Favier (1961) which demonstrated a comparable amino acid loss when a lysine–glucose solution was heated for 25 h at 120 °C, or for 6 h at 130 °C.

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Determination of Choline in Soybean Meal by Liquid Chromatography with the Ion-Exchange Membrane Detector

John G. Dorsey, Larry C. Hansen, and T. W. Gilbert*

A liquid chromatographic method for the determination of choline in feeds is described, using the ion-exchange membrane detector. An 8-h acid hydrolysis–extraction step is required to quantitatively extract the bound choline. Chromatographic analysis is accomplished in 20 min. Results compare favorably with an NMR method and are shown to be more reproducible and faster than popular ammonium reineckate methods.

Choline occurs in the cells of all living things. It may occur free in small quantities; however, it is usually bound in phospholipids (lecithins and sphingomyelin), in acetylcholine and other esters, or in phosphorylcholine. Choline is an important nutrient and serves many functions in the body such as methyl donor for transmethylation. A choline deficiency interferes with normal bone growth and fat metabolism. This importance of choline as an essential nutrient creates a need for suitable analytical methods.

Many methods for the determination of choline (as well as choline esters) are based on the use of ammonium reineckate as a precipitant. The original gravimetric method (Kapfhammer and Bischoff, 1930) was later modified to include a colorimetric comparison for quantitation (Beattie, 1936). Probably the most widely used method today for the determination of choline in feeds is the reineckate method of Lim and Schall (1964). As popular as these methods have been, they still suffer from the drawback that other compounds such as acetylcholine, other choline esters, and other quaternary nitrogen compounds will also form reineckates and, therefore, interfere with any determination where separation of these interfering compounds is not first accomplished.

Harkiss (1972) surmounted this problem by precipitating choline reineckate, converting it back to choline chloride, chromatographing on Whatman No. 1 paper, and locating the sample with Dragendorff reagent. Unfortunately, this method has a range of only 0.3 to 2.4 μg of choline chloride.

Other methods that have been reported for the determination of choline, mostly with biological samples, include pyrolysis GC (Schmidt and Speth, 1975), high-voltage electrophoresis (Brooker and Harkiss, 1974), thin-layer chromatography using a Dragendorff reagent (McLean and Jewers, 1972), mass spectrometry (Johnston, et al., 1968), photometry following ion-pair extraction (Eksborg and Persson, 1971), and radioisotopic measurement (Wang and Haubrich, 1975).

Chastellain and Hirsbrunner (1976) recently reported the simultaneous determination of betaine and choline in feeds by ^{1}H-NMR spectrometry. While being a significant advance over the Lim and Schall method, it still suffers from the inherently poor sensitivity of NMR and the difficulty of quantitating NMR peaks in aqueous solutions.

The poor volatility of choline makes liquid chromatography the analytical method of choice. Unfortunately, the lack of useful UV-visible absorption bands has heretofore precluded the use of this method. We have recently described the separation and quantitation of some simple quaternary ammonium ions by liquid chromatography (Dorsey et al., 1978) using a detector developed in this laboratory (Gilbert and Dobbs, 1973).

This paper describes the application of this method to the determination of choline in soy meal. Extraction conditions are described and discussed in terms of recovery. The method is compared to both the Lim and Schall (1964) and Chastellain and Hirsbrunner (1976) methods. Precision, linearity, detection limits, and speed of analysis are discussed.

EXPERIMENTAL SECTION

Chromatographic System. The liquid chromatography system used and the ion-exchange membrane detector have been described previously (Dorsey et al., 1978).

Aminex A-4 resin (Bio-Rad Laboratories, Richmond, CA), 16–24 μm, an 8% DVB cross-linked strongly acidic cation-exchange resin with sulfonic acid functional groups, was equilibrated three times with the mobile phase and then slurry packed in a Chromatronix LC-9M column, 9.0 mm i.d. (LDC, Riviera Beach, FL). When the column was filled, the resin bed was compressed by pumping the mobile phase through the column at maximum flow rate for at least 1 h, and then the column was adjusted to this final bed height of 20 cm. The mobile phase was 70:30 acetone/water which was also 0.035 M ethylene diammonium chloride (en·2HCl) and was vacuum degassed.

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Soy Extraction. Two different soy samples were used: a commercially prepared soybean meal, where the oil was removed by extraction, the lecithin gum isolated and then remixed with the meal (Woodson-Tenent Laboratories, Memphis, TN); and 100% soya grits (El Molino Mills, City of Industry, CA, stock no. 610), which were ground in a Waring blender. Accurately weighed samples were placed in a Whatman cellulose extraction thimble (80 × 33 mm i.d., Whatman, Inc., Clifton, NJ). The thimble was placed in a medium-size Soxhlet extractor (40 mm i.d.) with an Allihn type condensor and an extraction–hydrolysis mixture of 75 mL of 1.0 N HCl and 175 mL of methanol. Two glass beads were placed in the flask to prevent bumping, and the meal was extracted with this refluxed methanol–HCl mixture for 8 h. Most of the solvent was removed with the use of a Buchi Rotovapor R (Rinco Instrument Co., Greenville, IL), leaving approximately 10 mL of oily liquid. This material was mixed with 50 mL of water and filtered into a large separatory funnel. This solution was extracted twice with five times its volume of anhydrous ether, and the aqueous phase retained. This was returned to the rotary evaporator and concentrated again to about 10 mL. It was then quantitatively transferred to a 50-mL volumetric flask and diluted to volume with water. The final solution was stored at 4 °C until analysis.

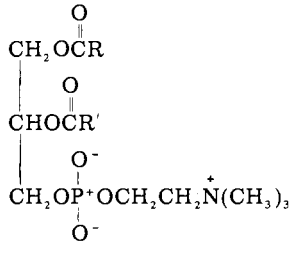
Chromatographic Analysis. An excess of ammonium reineckate solution (ca. 10 mL) was added to a sintered glass funnel, 5.0 mL of the soy extract was added, and the resulting precipitate was collected. The precipitate was then dissolved in 3.0 mL of the chromatographic mobile phase, and 0.276-mL samples were injected onto the ion-exchange column. The injection volume was that of an available loop and is by no means critical. Choline standards were handled in the same manner.

Standard Choline Analyses. The accepted reineckate–choline analysis was performed as described by Lim and Schall (1964). Absorbance measurements were made using a Perkin-Elmer 139 spectrophotometer. The ^{1}H-NMR analysis reported by Chastellain and Hirsbrunner (1976) was performed on the soy extract without further concentration, using a Varian A-60.

Reagents. The en·2HCl was prepared using the procedure of Arguello and Fritz (1977). Choline chloride was 99% (Sigma Chemical, St. Louis, MO) and was used as received. The *tert*-butylamine hydrochloride was made by mixing 150 mL of concentrated HCl with 75 mL of *tert*-butylamine (Eastman Chemical, Rochester, NY) which had been dissolved in 250 mL of water. Most of the solvent was rapidly removed with a rotary evaporator and the solution was then suction filtered. The salt was washed with CCl_4 and air-dried. Saturated ammonium reineckate solutions were prepared by shaking 1.5 g of ammonium reineckate in 50 mL of distilled water for ca. 10 min and filtering.

RESULTS AND DISCUSSION

Choline Extraction. The choline present in plant material is for the most part bound in lecithin with little, if any, free choline. Lecithin is represented by structure I, where R and R′ are fatty acids. Choline can be freed



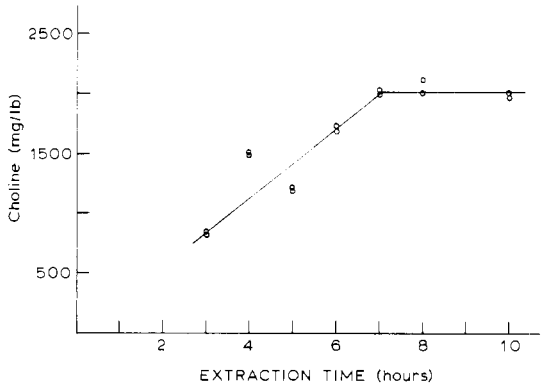


Figure 1. Extraction time for soy meal vs. choline, reported as milligrams of choline chloride/pound of soy meal.

from lecithin through either acid or base hydrolysis. Complete hydrolysis is essential if accurate results are to be obtained.

Fritz (1965) reported on a collaborative study by 12 laboratories involving a 4-h acid hydrolysis–extraction and subsequent reineckate analysis of a mixed feed. The results were very erratic, and it was suggested that much of the difficulty was in the extraction–hydrolysis step. Glick (1944) has demonstrated that cake formation in the extraction thimble can also hinder extraction.

To find the length of time necessary for complete extraction of the choline, samples of the commercial soy meal were extracted for various lengths of time from 3 to 10 h. Figure 1 shows the results. Duplicate choline determinations were made on each soy extract after the specified extraction time and are shown as separate data points. The choline values were obtained using a preliminary chromatographic method before development of the choline isolation step. Nevertheless, the relative values should be valid and they show that an 8-h extraction is sufficient for complete hydrolysis of the choline.

Furfural is a major product of the acid hydrolysis of almost any plant material (McCullough, 1972). To remove this compound along with any residual methanol and other organic substances from the hydrolysis–extraction mixture, two ether extractions were used. It was demonstrated that ether would not extract any of the choline by extracting a 1.0 M choline solution with an equal volume of ether, followed by back-extraction of the ether with water. The water was tested for choline with a saturated ammonium reineckate reagent. No precipitate was observed compared to easily noticeable silver flecks when the reagent is added to a 10^{-3} M solution of choline. Thus, less than 0.1% of any choline present in the aqueous phase would be ether extracted.

During some of the early experiments with the Soxhlet apparatus it was noted that the volume of the extraction mixture in the boiling flask would gradually decrease with time. When the liquid level dropped below the point where the heating mantle was located, the sides of the flask would experience localized heating and some of the extracted material was apparently decomposed. A hard, black substance was deposited on the walls of the flask which was not soluble in water, methanol, or ether. Consequently, to avoid low recoveries it is imperative that the volume of the extraction mixture be sufficiently large to remain above the level of the heating mantle.

The commercial soybean meal was also used to determine the reproducibility of choline hydrolysis after an extraction time of 8 h. The results are shown in Table I. These values were also obtained using the preliminary chromatographic method.

Table I. Reproducibility of Extraction Technique on a Commercial Soy Meal

extraction no.	no. of sample injections	av choline chloride, mg/lb
1	3	1998
2	2	1995
3	3	1984
4	3	1971
5	2	2111

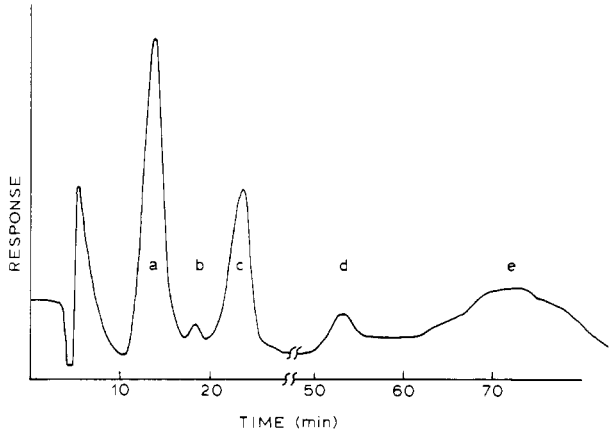


Figure 2. Initial separation of soy extract. (a) Na^+ and Li^+; (b) choline; (c) K^+ and unknown; (d) Mg^{2+}; (e) Ca^{2+}. Sample: 0.456 mL of extract. Mobile phase: 0.035 M en·2HCl. Flow rate: 2.0 mL/min. Column: 17 × 0.90 cm Aminex A-4.

Chromatographic Analysis. Initial trials were made by injecting samples of the soy extract onto the ion-exchange column with 0.035 M en·2HCl as the mobile phase. To do this it was necessary to raise the pH of the extract to that of the mobile phase (ca. pH 4.4). Figure 2 is the chromatogram resulting from this attempt. The negative and then positive peak at 4 min is the "exchange peak" and has been discussed previously (Gilbert and Dobbs, 1973). Peak "a" at 14 min is Na^+ and Li^+, from LiOH, which was used to raise the pH of the extract. Choline is the small peak, "b", at 18.5 min, followed by K^+ and an unidentified component both eluting at 24 min, peak "c". Then at 53 and 72 min come Mg^{2+} and Ca^{2+}, peaks "d" and "e". The mineral content of air-dried soybeans includes 1.01% K, 0.34% Na, 0.22% Mg, and 0.21% Ca (Markley and Goss, 1944) and the extraction conditions necessary to extract the choline also extract the inorganic components. While the choline peak is well resolved and easily quantitated, the 80-min analysis time is unacceptable for a routine procedure. For this reason an isolation step for the choline was required.

It is known that reineckate complexes of quaternary ammonium ions are soluble in acetone, and ion-exchange processes in mixed solvents have been well discussed (Helfferich, 1962). After determining the minimum concentration of acetone necessary for good solubility of the reineckate complex to be 70%, the ion-exchange resin was equilibrated with this mobile phase and the column repacked. Figure 3A is a chromatogram of the soy extract after precipitation and redissolution of the reineckate complex. The peak at 4 min is the "exchange peak" and the choline is eluted at 17 min, peak "a". Any difference in acetone concentration between the sample and the mobile phase will give rise to a peak at 13.5 min (Figure 3B, peak "a", but this does not interfere with quantitation of the choline peak.

The retention mechanism is strictly an ion-exchange reaction between the choline and the ion-exchange resin, and is not affected by the reineckate. The reineckate was

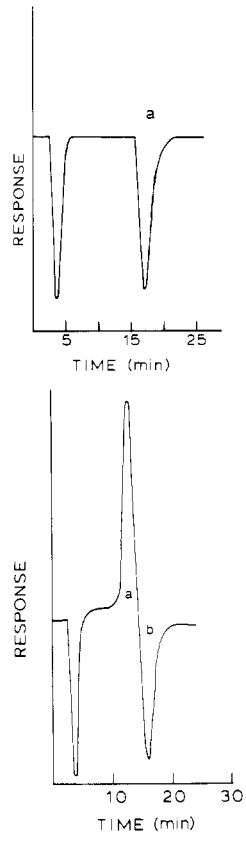


Figure 3. (A) Final choline separation; choline (a). (B) Acetone (a); choline (b). Sample: 0.276 mL of reineckate solution precipitated from soy extract. Mobile phase: 70:30 acetone/water which is also 0.035 M en·2HCl. Flow rate: 2.0 mL/min. Column: 20 × 0.90 cm Aminex A-4.

unretained and the red band could be seen coming through at the void volume of the column. Samples of pure choline chloride showed the same retention time as samples of the complex, further confirming the reineckate has no role in the separation.

We have previously established the detector to be linear over at least 2.5 orders of magnitude, with a reproducibility of better than 1% standard error during any given period of operation (Dorsey et al., 1978). While the detector is very reproducible during any given period of operation, day-to-day variations of 10–20% can occur if the detector cell is removed from the thermomechanical analyzer at the end of a day's operation. This variation most likely arises from small differences in positioning and adjustment of the membrane, rather than in any inherent imprecision in the membrane detector itself. This removal is necessary as the ion-exchange membrane cannot be allowed to dry out. This problem is overcome by leaving the detector cell in place and allowing the system to pump continuously at a slow flow rate.

For this method of analysis the upper end of the linear range is limited by the solubility of the reineckate complex in the mobile phase, but with the ability to adjust the volume of soy extract precipitated and the mobile phase used for redissolution almost any original concentration can be made to fall within the limits of analysis. For the detector itself, the quantity of choline, defined as the concentration of sample that produces a signal equal to twice the base line noise, is about 3×10^{-5} g/mL. The calibration plot showed a negative intercept (Figure 4), implying a constant loss of choline at some point in the procedure. The most likely place for this would be the filtering and redissolving of the precipitate. Nevertheless,

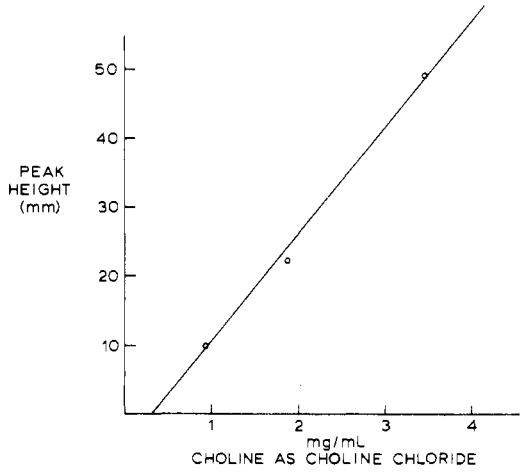


Figure 4. Calibration plot for final chromatographic determination of choline. Samples: 0.276 mL of reineckate solution precipitated from choline standards. Mobile phase: 70:30 acetone/water, which is also 0.035 M en·2HCl. Flow rate: 2.0 mL/min. Column: 20 × 0.90 cm Aminex A-4.

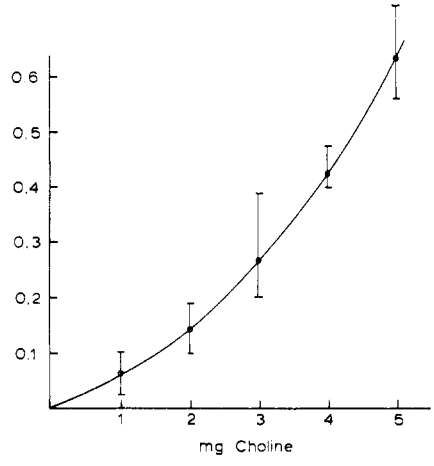


Figure 5. Calibration curve for ammonium reineckate method of choline analysis. Milligrams of choline vs. absorbance at 526 nm. Error bars represent the range of values obtained on each standard.

the calibration plot was linear with a correlation coefficient, r, of 0.9984, and therefore useful for quantitative purposes. Each data point is the average of two injections, but all six points were used for the regression analysis.

Following the above procedure, the choline content of the soya grits sample was determined to be 1440 mg/lb of soy. These units are customary for this determination and in this instance correspond to 0.317% by weight. This value is the average of three replicate analysis with individual values of 1470, 1360, and 1490 mg/lb. The standard deviation, s, was 70, or 4.9%.

Ammonium Reineckate Analysis. The previously extracted soy was analyzed by the popular ammonium reineckate method (Lim and Schall, 1964). Figure 5 shows the calibration curve for this method. Each data point is the average of six–ten analyses, and this curve is an indication of the difficulties encountered with this method. The poor precision shown by choline stock solutions is greatly magnified when a determination of choline in a difficult matrix, i.e., soy extract, is attempted. This imprecision is exemplified by results reported by a commercial laboratory that routinely performs this analysis. On four replicate determinations of soybean meal, they obtained values of 1162, 998, 876, and 1075 mg of choline/lb. These data are included here only as an indication

Table II. Choline Determination by Ammonium Reineckate

1 mL of soy extract		1 mL of soy extract + 1 mg of choline	
AU	mg of choline	AU	mg of choline
0.105	1.6	0.150	2.05
0.095	1.5	0.175	2.3

of the precision of the traditional reineckate method and are not intended as a comparison of final results.

More and Kenner (1970) have further noted that the absorbance of solutions of reineckate salts in acetone or in aqueous acetone varies with time, adding another complicating factor to the analysis.

Table II shows the data obtained from the soy extract. A 5-mL sample of the extract was spiked with 5 mg of choline to determine the recovery from the soy matrix. The spiked sample was allowed to stand for 90 min, and 1.00-mL aliquot portions were added to the Florisil column and analyzed. An average recovery of only 62.5% was found. Using this factor, and the average of duplicate analyses, a value of 1500 mg of choline/lb of soy was determined.

NMR Analysis. The NMR analysis described by Chastellain and Hirsbrunner (1976) was performed on the soy extract without further concentration. An accurately weighed amount of *tert*-butylamine hydrochloride was added as an internal standard, such that its final concentration in solution was about the same as that of choline. The signals from the trimethylammonium groups are then used for quantitation. A value of 1480 mg/lb was determined for our soy extract by this method.

CONCLUSION

A precise method has been described for a difficult analytical problem. The value of 1440 mg/lb compares very favorably with values of 1500 and 1480 mg/lb obtained by two independent methods. This method should be directly applicable to choline determination in any type foodstuff. The precipitation step eliminates any interferants other than quaternary ammonium compounds, and the chromatographic step provides separation of the choline from any other quaternary nitrogen compounds that might be present. Although an 8-h extraction is required before any method of choline analysis can be performed, the technique described here is faster and has significantly lower detection limits than other popular methods. While the precision of the chromatographic step is very high, the precision of the analysis is limited by the extraction step, and it is in this area that we feel further research should be directed.

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Flocculants in the Separation of Green and Soluble White Protein Fractions from Alfalfa

Benny E. Knuckles,* Richard H. Edwards, George O. Kohler, and Lester F. Whitney

Fifty-four commercial flocculants were tested for their ability to improve the separation of the green chloroplastic protein fraction from alfalfa juice. From screening tests, it was determined that ten of the cationic flocculants improved the separation by centrifugation. Three of these flocculants were added at levels up to 1% without causing precipitation of the soluble white protein. At a flocculant level of 1%, a continuous high-speed centrifuge could be used to sediment the chloroplastic fraction although the processing rate was low (≤11.4 L/min). The flocculants also improved the separation of the chloroplastic fraction by membrane filtration. The permeate flux during ultrafiltration of flocculant treated juice was double that of untreated juice. The soluble protein concentration in the permeate from the treated juice was also higher than that from the untreated control.

Although leaf protein concentrates (LPC), prepared as described by Pirie (1971), have been fed to children in several trials with minimal acceptance problems (Kamalanathan and Devadas, 1975; Olatunbosun et al., 1972; Waterlow and Cruickshank, 1961), its green color and grassy taste has prevented its acceptance by the general population. A critical step in the preparation of a soluble white edible LPC is the removal of the green chloroplastic material. This material has been removed from the soluble white protein by centrifugation or ultrafiltration.

Centrifugal forces needed to separate the chloroplastic material from fresh alfalfa juice is very high (RCF_{max} 100000g for periods (>1 h). Machines capable of producing these conditions on a continuous large-scale basis are not available. Therefore, centrifugal separation on a large scale requires some type of pretreatment. Usually, a mild heat pretreatment (40–70 °C) is used to agglomerate the green fraction so that it can be easily separated from the soluble protein (Byers, 1967; Bickoff and Kohler, 1974; Cowlishaw et al., 1956; de Fremery et al., 1973; Edwards et al., 1975; Henry and Ford, 1965; Lexander et al., 1970; Subba Rau et al., 1969). The temperature necessary to agglomerate the green fraction and the amount of soluble protein which coagulates at those temperatures depend upon the type of plant (Bahr et al., 1977). When alfalfa extracts are heated to 56–65 °C, the green material can be separated in large-scale continuous centrifuges but, at these temperatures, 20–40% of the soluble protein is coagulated and separated as part of the green fraction (de Fremery et al., 1973; Edwards et al., 1975).

Ultrafiltration, which has been used to concentrate whey, skim milk, and extracts from seed and leaf tissue (Horton et al., 1972; Knuckles et al., 1975; Lawhon et al., 1973; McDonough et al., 1971; Peri et al., 1973; Porter and Michaels, 1971; Tragardh, 1978), has also been used to separate the chloroplastic and soluble protein fractions (Eakin, 1976; Eakin et al., 1978; Singh et al., 1974; Whitney and Bernardo, 1977). During ultrafiltration of fresh alfalfa juice, permeation rates decrease rapidly due to gel formation and concentration polarization. The permeation rate can be improved by use of polyelectrolytes (organic flocculants) (Eakin, 1976).

Flocculants enhance the agglomeration of suspended solids in aqueous solutions. They have been used to separate or fractionate protein from plant extracts (Anelli et al., 1977; Horisberger and Olofsson, 1976). But they are most widely used in the removal of suspended solids from municipal water and waste water (Daniels, 1973; Cohen et al., 1958; Schaffer, 1963).

This paper reports the results of a laboratory study of the effect of commercially available flocculants on the separation of the chloroplastic and soluble protein fractions in alfalfa extracts. The study includes the effects upon centrifugal and ultrafilter separations.

EXPERIMENTAL SECTION

Preparation of Juice. Chopped alfalfa, treated with

Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, California 94710 (B.E.K., R.H.E., G.O.K.), and the Food Engineering Department, University of Massachusetts, Amherst, Massachusetts 01003 (L.F.W).