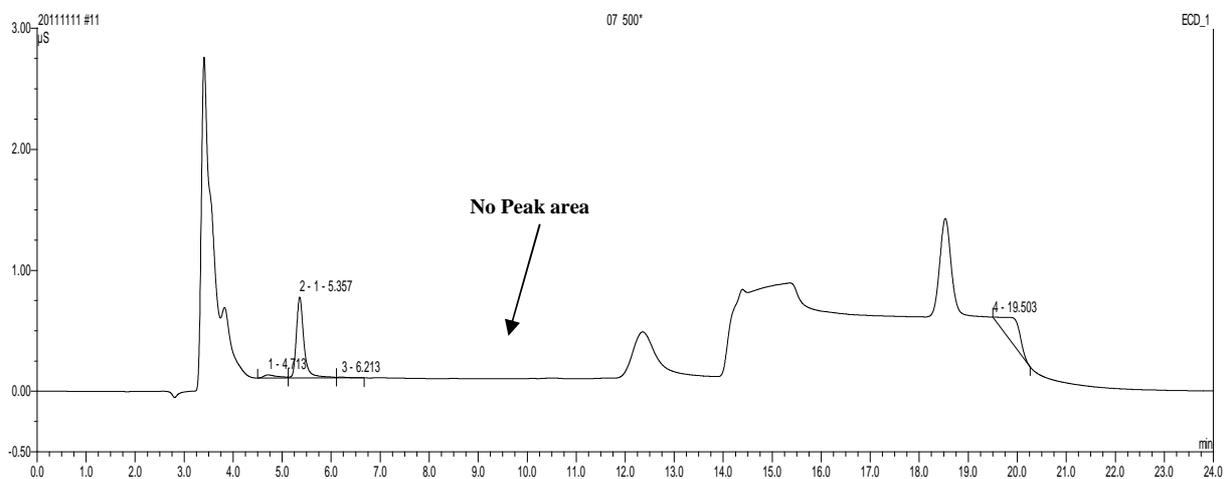


Figure 1. A. Detection of NCG in rumen fluid (Blank sample contain only rumen fluid without substrate and NCG)

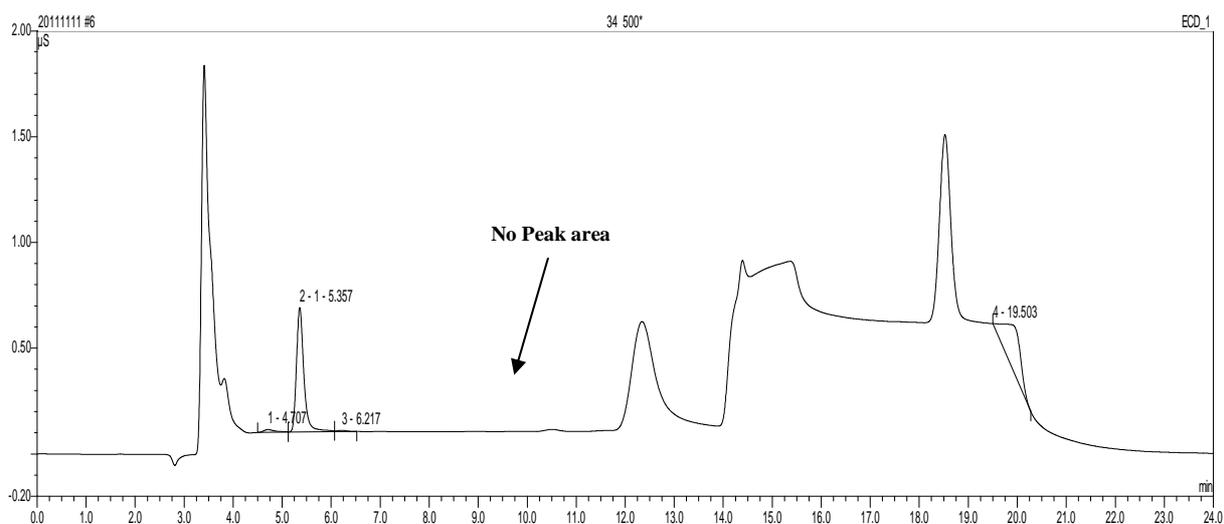


Figure 1. B. Detection of NCG in rumen fluid (Control sample contain only rumen fluid and substrate without NCG)

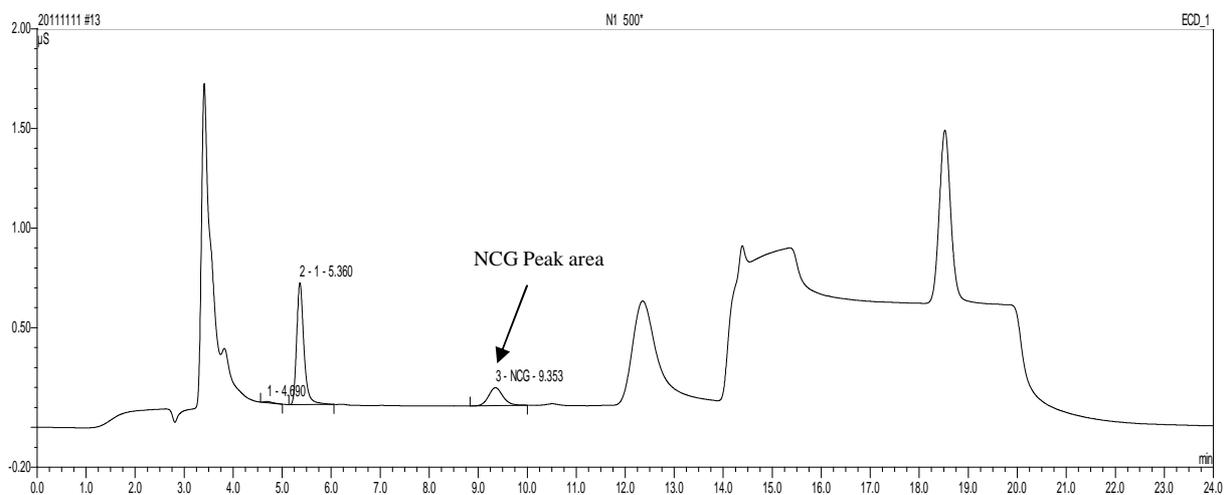


Figure 1.C. Detection of NCG in rumen fluid (sample contain 1mmol/L NCG + Substrate + Rumen fluid) during 2 hours incubation.

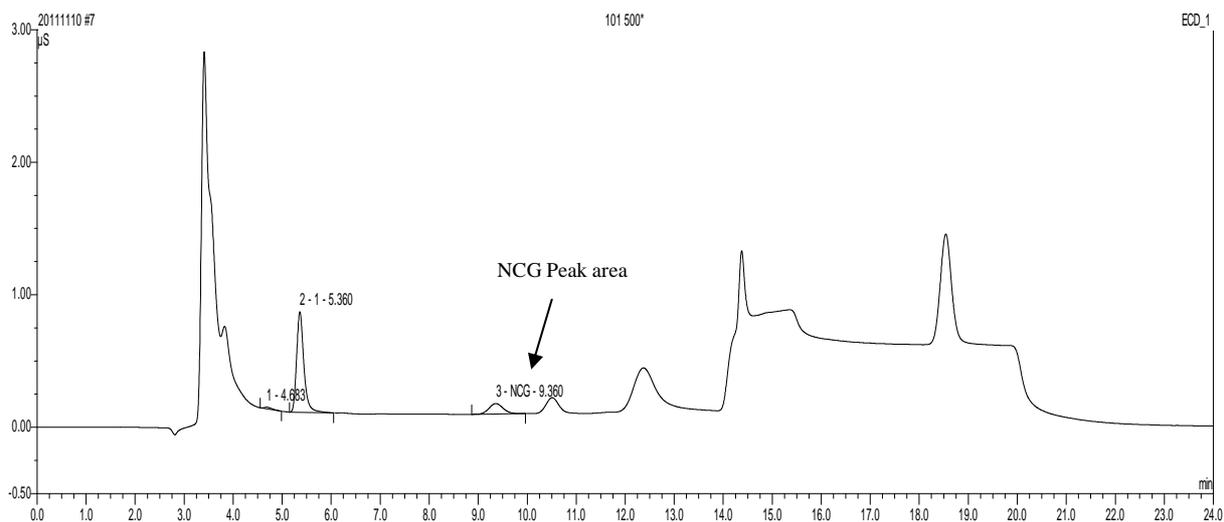


Figure 1.D. Detection of NCG in rumen fluid (sample contain NCG+ Substrate + rumen fluid) during 24 hours incubation

Table 1. Detection limit of NCG in rumen fluid, at various time intervals .

Incubation time (Hours)	Peak area	Relative standard Deviation %	NCG Concentration $\mu\text{g mL}^{-1}$	% Disappearance
00	0.0000	0.00	190.2 + 0.00	0.00%
02	0.0328	4.41	175.3 \pm 1.63	7.8
04	0.0309	2.10	165.9 \pm 1.35	12.8
06	0.0307	4.71	164.9 \pm 4.28	13.3
12	0.0304	1.50	163.2 \pm 1.92	14.2
24	0.0291	1.89	156.4 \pm 4.27	17.8

The values are expressed as mean \pm SE and % average degradation. NCG concentration of $190.2 \mu\text{g mL}^{-1}$ was taken as initial concentration

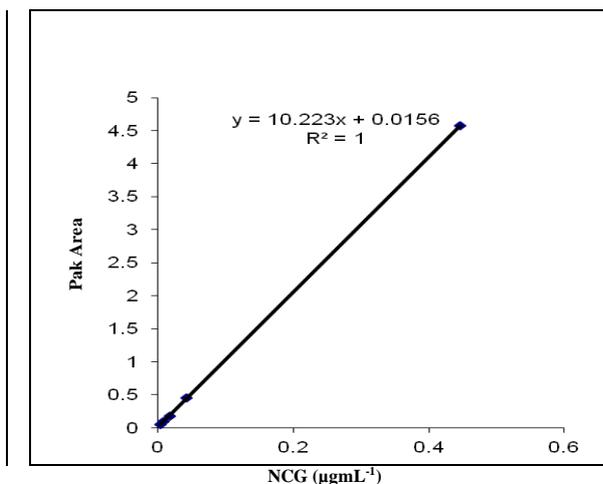


Figure 2. NCG Standard Curve Line

Conclusion

A simple cation-exchange chromatographic technique with non-suppressed conductivity detection for determination of NCG in rumen fluid was developed. NCG can be detected in rumen fluid without lengthy separation procedure. The run time of measurement of NCG in rumen liquid was not more than 11min. The method can be used for routine analysis of NCG in feed additive of ruminant animals.

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