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Research Article

A dual choline/phosphocholine colorimetric method for measuring the relative strength of inhibitors of choline kinases of Gram-positive pathogens

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Abstract

Using chemical and artificial preservation methods to keep food safe is becoming an issue with consumers. Therefore, it is important to discover antimicrobials from natural sources for use in food safety applications. The objective of study was to establish screening system for natural inhibitors of choline kinase (ChoK), a known antimicrobial target. A previously developed dual choline/phosphocholine colorimetric method was used to determine the relative strength of 3 choline kinase inhibitors: Hemocholinium-3, RSM-928A, and MN 58. Whole cell extracts containing the choline kinase of *Streptococcus pneumoniae* (sChoK) was used as a model. The measured IC50 values of these drugs were >2700 µM, 0.54 µM, and 170-225 µM, respectively. Importantly, not every step of the colorimetric method could be used in the case of every inhibitor, since each had its own particular reactive profile with the colorimetric dyes, which could have led to confounded measurements. However, in every case, the system was flexible enough to measure choline or phosphocholine, if not both metabolites. We establish here that this dual choline/phosphocholine system is flexible enough to measure the IC50 any possible inhibitor. This colorimetric method is an ideal benchtop method for screening for natural inhibitors of bacterial ChoKs. Practical applications: This system is an easy to implement method for screening inhibitors of choline kinase isoforms of Gram-positive bacteria in enzymatic reactions in which whole cell extracts are the source of the enzyme. Choline kinases from food pathogens, such as *Staphylococcus aureus*, and *Bacillus cereus*, could be assessed with this method. This method is highly advantageous for screening putative choline kinase inhibitors from natural sources for development as food preservatives.

Keywords: pathogens, choline kinase, enzyme inhibition, colorimetry, choline, phosphocholine, natural antimicrobials.

Abbreviations: ADP – adenosine diphosphate; ATP – adenosine triphosphate; Cho – choline; ChoK – choline kinase; CTA – cell wall teichoic acid; DMCO – dimethyl sulfoxide; HC-3 – hemocholinum-3; hChoK – human choline kinase; IPTG - Isopropyl β-D-1-thiogalactopyranoside; LB – Luria Broth; LTA – lipoteichoic acid; MIC: minimum inhibitory concentration; sChoK – S. pneumoniae choline kinase; Pcho - phosphocholine

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Introduction

Due to the increase in the number of foodborne outbreaks caused by pathogenic microorganisms such as *Staphylococcus aureus* and *Bacillus cereus*, there has been an concomittant increase in concerns over food safety (Tajkarimi, Ibrahim et al. 2010, Gyawali and Ibrahim 2012, Gyawali and Ibrahim 2014). In addition, the misuse of antibiotics has resulted in the rise of antibiotic resistant pathogens that are becoming more tolerant to several food processing and preservation methods (Gyawali & Ibrahim 2014). Meanwhile, consumers are concerned about the use of chemical and artificial antimicrobial compounds and preservatives. In order to promote consumer acceptance, the use of natural antimicrobials to control microorganisms has been increasing and being incorporated into food systems. Choline kinase (ChoK) is an enzyme that catalyzes the production of phosphocholine (PCho) from choline (Cho) and ATP (Wittenberg and Kornberg 1953). ChoK enzymes play a key role in cell growth and division in eukaryotic cells (Lacal Sanjuan 2015) and are also oncogenic drug targets for cancer cells (Lacal 2001) as well as for parasites such as *Plasmodium falciparum* (Zimmerman, Moneriz et al. 2013). The role ChoK plays in bacterial cell division and growth is less clear, although it is known to be involved in the pathway leading to the production of lipoteichoic acid (LTA) and cell wall teichoic acid (CTA) (Whiting and Gillespie 1996, Grundling and Schneewind 2007). Experimentally unconfirmed sequence predictions suggest that other Gram-positive pathogens, such as *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum* also produce ChoK isoforms. Importantly, the choline kinase of *Streptococcus pneumoniae* (sChoK) was recently established as a drug target (Zimmerman and Ibrahim 2017) and has since served as model of ChoK inhibition for Gram-positive bacteria. Inhibiting sChoK was found to effectively slow cell growth and division in this species. However, the only known inhibitor of sChoK is Hemicholinium-3 (HC-3), which was found to only weakly inhibit sChoK. Stronger inhibitors need to be found that can be used to prevent the growth of food-borne pathogens that express bacterial ChoKs. Ideally these should come from natural sources in order to be acceptable for use in food systems. In order to establish a screening system for promising natural antimicrobials that inhibit bacterial ChoKs, we have developed a system to gauge the relative strength of ChoK inhibitors using a colorimetric system that had been designed to monitor enzymatic activity (Zimmerman and Ibrahim 2018).

Materials and Methods

Materials

All chemical reagents were purchased from Sigma-Aldrich, unless otherwise noted.

Methods

Colorimetric quantification of Cho consumption and PCho production.

We used a colorimetric method that has recently been described (Zimmerman and Ibrahim 2018). This method has three steps that were performed on each sample (1) a charcoal filtration step for removal of ATP and ADP, (2) a triiodide step for quantification of Cho, and (3) Molybdenum blue dye (MBD) step for quantification of PCho (Fig. 1 for schematic).

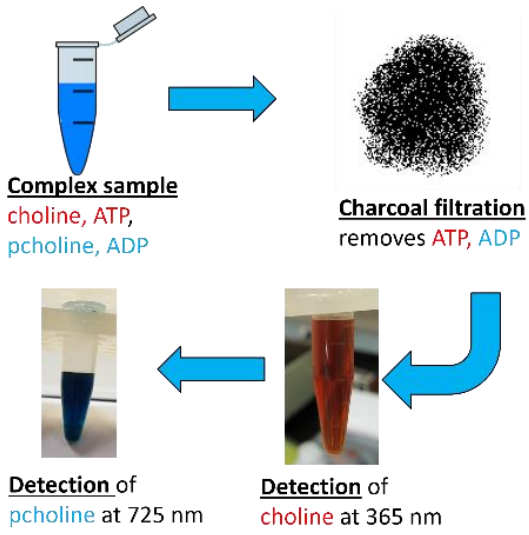


Figure 1. Simplified schematic of the charcoal-triiodide molybdenum method for measuring choline kinase activity**.**

Preparation of Choline Kinase Enzyme Extracts.

The *S. pneumoniae* LicA gene expressing sChoK had been previously cloned into the pET28a plasmid (Wang, Jiang et al. 2015). The method of extract preparation has previously been described (Zimmerman and Ibrahim 2018). In brief, the plasmid was transformed into BL21 (DE3) cells (New England Biolabs). These cells were then used to inoculate 10 mL of Luria Broth (LB) which was incubated overnight at 37°C. The next morning, 10 mL of fresh LB was inoculated to 2% with the overnight culture and then incubated at 37°C until an O.D.600 of 0.6 was reached. The culture was cooled on ice, and IPTG was added to a final concentration of 1 mM to induce production of sChoK. The culture was then incubated overnight at room temperature after which it was centrifuged for 10 min at 3500 g, resuspended in 1 mL 100 mM Tris pH 8 and transferred to an Eppendorf tube. Three mg of Glasperlen beads (*Sartorius Stedim*) were added to the resuspension and lysis was performed using a Bead-Beater 16 (Biospec products). The lysate was centrifuged at 20,000 g for 30 min at 4°C. The supernatant was removed, aliquoted into Eppendorf tubes, and stored at −20°C. An aliquot was defrosted on ice when needed for use in an enzymatic reaction.

Preparation of Colorimetric solutions/dyes.

Preparation of Activated Charcoal. Activated charcoal was suspended in 0.1 N HCl for a total of 2.5 g.50 mL^{-1} and then mixed by inversion and centrifuged at 4000 g for 20 min at 4°C. The supernatant was removed and the charcoal pellet was resuspended in fresh 0.1 N HCl. This sequence was repeated 3 times, and the charcoal was resuspended in 50 mL 0.1 N HCl.2.5 g^{-1}. The suspension was stored at 4°C until needed.

Preparation of Triiodide dye. A solution of potassium triiodide was prepared using the following reagents (per 100 mL deionized water): 15.7 g of reagent grade iodine and 20 g of reagent grade potassium iodide. The solution was stored at 4°C until immediately before use.

Preparation of MBD dye. Molybdenum blue dye (MBD) was prepared fresh daily two hours before use. One and two-tenths of a gram of Phosphomolybdic acid hydrate (Sigma-Aldrich, St. Louis, MS, USA) and 0.2 g Stannous chloride (Fisher Scientific, Hampton, NH, USA) were dissolved in 2.5 N HCl by vortexing for 1 min. Twenty mL of deionized water were added, and the mixture was vortexed for 1 more minute. The resulting solution was then filtered using a 0.2 µM GPF/CA membrane non-sterile syringe filter (Phenomenex, Torrance, CA, USA).

sChoK Enzymatic Reaction.

Four microliters of ChoK enzyme extract were added per 10 mL reaction buffer (RB: 100 mM Tris, 10 mM $MgCl_2$, 1 mM Cho, 1 mM ATP). Five mL reaction mixes with various concentrations of HC-3, MN58b and RSM-932A were prepared by serial dilution. The reactions were incubated in a water bath at 37°C for 60 min. One mL samples were removed in triplicate from each reaction mix after the incubation and placed in Eppendorf tubes on ice for 1 min. The samples were then immediately heated to 95°C in a heat block for 3 min to stop the reaction. Samples were then placed on ice again for a minimum of 10 min.

Colorimetric Procedure. Activated Charcoal Filtration of ATP/ADP. Three-hundred µL of this suspension were added to the enzymatic reaction sample and mixed by inversion at 1 min intervals for 10 min and then centrifuged in tubes at 20 000 g 4°C for 1 h to remove the charcoal and denatured protein. One mL of supernatant from each sample was transferred to a clean 1.5 mL Eppendorf tube. The Cho in these samples was then quantified.

Triiodide Quantification of Cho. Four-hundred microliters of triiodide solution were added to each charcoal supernatant sample after which the mixture was immediately placed on ice for 1 hour. The samples were then centrifuged at room temperature for 15 min at 20,000 g. One mL of each sample in triiodide solution was then set aside in fresh Eppendorf tubes for the subsequent PCho quantification. The remainder of the supernatant was discarded without disturbing the pellet which exhibited a dark red color. Because chlorine iodide decays quickly (Appleton, La Du et al. 1953), 1 mL 1,2-Dichloroethane, was immediately added. The pellets were then dissolved by vortexing, resulting in an intense orange color (Fig. 2B). Some charcoal fines were occasionally left over from the previous step; however, these did not dissolve and were left for 1 min to settle to the bottom of the tube before continuing with the procedure. As previously reported, the residual triiodide solution did not interfere with subsequent measurements. Forty µL of each sample were aliquoted into wells of Greiner Bio-one CellStar® U-bottom 96 well-plates. One hundred sixty µL of 1,2-dichloroethane were then added to each sample-containing well. Two hundred µL of 1,2-dichloroethane were added to an empty well in the plate and used as the blank. Absorbance was measured at 365 nm in a BioTek Synergy HT microplate reader. Standard curves were constructed using defined quantities of Cho (Fig. 2C).

MBD Quantification of PCho. Four hundred µL of MBD were added to 1 mL enzymatic assay samples in triiodide. The samples were placed on ice for 2 h and then centrifuged at 20 000 g for 3 min at room temperature. The pellets had a dark blue color. The supernatants were discarded, and 1 mL of a 1:1 solution of 2.5 N HCl: acetone was added to the pellet resulting in a blue solution whose intensity increased with concentration (Fig. 2B). Two hundred µL of each resuspension were aliquoted onto a well in a Greiner Bio-one CellStar® U-bottom 96-well plate. Absorbance readings were immediately made at 725 nm using a BioTek Synergy HT microplate reader. Standard curves were constructed using defined quantities of PCho (Fig.2D).

Results and Discussion

Quantification of choline and pcholine. Using fixed amounts of Cho and PCho, we observed increases in color intensities with the triiodide and MBD dyes respectively (Fig. 2A, 2B). This was reflected in absorbance values, which increased linearly with Cho and PCho concentration (Fig. 2C, 2D). Calculating IC_{50} of

sChoK inhibitors HC-3, RSM-932A and MN58b. Increasing amounts of each drug were added to enzymatic reactions containing the substrates and extracts containing sChoK until a maximum % inhibition was reached with each drug, as determined in comparison to a drug-free control reaction. Data was fitted into sigmoidal curves (Fig. 3) and the IC50s were determined from these curves.

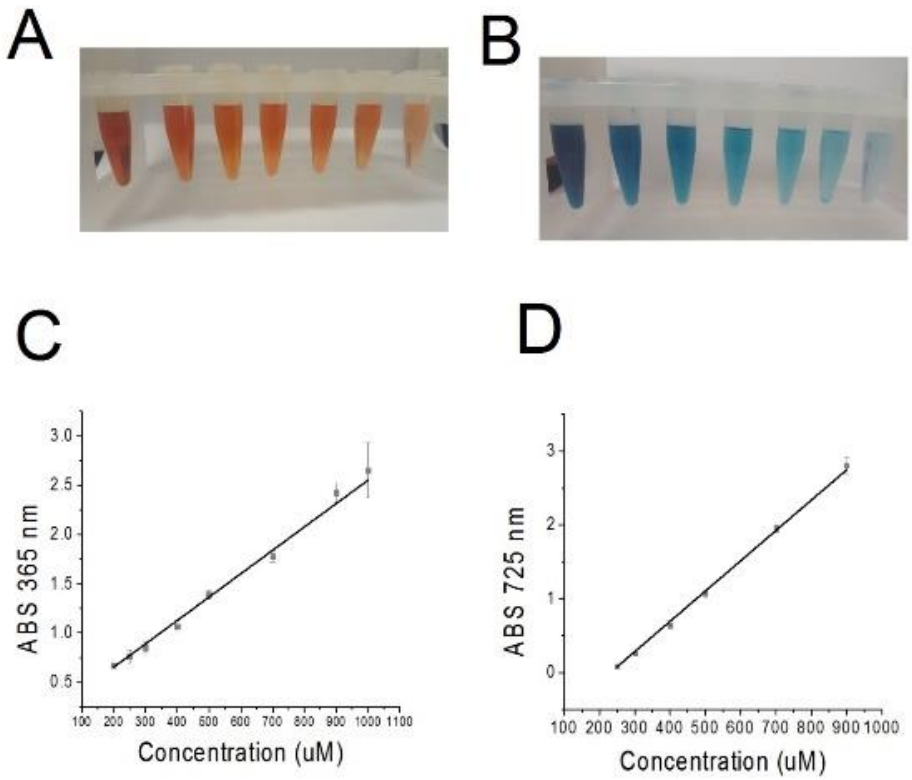
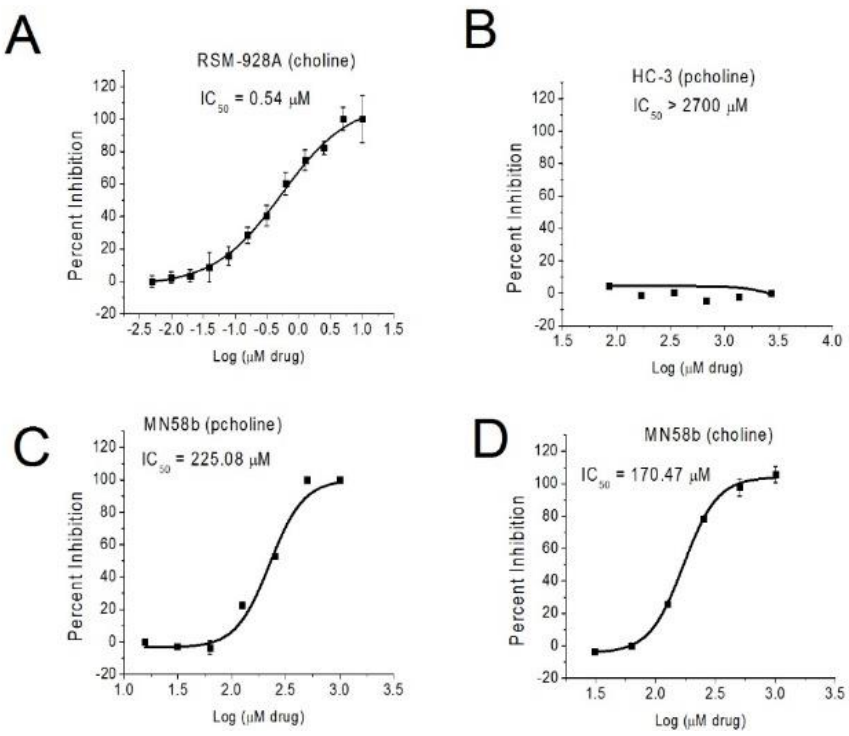


Figure 2. (A) Detection of fixed amounts of choline (left to right, 900 µM, 500 µM, 400 µM, 300 µM, 250 µM, and 200 µM) using the triiodide solution. (B) Detection of fixed amounts of PCho (left to right, 900 µM, 500 µM, 400 µM, 300 µM, 250 µM, and 200 µM) using the MBD solution. The intensity of the blue increases as the concentration of PCho increases. An increase in PCho production is in turn indicative of weaker inhibition. (C) Standard curve relating the absorbance of triiodide reagent measured at 365 nm at each concentration of Cho. (D) Standard curve relating the absorbance of molybdenum reagent measured at 725 nm at each concentration of PCho.

Quantification of Choline and Pcholine. Using fixed amounts of Cho and PCho, we observed increases in color intensities with the triiodide and MBD dyes respectively (Fig. 2A, 2B). This was reflected in absorbance values, which increased linearly with Cho and PCho concentration (Fig. 2C, 2D).

Calculating IC50 of sChoK inhibitors HC-3, RSM-932A and MN58b. Increasing amounts of each drug were added to enzymatic reactions containing the substrates and extracts containing sChoK until a maximum % inhibition was reached with each drug, as determined in comparison to a drug-free control reaction. Data was fitted into sigmoidal curves (Fig. 3) and the IC_{50}s were determined from these curves.



Data were expressed as Mean ± SD (n = 3).

Figure 3. Determinations of the IC_{50} of RSM-932A, HC-3, and MN58b. The inhibitory effect of RSM-932A on choline kinase was studied by measuring choline production of sChoK using a charcoal-triiodide combination. Meanwhile, the effect of HC-3 could only be measured using the molybdenum method. The effects of MN58b could be measured using the full charcoal-triiodide-MBD method so that both Cho and Pcho could be quantified.

Determining the reactivity of 3 choline kinase inhibitors with charcoal, triiodide, and MBD. Three inhibitors were chosen for this study. The first, hemicholinium-3 (HC-3), had already been demonstrated to inhibit the choline kinase of the gram-positive bacteria *S.pneumoniae* (Zimmerman and Ibrahim 2017).

Two more inhibitors, MN58b (Hernandez-Alcoceba, Saniger et al. 1997) and RSM-932A (Lacal and Campos 2015) were chosen for being well-characterized inhibitors of human choline kinase (hChok) and parasite choline kinases (Zimmerman, Moneriz et al. 2013). As a first step we had to determine two things about the inhibitors: 1) Did the inhibitors interact with either the triiodide or MBD dye, that is, did any have the potential to confound either the Cho or PCho measurements? (2) If there was an interaction, could the activated charcoal filter them out? As summarized in Table 1, we found that each inhibitor had its own reactivity with regard to each component of the colorimetric system. HC-3 was reactive with the triiodide dye but not the MBD dye.

Table 1. Reactivity of the drugs RSM-932A, MN58b, HC-3 and the reagent DMSO with the different components of the colorimetric detection system, as which components were chosen to assess strength of inhibition of choline kinase. In the case of MN58b, the charcoal filtered the drug before it could react with either the triiodide or molybdenum solution, which meant that both Cho and PCho could be assessed in the enzymatic reaction.

Reactivity/ Reagent	**HC-3**	**MN58b**	**DMSO**	**RSM-932A (in DMSO)**
charcoal	Partial only	YES	NO	Yes
Triiodide	YES	YES	NO	NO
Molybdenum	NO	YES	YES	YES
Method for IC50	**Molybdenum only**	**Triiodide and Molybdenum**	**Triiodide only if DMSO used**	**Triiodide only (due to DMSO)**

Meanwhile HC-3 could only be partially filtered by the activated charcoal step at the highest concentration tested in the enzymatic reactions (2700 μM). As a consequence, only MBD dye quantification of PCho could be used to determine the strength of inhibition of HC-3. Meanwhile, while MN58b was reactive with the triiodide, it was filtered out by the activated charcoal at the concentrations used. Therefore, both the triiodide solution and the MDB dye could be used to determine inhibitor strength in the case of this inhibitor at the concentrations tested.

DMSO at 5% v/v was shown to react with the MBD dye and therefore confound the measurements. This was important, because, due to its low solubility, RSM-932A had to be first solubilized in DMSO before being added to the reaction mix. Therefore DMSO could also be a confounding factor, and RSM-932A activity could only be assessed using the triiodide quantification of Cho. Due to the redundancy of the activated charcoal-triiodide-MBD system of parallel quantification of Cho and PCho, choline kinase inhibitors could always be assessed in an enzymatic reaction, no matter what the confounding effects of those inhibitors were. We could now proceed with measuring the IC_{50} of HC-3, MN58b, and RSM-932A in order to compare their strength of inhibition.

Determining the relative strength of 3 choline kinase inhibitors.

As can be seen in Fig. 2A, the IC_{50} of RSM-932A was determined using the triiodide measurements of Cho alone and the result was an IC_{50} of 0.5 μM. This makes RSM-932A the strongest inhibitor of sChoK ever described and therefore the most likely candidate for further development against Gram positive pathogens. Importantly, the preclinical studies for RSM-932A have already been carried out (Lacal and Campos 2015), and the phase I clinical trial of this drug has been completed (https://clinicaltrials.gov/ct2/show/NCT01215864). This makes RSM-932A a promising candidate for development of an antibiotic therapy for Gram-positive infections. Meanwhile, the IC_{50} of HC-3 could not be

determined under the under the conditions of the enzymatic reaction used here (Fig. 2B). Although HC-3 is known to be an inhibitor of sChoK (Zimmerman and Ibrahim 2018), inhibition could only be observed with very low titers of enzyme in the enzymatic reaction. Nevertheless, we can state that the IC_{50} > 2700 μM, which the highest concentration of HC-3 that could be tested due to limitations of solubility. This makes HC-3 by far the weakest inhibitor of sChoK known so far. The IC_{50} of MN58b differed slightly depending on whether the triiodide or the MBD measurements were relied upon (Fig. 2B, 2C), either 170 μM or 225 μM, respectively. Comparing the IC50s of the 3 choline kinase inhibitors to minimum inhibitory concentrations (MICs) in bacterial cultures, RSM-928 had the strongest effect on choline kinase activity, followed by MN58b, with HC-3 being a distant third. This corresponded will with the MICs measured in active cultures of *S. pneumoniae*. which showed MICs of 0.2 μM, 10 μM and 5400 μM for RSM-928, MN58b, and respectively. We had analogous results with *S. aureus*. Here we have shown that at a concentration 10 μM, the effectiveness of these drugs follow the follows the pattern RSM-928> MN58b > HC-3.

Future challenges

There are many challenges to establishing choline kinase inhibitors as a broad spectrum inhibitor of food pathogen growth for use in food safety applications. For example, the existence of choline kinase activity in Gram positive food pathogens needs to be established. Though choline kinase is predicted by sequence analysis to exist in many Gram-positives, choline kinase activity has so far only been firmly established in *S. pneumoniae.* The colorimetric method described here should be very helpful in this respect, as it has been designed to be used with bacterial extracts as the source of enzyme. A second challenge is establishing that choline kinase is indeed a target in food pathogen species. Again the colorimetric method described here will be critical for this goal. A third challenge is discovering natural sources of bacterial choline kinase inhibitors. Inhibitors of human choline kinase from natural sources do exist (Estevez-Braun et al. 2015), it remains to be seen if similar inhibitors can be found for bacterial versions of the enzyme. Testing such inhibitors will also be carried out using the colorimetric method.

Conclusions

The only known inhibitor of sChoK documented up until now is HC-3 (Zimmerman and Ibrahim 2017). To these we add two newly characterized inhibitors of sChoK: RSM-932A and MN58b. Both are far stronger inhibitors than HC-3, particularly RSM-932A (IC_{50} = 0.5 μM), which is consistent with the relative strengths of these drugs against *S. aureus* and *S. pneumonia* growth. However, in order to develop choline kinase as a target for antimicrobials for use in food as a prophylactic against food-borne pathogens, natural sources of choline kinase inhibitors need to be explored and screened for strength of inhibition The charcoal-triiodide-MBD method is an efficient method for measuring choline kinase activity via quantification of Cho and PCho. More importantly, we have demonstrated here that this is a redundant and flexible method for measuring choline kinase inhibition. This means if a drug is reactive with one component of the colorimetric system, it cannot by definition, react with another component. As a consequence, no matter the inhibitor tested, either Cho, PCho or both metabolites can be measured to characterize strength of inhibition. This makes the colorimetric method an ideal benchtop method for medium throughput

screening of choline kinase inhibitors and for measuring the relative strength of each inhibitor. This method will critical to the development of natural ingredients to block the growth of Gram-positive food-borne pathogens via inhibition of the target enzyme choline kinase.

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