ARTICLE

# Modified Reinecke's salt spectrophotometric method for quantifying choline chloride in feed additive

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**ABSTRACT**

Rampant choline adulteration in feedstuffs has been a huge problem in many parts of Asia due to the lack of a reliable standard method to quantify choline chloride (CC), the most common form of choline additive in feeds. Contemporary methods are either expensive, non-selective or require elaborate and sophisticated instrumentation. In this work, Reinecke's salt spectrophotometric method was modified and extensively validated to quantify choline chloride in a feed additive sample. The existing method requires the use of a calibration material that might have questionable purity and longer time to prepare, and a solvent that is extremely volatile – issues that can be difficult to manage when analyzing many samples, which the modified Reinecke's salt method was able to address and resolve. Method modification includes the use of ammonium reineckate as standard calibration reagent, and 70% (w/v) acetonitrile solution in water as solvent for analysis. The modified method is highly selective to choline, and is simple and economical, requiring only either a single or double beam UV-Visible spectrophotometer to carry out analysis. The modified Reinecke's salt method was found to be linear from 0 to 1200 mg $L^{-1}$ choline chloride equivalent and has a calibration linearity coefficient ($r^2$) of 0.9995. Repeatability and intermediate precision studies gave RSD values of 0.30% and 0.50%, respectively, while recovery values ranged from 97.67 ± 2.34% to 105.39 ± 6.27% for different spike levels. Results from both precision and recovery studies were within the recommended range set by the Association of Official Analytical Chemists (AOAC). LOD and LOQ values were 2.83 mg $L^{-1}$ and 9.42 mg $L^{-1}$ CC, respectively, suggesting applicability of the method to samples with much lower CC content such as in finished feeds. Selectivity studies showed that there is no significant difference on the CC content in samples with and without amino acids present. Choline reineckate was stable both as dried solids and in 70% (w/v) acetonitrile solution in water for one week based on robustness studies. The modified method quantified 52.11 ± 0.85% (w/w) CC in a feed additive sample that reportedly contains 50% (w/w) CC. The result from the modified method was found to be both statistically different compared to the CC

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values obtained using $AgNO_3$ (Mohr) titration and HPLC method. Trueness of the value obtained from the HPLC "gold standard" method could not be established, to which the modified Reinecke's salt method gave a recovery value of 108.09%.

## INTRODUCTION

Choline is a quaternary ammonium compound and is recognized as an essential nutrient to animals (Ziesel and Blusztajn 1994). It is an important precursor for the biosynthesis of many biologically important compounds such as phosphatidylcholine, acetylcholine, and betaine (Schenkel et al. 2015). Choline is considered as an important dietary supplement to poultry feeds. For instance, about 50% of choline requirements of growing chicks must be supplied to meet the demands for their growth (Dilger et al. 2007). Insufficient choline supplementation may also lead to a decreased poultry egg production (Llerena et al. 2003).

Rampant adulteration of choline chloride content in feeds has been reported in parts of Asia and has significantly affected the feed industry (Zhang and Zhu 2007). This is usually carried out by adding inexpensive inorganic salts such as table salt (sodium chloride, NaCl) which cannot be discriminated against by the current method of choline chloride determination in most analytical laboratories. This existing analytical method for choline chloride is solely based on its chloride content, and completely disregards any amount of the main analyte in question i.e., choline through $AgNO_3$ titrimetric analysis, with the fundamental presumption that only choline chloride is the lone source of chloride ions in feedstuffs. Despite this method being extremely simple and fast to carry out, it is clearly non-selective to choline and provides very inaccurate, unreliable results. In a purely analytical method point of view, the only way to resolve this adulteration issue is by proposing a new method that accurately quantifies the choline content in feedstuffs. Such method may not be as lenient and straightforward as the current titrimetric method, nevertheless, it is a very important trade off to deliver accurate results of analysis.

In this work, we present a modified Reinecke's salt spectrophotometric method which uses high purity and commercially available ammonium reineckate as standard calibration reagent and 70% (w/v) acetonitrile solution in water as solvent for spectrophotometric analysis. The modified method was tested and is intended to use for a feed additive sample that contains unbound choline present as choline chloride. The method was extensively validated to establish its reliability, and the obtained parameters are presented. The authors believe that this modified method will be able to provide a long-term solution to the choline chloride adulteration predicament and will be accessible even to small scale analytical laboratories where sophisticated instrumentation may not be readily available. To the best of our knowledge, this study is the first known attempt to establish an inexpensive and selective method to quantify choline chloride in feedstuffs after many decades.

## MATERIALS AND METHODS

### Sample

A feed additive sample commonly used as raw material for commercial production of poultry feeds was provided by a private analytical testing laboratory sourced from a private individual/company and was used in this study. The sample came in as an unlabeled package containing about 500 g of yellow-brown colored finely milled corn cob which according to the source contains approximately 50% (w/w) CC.

### Modification of the Reinecke's Salt Spectrophotometric Method

The Reinecke's salt spectrophotometric method was adopted from the works of Szasz and Gimesi (1974) and was modified in terms of the standard calibration reagent and solvent for analysis. In terms of calibration reagent, the existing method used a choline reineckate precipitation procedure from choline chloride that requires individual precipitation and dissolution of every point/concentration in the standard curve, whereas in the modified method, high purity, commercially available ammonium reineckate is used to generate the standard calibration curve for quantifying the choline chloride content. The modified method uses a simple choline chloride extraction procedure using warm water since feed additive samples contain free (unbound) choline in the form of choline chloride and does not employ the $Ba(OH)_2$ extraction procedure which is only necessary for analyzing samples containing bound choline. In terms of solvent for analysis, the existing method used pure acetone to prepare both the standard solutions and samples to be analyzed, whereas in the modified method, 70% (w/v) acetonitrile solution in water is employed.

***Precipitating agent.*** The precipitating agent was prepared by dissolving 2.5 g ammonium reineckate monohydrate in 100 mL methanol. Freshly prepared methanolic ammonium reineckate solution was used in every analysis.

***Standard ammonium reineckate solution.*** Standard concentrations from 0 to 1200 mg $L^{-1}$ choline chloride equivalent (CCE) were prepared from ammonium reineckate monohydrate solids. Preparation of 25 mL of 1500 mg $L^{-1}$ CCE is given here as an example:

*Legends: CC - choline chloride; AR - ammonium reineckate*

$$\left(1500\frac{mg}{L}CC\right)x(0.025\ L)x\left(\frac{1\ g}{1000\ mg}\right)x\left(\frac{1\ mol\ CC}{139.622\ g\ CC}\right)x\left(\frac{1\ mol\ AR}{1\ mol\ CC}\right)x\left(\frac{336.41\ g\ AR}{1\ mol\ AR}\right)=0.0904\ g\ AR \quad (1)$$

Thus, 0.0904 g ammonium reineckate was dissolved in 25 mL of solvent to prepare a 1500 mg $L^{-1}$ CCE standard solution.

***Sample preparation.*** A 0.10 g sample was used, and choline chloride was extracted using 10 mL of warm distilled (40-45 °C) water. Extraction was done for 20 min with intermittent agitation. The mixture was filtered, and the filtrate was obtained.

***Choline precipitation.*** The collected filtrate was treated with excess precipitating agent and stood for one hour in an ice bath (~5 °C). The choline reineckate solids (lustrous pink crystals) were collected using a fritted glass filter and washed with small amounts of cold distilled water and 2-propanol. A method blank containing 10 mL distilled water was used.

***Spectrophotometric analysis.*** Choline reineckate solids were dissolved in 50 mL 70% (v/v) aqueous acetonitrile solution and the absorbance was read at 525.5 nm using a Shimadzu UV-1601 double-beam spectrophotometer (Shimadzu, Kyoto, Japan). The CC content of the sample was determined from the calibration curve and an average of six determinations was calculated.

### Method Validation

The validation methods employed in this study were based on the guidelines recommended by the AOAC Official Methods of Analysis (AOAC 2016), International Council for Harmonization of Technical Requirements (ICH 1994), and Australian Pesticides and Veterinary Medicines Authority (APVMA 2004).

***Linearity.*** Linearity of the method was evaluated from the absorbance of standard ammonium reineckate solutions (0 to 1200 mg $L^{-1}$ CCE) at 525.5 nm. Linear regression analysis was performed and the results ($r^2$, y-intercept, and slope) were obtained.

***Repeatability and intermediate precision.*** Within-day instrument's precision was obtained from the standard deviation of calibration curve at 525.5 nm for ten times. Intermediate precision of the instrument was also obtained from the standard deviation of calibration curve at 525.5 nm for ten consecutive days. Relative standard deviation (RSD) for both precision measurements were calculated using the following equation:

$$\text{RSD} = \frac{\text{std.dev.}}{\text{mean}} \text{ x } 100 \quad (2)$$

***Bias (recovery).*** Standard addition method via spiking was used to evaluate bias in the absence of a certified reference material (CRM). Known amounts of choline chloride (0.0125 g, 0.025 g and 0.050 g) were separately added to a 0.10 g sample. Percentage recovery of CC in low, middle, and high spiked samples were calculated. Each spiked sample was analyzed in triplicate (n=3) and the mean percent recovery for each spike was reported.

***Limit of detection (LOD) and limit of quantitation (LOQ).*** LOD and LOQ were obtained from the absorbance of 5.0 mg $L^{-1}$ CCE standard ammonium reineckate solution at 525.5 nm for ten times. The LOD and LOQ were calculated as follows (ICH 1994; APVMA, 2004):

$$\text{LOD} = \frac{\text{3 x std.dev.of absorbance readings}}{\text{slope of calibration curve}} \quad (3)$$

$$\text{LOQ} = \frac{\text{10 x std.dev.of absorbance readings}}{\text{slope of calibration curve}} \quad (4)$$

***Selectivity.*** Selectivity was evaluated by comparing the CC content of samples with and without added amino acids. 0.10 g sample was prepared and analyzed as previously described, and another 0.10 g sample was prepared and added with a cocktail of amino acids (glycine, L-isoleucine, L-asparagine, DL-aspartic acid and L-glutamine) containing 500 mg $L^{-1}$ concentration of each amino acid. Analysis was done in triplicate (n=3) and the CC contents were compared.

***Robustness.*** Robustness was evaluated from the stability of choline reineckate solids after immediate and delayed filtration procedures. 0.10 g sample was prepared and subjected to precipitation procedure as previously described. For the stability of solids from delayed filtration, the aqueous mixture was stored for various days in the refrigerator at 4 °C before filtering. For the stability of solids obtained after immediate filtration, the precipitates were dried and stored for various days in a desiccator. CC content in both setups was analyzed during the same day of precipitation and after 3 and 7 days of storage of either solid or aqueous mixture. Analysis for both setups was done in triplicate (n=3) and the standard deviation was calculated.

**$AgNO_3$ (Mohr) Titration**

The titration procedure was adopted from ASTM D512-12 for chloride determination in water (ASTM 2012). A similar sample preparation previously described was followed. One milliliter (1 mL) of filtrate from sample preparation was obtained and placed in a 250-mL Erlenmeyer flask and diluted to 100 mL using distilled water. $AgNO_3$ solution was previously standardized against a standard sodium chloride solution using $K_2CrO_4$ indicator prior to analysis. A 1-mL portion of $K_2CrO_4$ indicator solution was added and the solution was titrated with the standardized $AgNO_3$ solution until a golden yellow endpoint is reached. The volume of the titrant used to reach the endpoint was recorded. Distilled water was used as blank during the analysis. Average of three trials (n=3) was reported. A laboratory quality assurance standard (QAS) for chloride analysis was analyzed alongside with the sample.

**High Performance Liquid Chromatography (HPLC)**

The HPLC method for choline chloride quantification was adopted from the works of Hefni and colleagues (2015) with minor modification. Instead of using a 125-mm strong cation exchanger column employed in the original method, a 150-mm column of similar brand and composition was used. The rest of the original HPLC method was employed for this study.

***Standard choline chloride solution and sample preparation.*** Choline chloride solutions from 0 to 800 µM concentrations were prepared and distilled water was used as blank. The same sample preparation procedure previously described was used. One milliliter (1 mL) of the obtained filtrate was diluted to 100 mL using distilled water and was used for analysis.

***Derivatization of choline.*** Twenty microliters (20 µL) of either the diluted filtrate from feed additive sample or from standard choline chloride was placed in a 1.5 mL microcentrifuge tube and was added with 1 mL of acetonitrile. This was followed by the addition of 60 µL of 1.0 M NaOH solution and 20 µL of 1-naphthyl isocyanate. The mixture was vortexed and shaken in a mechanical shaker for 15 min at room temperature. Sixty microliters (60 µL) distilled water was added and vortexed, and the resulting mixture was centrifuged using a Vision VS-100BN mini centrifuge (Vision Scientific Co., Ltd., Daejeon-Si, South Korea) for two minutes. The mixture was allowed to stand for 10 minutes and a 200 µL portion of the supernatant was transferred into a clean 1.5 mL microcentrifuge tube for HPLC analysis.

***Analysis.*** Quantification of CC content was carried out using a Shimadzu LC-10AS liquid chromatograph coupled with a Shimadzu CTO-10A column oven (Shimadzu, Kyoto, Japan). Peaks were detected using a Prominence RF-20A fluorescence detector connected to a Prominence CBM-20A communications bus module data processor (Shimadzu, Kyoto, Japan). The chromatograms were viewed and analyzed in a desktop computer using the Shimadzu LC Solution software (Shimadzu, Kyoto, Japan). Twenty microliters (20 µL) of the derivatized sample were injected in a Phenosphere SCX strong cation exchanger column (150 mm x 4.6 mm x 5µm) (Phenomenex, California, USA). The column temperature was maintained at 40 °C and isocratic separation was performed using the prepared mobile phase (10 mM tetramethylammonium hydroxide, 20 mM glycolic acid, and 12% (v/v) water in acetonitrile). Excitation wavelength was set at 220 nm while emission wavelength was set at 350 nm. Flowrate was maintained at 1 mL $min^{-1}$ and the total run time for each analysis is 15 minutes. Linearity, precision, bias (recovery), LOD and LOQ were obtained to validate the method.

## RESULTS AND DISCUSSION

**Modification of Reinecke's Salt Spectrophotometric Method**

The spectrophotometric procedure for choline chloride analysis was adopted from the works of Szasz and Gimesi (1974) and was carefully modified. Similar choline extraction procedure from the existing method was implemented in this study since choline was present as chloride salt in the sample – a form that

is very soluble in water. Warm water (40-45 °C) was used for extraction to increase the rate of dissolution of salt. Acetone was used as solvent for analysis in the existing method. In this study, acetonitrile was explored as an alternative solvent for analysis. Acetone has a polarity index of 5.1, while the polarity index of acetonitrile is 5.8 (Snyder and Kirkland 1979). Both solvents are aprotic, and their polarities are very similar. Solubility values of choline reineckate in acetonitrile and acetone at room temperature previously reported in literature are 1.511 mg $mL^{-1}$ and 1.626 mg $mL^{-1}$, respectively (Argoudelis 1975). This similarity in solubility values, in addition to the close polarity indices of acetone and acetonitrile suggests that acetonitrile is a good alternative solvent. Furthermore, similar values of molar extinction coefficient (ξ) were reported for solutions of choline reineckate in acetone and acetonitrile**,** (Argoudelis 1975). Alternatively, an aqueous solution of acetonitrile can be used as solvent, as use of pure solvent can be very costly. Choline reineckate is soluble in 70% (v/v) aqueous acetonitrile solution, and this solvent was used for analysis in this study. Moreover, an aqueous solvent is more convenient for solution preparation since pure acetonitrile is highly volatile and may cause error in standard concentrations because of rapid solvent loss.

In this study, ammonium reineckate served both as a precipitating agent for choline and calibration standard for spectrophotometric analysis. It is commercially available in pure form and it replaces the use of solid choline chloride standard in the existing method. In the existing method, choline reineckate is cast out from standard solutions of choline chloride by ammonium reineckate precipitation, and the precipitates are collected and dissolved in acetone to generate a calibration curve. This means that different concentrations of the standard choline chloride solution need to be subjected to a precipitation procedure before a calibration curve can be constructed. This kind of method is particularly inconvenient when many samples of multiple replicates are needed to be analyzed. Choline reineckate is not industrially manufactured and thus no standard is readily available for use. Another option is to do a small-scale synthesis of choline reineckate, however, the purity of the synthesized product cannot be guaranteed to be suitable for standard use. For these reasons, reagent grade ammonium reineckate was used as a calibration standard. Choline chloride cannot be directly used as standard in spectrophotometric analysis since aqueous solution of this salt is colorless and does not absorb in the visible region. It absorbs in the ultraviolet (UV) region at approximately 208 nm (Krishnamoorthy et al. 2012), however, analysis at this wavelength can give inaccurate results since most solvents also absorb in this region. Instead, a lustrous pink choline reineckate may be precipitated out from choline chloride-containing samples which can then be dissolved in an aqueous acetonitrile solution. This resulting solution is suitable for UV-Vis spectrophotometric analysis since it is colored (pink solution) and therefore absorbs in the visible region. Choline chloride readily reacts with ammonium reineckate to form choline reineckate via double replacement (metathesis) reaction below. The structure of the reineckate anion is shown in Figure 1.

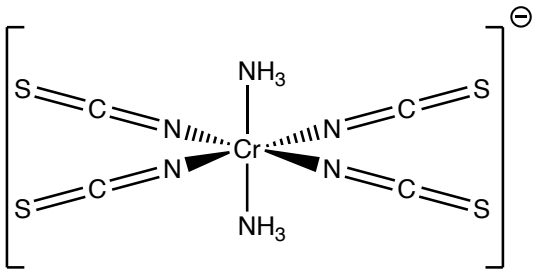


**Figure 1: Chemical structure of the reineckate anion.**

$$[\text{choline}]Cl_{(aq)} + NH_4[\text{reineckate}]_{(aq)} \longrightarrow \text{choline reineckate}_{(s)} + NH_4Cl_{(aq)}$$

The use of ammonium reineckate as standard may be explained through the absorption spectra of ammonium reineckate and choline reineckate in the visible region. Both solutions of ammonium reineckate and choline reineckate in 70% (v/v) aqueous acetonitrile absorb at 397 nm and 525.5 nm (Figure 2). The absorption of both compounds is neither due to the choline ion nor the ammonium ion, but from the chromium complex in the reineckate ion (Szasz and Gimesi 1974; Ahmed and Elbeshlawy 1995; Ragab and Amin 2004; Al-Gahnnam 2007). With this knowledge, any reineckate-containing compound such as ammonium reineckate may be used as standard to quantify the reineckate concentration in the solution of choline reineckate. This quantity can be easily converted to choline chloride concentration through stoichiometry. Standard ammonium reineckate solutions in this work were expressed in choline chloride equivalent (CCE) concentration, a unit concentration that is stoichiometrically equivalent to choline chloride.

Spectrophotometric analysis was carried out at 525.5 nm, a slight difference from 526 nm used in the existing method. Maximum wavelength of absorption ($\lambda_{max}$) at 525.5 nm has a higher absorption response compared to 397 nm (Figure 2) and thus, this wavelength is more suitable and selected for analysis.

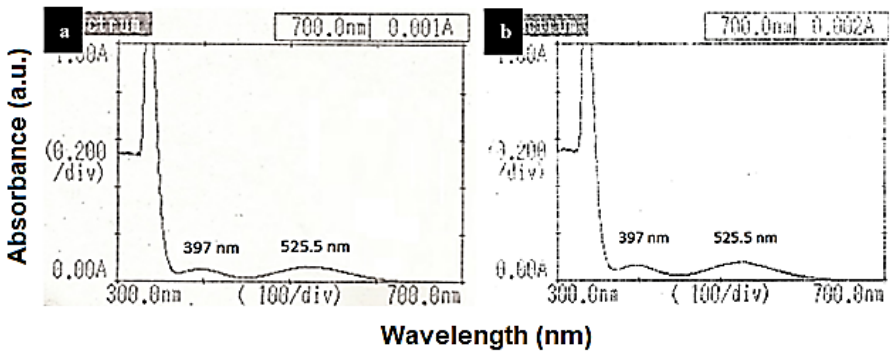


**Figure 2: Visible spectra of (a) 100 mg $L^{-1}$ CCE ammonium reineckate and (b) 100 mg $L^{-1}$ CCE choline reineckate solutions.**

**Method Validation**

Among the figures of merit established for the modified method were linearity, precision (repeatability and intermediate precision), bias (recovery), LOD and LOQ, selectivity, and robustness. Very strong correlation between concentration and response was obtained from 0 to 1200 mg $L^{-1}$ CCE ($r^2 = 0.9995$) and this concentration range was used to construct the standard calibration curve used for analysis, i.e., estimation of choline chloride content (Figure 3). Although the method is found to be considerably linear up to 1500 mg $L^{-1}$ CCE (Figure S1 Supporting Information), higher linearity is obtained when the plot is truncated to 1200 mg $L^{-1}$ CCE.

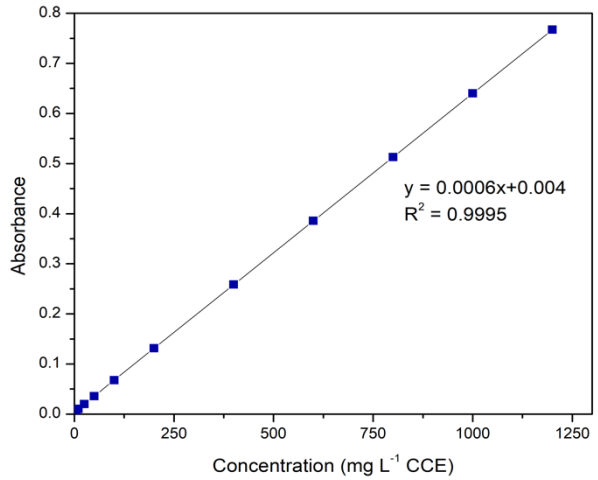


**Figure 3: Absorbance vs. concentration plot of standard ammonium reineckate solutions at 525.5 nm.**

Evaluation of within-day precision (repeatability) gave an RSD value of 0.30%, calculated from the slopes of the calibration curve performed 10 times. A repeatability control chart was constructed and is shown in Figure 4. Determination of inter-day (intermediate) precision gave an RSD value of 0.50%, computed from the slopes of the calibration curve performed for 10 consecutive days. An intermediate precision control chart was also constructed and is presented in Figure 5. RSD values from both repeatability and intermediate precision studies were within the recommended value of AOAC (<1.3-1.9%) for 50% analyte concentration, showing that the modified method exhibits high precision. Moreover, both precision control charts show that the slope of calibration was always within the mean ± 2SD. Recovery values shown in Table 1 were 97.67 ± 2.34%, 104.84 ± 5.32%, and 105.39 ± 6.27% for low, middle, and high spike, respectively. The recommended recovery range for samples containing 50% (w/w) analyte is 98-102% (AOAC 2016; Krishnamoorthy et al. 2012). Although the individual mean values for each spike may be viewed as outside the recommended range, the true mean value for each spike still falls within the recommended range considering the uncertainty values reported. A bar plot was created for clarity and is shown in Figure 6.

LOD and LOQ values (ICH 1994; APVMA 2004) were calculated from the lowest concentration of standard ammonium reineckate solution that gave a response in the spectrophotometric procedure (5.0 mg $L^{-1}$). LOD and LOQ of the method were 2.83 mg $L^{-1}$ CC and 9.42 mg $L^{-1}$ CC, respectively. These values were very small, considering a large amount of choline chloride present in the feed additive sample (~50% (w/w)). Low detection and quantification values mean that the method may also be used in samples containing much smaller amounts of choline chloride such as finished feeds, where choline chloride inclusion typically ranges from 0.5–1.0 g $kg^{-1}$ of feed (Workel et al. 2002).

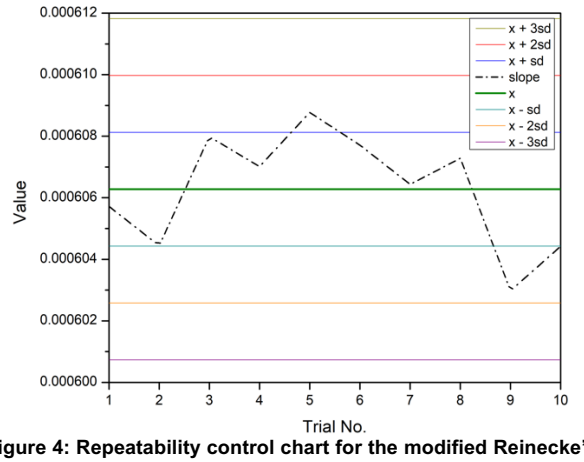


**Figure 4: Repeatability control chart for the modified Reinecke's salt spectrophotometric method. Mean value of the slopes is 6.06 x $10^{-4}$; an upper slope limit (mean + 3*std. dev.) of 6.12 x $10^{-4}$ and a lower slope limit (mean - 3*std. dev.) of 6.01 x $10^{-4}$ were obtained.**

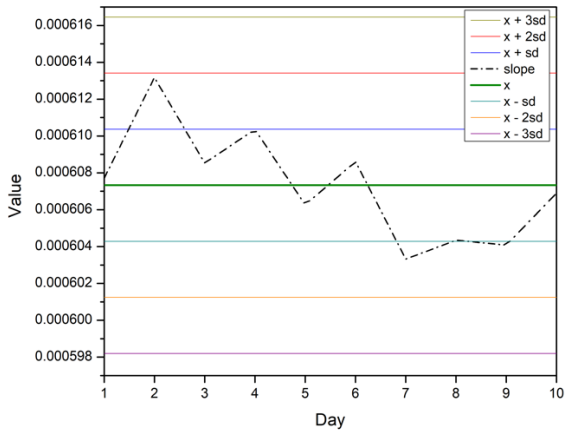


**Figure 5: Intermediate precision control chart for the modified Reinecke's salt spectrophotometric method. Mean value of the slopes is 6.07 x $10^{-4}$; an upper slope limit (mean + 3*std. dev.) of 6.16 x $10^{-4}$ and a lower slope limit (mean - 3*std. dev.) of 5.98 x $10^{-4}$ were obtained.**

**Table 1: Percent recovery values for different amounts of choline chloride (spike) added in feed additive sample.**

| Mass of Choline Chloride Added, g | Percent Recovery[a], % (w/w) |
|---|---|
| 0.0125 | 97.67 ± 2.34 |
| 0.025 | 104.84 ± 5.32 |
| 0.05 | 105.39 ± 6.27 |

[a]mean ± std. dev. (n = 3)

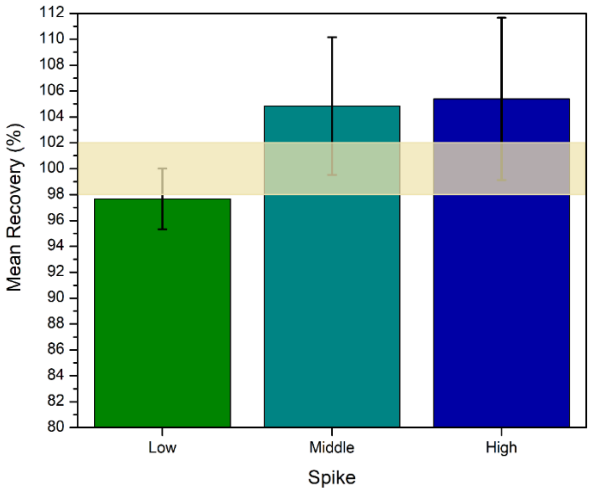


**Figure 6: Mean recovery values for low, middle, and high spike samples. Highlighted area shows the recommended range by AOAC for 50% (w/w) analyte concentration.**

Selectivity of the method was studied in the presence of amino acids – substances that could potentially interfere the analysis by binding with reineckate ions, specifically their cationic forms in solution. Reinecke's salt was once extensively used to precipitate primary and secondary amines (Dakin 1935), including amines derived from amino acids such as proline and hydroxyproline. No known studies had shown the potential precipitation of other amino acids as reineckate salts, and since amino acids are usually incorporated to feedstuffs to fortify their nutritional values, the possibility of interference with the modified method must be evaluated. Among the amino acids tested were essential amino acids for poultry such as glycine and L-isoleucine (Kim et al. 2017) and others were selected to vary the acidity/basicity of amino acids at neutral pH. Results in Table 2 show that the method is not significantly affected by different amino acids based on the small difference in CC content quantified from samples with added amino acids (52.24% (w/w)) and without added amino acids (51.50% (w/w)). These values were close to the reported 50% (w/w) CC content of the feed sample, and comparison of two means using t-test revealed that there is no significant difference between these CC content values, as shown by the calculated t-value (0.6082) that is less than the tabulated value (2.132) at $P < 0.05$ ($df = 4$). Even though amino acids other than proline and hydroxyproline were initially expected to interact with the reineckate ion, no interference in the analysis was observed which could be due to the different length and spatial orientation of those amino acids that might not be preferential for precipitation as a reineckate salt to occur. Accessibility of the primary or secondary nitrogen atoms is crucial to reineckate precipitation which may not be readily accessible for those amino acids tested in this study and thus, no interference was detected.

**Table 2: Choline chloride content of feed additive sample with and without amino acids.**

| Sample | Choline Chloride Content[a], % (w/w) | Calculated t value | Tabulated t value ($p < 0.05$, df[b] = 4) |
|---|---|---|---|
| without amino acids | 51.50 ± 1.94 | 0.6082 | 2.132 |
| with amino acids | 52.24 ± 0.80 | | |

[a]mean ± std. dev. (n = 3)
[b]df – degrees of freedom

Robustness studies were also performed, and the results are presented in Tables 3 and 4. Evaluation of robustness was done up to 7 days of storage for both liquid and solid forms to simulate the average usual time a typical analytical laboratory takes to finish an analysis and make results available to the client. Table 3 shows that choline reineckate solids were stable for one week when stored at 4 °C as aqueous mixture before filtration (delayed filtration), with only a slight lowering of the CC content from 51.98% (w/w) to 50.80% (w/w) within a week. Based on the t-test performed at $P < 0.05$ ($df = 4$), no significant difference was found in the calculated CC content between same-day analysis and up to 7 days of storage. The slight lowering in CC content observed within a week can be attributed to the prolonged precipitate contact with the mother liquor which promotes redissolution of precipitates, otherwise known as peptization. There was also no significant difference in CC contents of sample when the precipitates were immediately filtered, air-dried, and stored in a desiccator at room temperature prior to analysis (Table 4) as supported by the t-test performed at $P < 0.05$ ($df = 4$) between same-day analysis and up to 7 days of storage in which CC contents ranged from 51.25–51.98% (w/w). Although both storage methods did not significantly affect the calculated CC content within a week, the results are suggesting that immediate filtration and storage as dried choline reineckate is more desirable than storing the solids in contact with solution since the extent of change in calculated CC values is lower in the former. All CC contents estimated from robustness studies were also very close to the reported 50% (w/w) CC content of the feed sample.

**Table 3: Choline chloride content of feed additive sample when choline reineckate is stored in contact with solution prior to analysis (delayed filtration).**

| No. of Days Prior to Analysis | Choline Chloride Content[a], % (w/w) | Calculated t-value (vs. same day) | Tabulated t value ($p < 0.05$, df[b] = 4) |
|---|---|---|---|
| same day | 51.98 ± 1.07 | - | 2.132 |
| 3 days | 51.05 ± 0.89 | 1.1567 | |
| 7 days | 50.80 ± 0.87 | 1.4917 | |

[a]mean ± std. dev. (n = 3)
[b]df – degrees of freedom

**Table 4: Choline chloride content of feed additive sample when choline reineckate is stored as dried precipitate prior to analysis (immediate filtration).**

| No. of Storage Days Prior to Analysis | Choline Chloride Content[a], % (w/w) | Calculated t-value (vs. same day) | Tabulated t value ($p < 0.05$, df[b] = 4) |
|---|---|---|---|
| same day | 51.98 ± 1.07 | - | 2.132 |
| 3 days | 51.27 ± 0.11 | 1.1419 | |
| 7 days | 51.25 ± 0.32 | 1.1240 | |

[a]mean ± std. dev. (n = 3)
[b]df – degrees of freedom

Finally, using the modified Reinecke's salt spectrophotometric method, 52.11 ± 0.85% (w/w) CC was quantified in feed additive the sample, close to the unofficial reported value by its source which was approximately 50% (w/w) CC. All in all, the modified method provides a satisfactory approximation of CC content in the feed additive sample and its reliability is supported by the established figures of merit presented above.

**Modified Reinecke's Salt Method versus $AgNO_3$ titration and "Gold Standard"**

$AgNO_3$ (Mohr) titration (ASTM, 2012) is the current method of choice to analyze choline chloride content in most analytical laboratories. However, this method is non-selective to choline since it directly quantifies the chloride content of the sample which is stoichiometrically converted to CC content. The titration method quantified 56.71 ± 0.63% (w/w) CC in the feed

additive sample and is found to be highly statistically different when compared against the HPLC method (Table 5). This is attributed to the non-selectivity of this method to choline which cannot discriminate choline chloride from other choline chloride sources such as table salt (NaCl) – the most common choline adulterant in feedstuffs among others, that may be present in the sample. The value for the recovery of the in-house laboratory QAS using this method was 109.30 ± 0.24%. There is also an extremely significant difference between the CC values obtained by the titration method and the modified method as shown by the large value of computed t-value (Table 5). Based on these results, there is a good indication that the modified method has indeed greater selectivity for choline than the titrimetric analysis, as it directly quantifies the choline content in the sample and not the amount of chloride which prevents over estimation of the CC content.

**Table 5: Comparison of choline chloride contents quantified in the feed additive sample using different methods of analysis.**

| Method of Analysis | Choline Chloride Content, % (w/w) | Calculated t-value (vs. HPLC gold standard) | Calculated t-value (vs. modified method) | Tabulated t value ($p < 0.05$) |
|---|---|---|---|---|
| HPLC Analysis | 48.21 ± 3.50[a] | - | 2.6514 | - |
| Modified Reinecke's Salt | 52.11 ± 0.85[a] | 2.6514 | - | 1.8125[c] |
| $AgNO_3$ (Mohr) Titration | 56.71 ± 0.63[b] | 4.0375 | 8.1557 | 1.8946[d] |

[a]mean ± std. dev. (n = 6)
[b]mean ± std. dev. (n = 3)
[c]df = 10
[d]df = 7

In the HPLC method, the cationic urethane choline derivative appears at approximately 14 min of run time, as shown in Figure 7a. A blank run shows that there is no peak at the 14 min mark when choline is absent (Figure 7b), confirming that the peak at this region is due to the urethane choline derivative. Linearity of the HPLC method was established from 0 to 1000 µmol $L^{-1}$ and had a $r^2$ of 0.998. Precision of the method, however, gave an RSD value of 7.25%, outside the recommended range by the AOAC (1.3-1.9%) (AOAC, 2016). The mean recovery value was 97.73 ± 7.53% (w/w) CC. Calculated LOD and LOQ were 8.82 µmol $L^{-1}$ CC and 29.41 µmol $L^{-1}$ CC, respectively. In theory, the HPLC method is a more established method and may be used as a "gold standard" against new analytical methods.

Using the HPLC method, 48.21 ± 3.50% (w/w) CC content was quantified in the feed additive sample. This value is lower than the CC content obtained from the modified Reinecke's salt method and is totally expected from the HPLC method since its selectivity to choline is considered higher compared to the spectrophotometric method. This may be attributed to the higher binding affinity of the isocyanate to choline compared to that of the reineckate ion, which makes this method less susceptible to interference effects due to higher selectivity to the analyte.

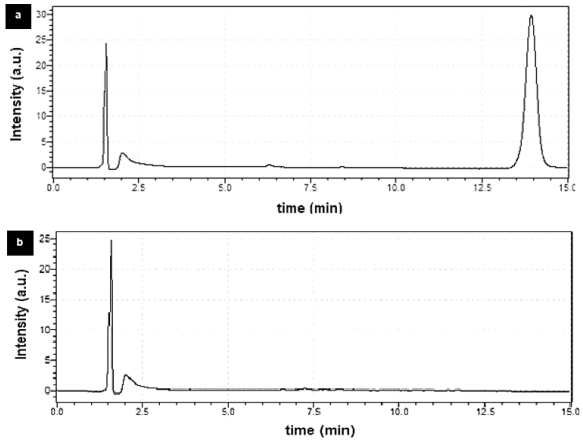


**Figure 7: HPLC chromatogram of (a) choline derivative (1000 µmol $L^{-1}$ choline chloride) and (b) method blank showing the absence of sharp urethane choline derivative peak at 14 min run time.**

Statistical analysis shows that there is a significant difference between the CC contents obtained from HPLC method and the modified Reinecke's salt method (Table 5), however, it should be noted that the precision of the considered "gold standard" is lower than the modified method and was found to be outside the recommended AOAC range for RSD value. This makes it difficult to tell whether the values obtained from the "gold standard" may be considered as exactly true value. The modified Reinecke's salt method gave a recovery value of 108.09% against the HPLC "gold standard" method (calculated using $CC_{modified}/CC_{HPLC}$ x 100) and is outside the recommended range set by AOAC, and this result may be attributed again to the trueness of the value obtained using the HPLC method. A summary of all the CC contents obtained from different analytical methods is given in Table 5, and all values obtained in individual replicates are presented in Table S1 in the Supporting Information. The CC value obtained using the modified Reinecke's salt method can still be considered a good estimate of CC content in the feed additive sample considering the method's simplicity, lower selectivity compared to HPLC, and lower cost of analysis.

## CONCLUSION

In summary, Reinecke's salt spectrophotometric was modified and extensively validated to quantify choline chloride in a feed additive sample. The modified Reinecke's salt method is an enhancement of the original existing method in terms of convenience, analysis time, and cost of analysis. It provides good estimation of choline chloride content, supported by satisfactory figures of merit for method validation. Low values of LOD and LOQ suggests that the method may also be applicable to samples containing lower amount of choline chloride such as finished feeds. This method is more selective to choline compared to $AgNO_3$ titration which is commonly used in analytical testing laboratories, and it offers an alternative method which addresses and potentially solves the rampant choline chloride adulteration in feedstuffs. More importantly, this method is designed for and tested in feed additive and gives a good estimate of choline chloride content without the need of highly elaborate and sophisticated instrumentation.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## CONTRIBUTIONS OF INDIVIDUAL AUTHORS

H.E.T. Mendoza was involved in the conception and design of study, acquisition, analysis and/or interpretation of data, and writing and drafting of the manuscript. M.S. Rodriguez and V.P. Migo were involved in the conception and design of study, analysis and/or interpretation of data, and manuscript revision.

## REFERENCES

[AOAC] Association of Official Analytical Chemists. Official methods of analysis of AOAC International. 16th ed. Maryland, USA: AOAC International, 1995.

[AOAC] Association of Official Analytical Chemists. Official methods of analysis of AOAC International. Appendix F (Guidelines for Standard Method Performance Requirements). Maryland, USA: AOAC International, 2016.

[APVMA] Australian Pesticides and Veterinary Medicines Authority. Guidelines for the validation of analytical methods for active constituent, agricultural and veterinary chemical products. Kingston, Australia: APVMA, 2004.

[ASTM] American Society for Testing and Materials. D512-12: Standard test method for chloride ion in water, test method B (silver nitrate titration). Pennsylvania, USA: ASTM International, 2012.

Ahmed MA, Elbeshlawy M. Colorimetric and atomic absorption spectrometric determination of some heterocyclic nitrogenous compounds of pharmaceutical interest, Polym Plast Technol Eng 1995: 28:14, 2535-2545. https://doi.org/10.1080/00032719508004033

Al-Ghannam SM. Spectrophotometric and atomic absorption spectrometric determination of cephalexin and cephradine in dosage forms. J Food Drug Anal 2008; 16(2). https://doi.org/10.38212/2224-6614.2368

Argoudelis CJ, Tobias J. The determination of small quantities of choline reineckate. Anal Biochem 1975; 64: 276-280. https://doi.org/10.1016/0003-2697(75)90429-7

Dilger RN, Garrow TA, Baker DH. Betaine can partially spare choline in chicks but only when added to diets containing a minimal level of choline. J Nutr 2007; 137(10): 2224-2228. https://doi.org/10.1093/jn/137.10.2224

Dakin HD. Reinecke salt. Organic Synt 1935; 15: 74. https://doi.org/10.15227/orgsyn.015.0074

Hefni M, McEntyre C, Lever M, Slow S. A simple HPLC method with fluorescence detection for choline quantification in foods. Food Anal Methods 2015; 8(9): 2401-2408. https://doi.org/10.1007/s12161-015-0131-9

Hirsch J. Prediction of choline chloride concentration on silage by FT-NIR spectroscopy. Thermo Fisher Scientific 2007, Madison, WI, USA. Retrieved from https://assets.thermofisher.com/TFS-Assets/CAD/Application-Notes/D14194~.pdf on 30 August 2015

[ICH] International Conference for Harmonization of Technical Requirements. Validation and analytical procedures: text and methodology Q2(R1). Geneva, Switzerland: ICH, 1994.

Krishnamoorthy G, Priyadarshini CD, Senthamarai R. Spectrophotometric method of choline bitartrate in bulk and its tablet formulation. Asian J of Pharm Anal 2012; 2(4): 114-115.

Kim H, Do HW, Chung H. A comparison of the essential amino acid content and the retention rate by chicken part according to different cooking methods. Korean J Food Sci Anim Resour 2017; 37(5): 626-634. https://doi.org/10.5851/kosfa.2017.37.5.626

Llerena JJ, Abad E, Caixach J, Rivera J. An episode of dioxin contamination in feedstuff: the choline chloride case. Chemosphere 2003; 53: 679-683. https://doi.org/10.1016/s0045-6535(02)00825-1

Mazzetti S, Bracco C, Regge D, Caivano R, Russo F, Stasi M. Choline-containing compounds quantification by 1H NMR spectroscopy using external reference and noise measurements. Phys Med 2013; 29: 677-683. https://doi.org/10.1016/j.ejmp.2012.07.002

Pal S, Sharma MJ, Danielsson B, Willander M, Chatterjee R, Bhand S. A miniaturized nanobiosensor for choline analysis. Biosen Bioelectron 2014; 54: 558-564. https://doi.org/10.1016/j.bios.2013.11.057

Ragab GH, Amin AS. Atomic absorption spectroscopic, conductometric and colorimetric methods for determination of fluoroquinolone antibiotics using ammonium reineckate ion-pair complex formation. Spectrochim Acta A 2004; 60: 973-978. https://doi.org/10.1016/S1386-1425(03)00327-5

Schenkel LC, Sivanesan S, Zhang J, Wuyts B, Taylor A, Verbrugghe A, Bakovic M. Choline supplementation restores substrate balance and alleviates complications of Pcyt2 deficiency. J Nutri Biochem 2015; 26(11): 1221-1234. https://doi.org/10.1016/j.jnutbio.2015.05.014

Snyder LR, Kirkland JJ. Introduction to modern liquid chromatography, 2nd ed. New York: John Wiley and Sons. 1979: 863p.

Szasz G, Gimesi O. Determination of choline and trimethylamine in seeds and premixes. Period Polytech Chem Eng 1974; 18(3): 203-210.

Workel HA, Keller T, Reeve A, Lauwaerts A. Choline – the rediscovered vitamin for poultry. 2002. Retrieved from https://thepoultrysite.com/articles/choline-the-rediscovered-vitamin-for-poultry on 30 August 2015

Zeisel SH, Blusztajn JK. Choline and human nutrition. Annu Rev Nutr 1994; 14: 269-296. https://doi.org/10.1146/annurev.nu.14.070194.001413

Zhang J, Zhu Y. Determination of betaine, choline and trimethylamine in feed additive by ion-exchange liquid chromatography/non-suppressed conductivity detection. J Chromatogr A 2007; 1170: 114-117. https://doi.org/10.1016/j.chroma.2007.09.014

## SUPPLEMENTAL INFORMATION

**Table S1: Summary of choline chloride content quantified in feed additive sample in various replicates using the modified Reinecke's salt spectrophotometric, HPLC (gold standard), and $AgNO_3$ (Mohr) titration methods.**

| METHOD | CHOLINE CHLORIDE CONTENT, % (w/w) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Modified Reinecke's Salt | 51.46 | 53.05 | 52.20 | 51.62 | 53.19 | 51.14 |
| HPLC Analysis | 44.58 | 50.66 | 46.39 | 45.19 | 53.62 | 48.81 |
| $AgNO_3$ Titration | 56.00 | 56.91 | 57.21 | - | - | - |

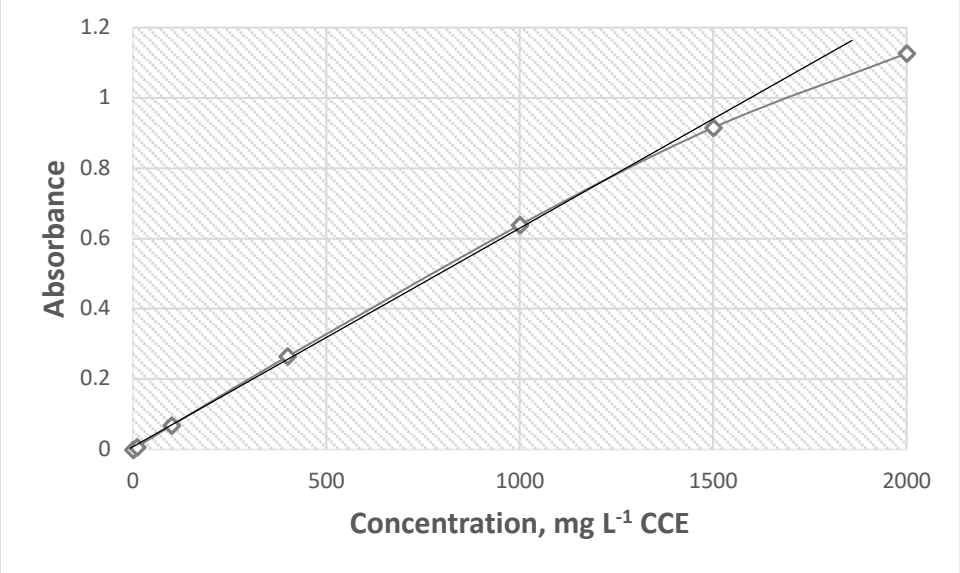


**Figure S1: Absorbance of standard ammonium reineckate solutions from 0 to 2000 mg $L^{-1}$ CCE at 525.5 nm.**