DETERMINATION OF CHOLINE AND TRIMETHYLAMINE IN SEEDS AND PREMIXES

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In biological substances, choline is present either combined with phospholipides or in the form of acetylcholine. Some substances, e.g. blood plasma, also contain free choline. Thus the total choline content of biological substances can only be determined after preliminary hydrolysis.

To extract bound choline, 96% ethanol, methanol and solvent mixtures (ethanol—ether, ethanol—chloroform) are proposed in the literature. After removal of the solvent, the choline base is released with an aqueous solution of barium hydroxide [1].

The chemical term for choline is β-hydroxyethyl-trimethylammonium-hydroxide:

$$[(CH_3)_3N^+—CH_2—CH_2—OH]OH^-$$

Choline is a strong base, forming a salt even with the carbon dioxide of air. It is readily soluble in water, methanol and ethanol, less soluble in acetone and chloroform and insoluble in benzene, toluene and carbon tetrachloride.

When heated in a strong basic solution, it is decomposed into trimethylamine and glycol. Numerous methods for the determination of free choline are reported in the literature. For its separation or gravimetric determination, its compounds insoluble in water formed with Au(III), Cd(II) or Hg(II) ions, with silicotungstic acid or with ammonium reineckate are used [2—5]. However, these precipitating agents are non-selective and can be used only for the determination of greater amounts of choline.

To determine small choline quantities, spectrophotometric procedures are known. The choline compound formed with ammonium reineckate, after filtration and wash, is dissolved in acetone and the light absorption of the solution is measured [6]. Several modified forms of this procedure are known. They differ in preliminary and purification operations and in the wave-length applied in spectrophotometry [7, 8].

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With samples containing higher percentages of choline, the solution of choline reineckate in acetone utilized for spectrophotometry having a concentration (expressed as choline chloride) of 100 to 500 $\mu g/cm^3$, measurement is performed at 526 nm.

The method is more sensitive at 310 nm, where extinction measurement can be used to determine choline in the 5 to 50 $\mu g/cm^3$ concentration range.

The selectivity and accuracy of the methods based on spectrophotometry of choline reineckate is satisfactory. Their disadvantage is that complete precipitation of choline reineckate is very time-consuming (about 12 hours).

KUSHNER [9] proposed a rapid and sensitive spectrophotometric determination. To the solution containing free choline, an aqueous solution of potassium iodide and iodine is added. The reaction yields choline periodate. Ethylene dichloride is then added to the solution and thorough mixing is provided by passing a nitrogen stream through the liquid. The two phases are then separated and the extinction of iodine in the organic phase is measured at 365 nm. The calibration curve is prepared in the concentration range of 0.1 to 5 μg choline per cm^3 ethylene dichloride. The disadvantage of this procedure is that all substances reacting with iodine interfere. Therefore it is unsuitable for determining choline in premixes. It is, however, suitable for determining the choline content of the hydrolysates of substances containing phospholipides with satisfactory accuracy.

A volumetric method for the selective determination of choline has also been developed. This is based on the oxidation of choline in a strong basic medium with potassium permanganate. This reaction yields trimethylamine which is distilled over into sulphuric acid and excess acid is back-titrated [10]. This method has been modified by many researchers. At present, the method developed by RUBIN and RITTER [11] is in general use. The principle of this method was utilized in our work related to the determination of choline in seeds and premixes and to the determination of free trimethylamine in premixes. Details will be given in the experimental part.

Submicro quantities of choline have been determined by pyrolytic decomposition and subsequent gas-liquid chromatography [12]. The sensitivity of this method is 200 μg choline chloride.

Paper chromatography [13, 14] and thin-layer chromatography [15] can be applied in many cases for the separation and detection of quaternary ammonium salts.

Experimental

Determination of bound choline in seeds

Our experiments included the determination of bound choline in wheat groats, bran, oat groats, maize groats, alfalfa flour and soy groats. These vegetable substances are constituents of premixes, therefore the knowledge of their choline content is of importance for modern feeding.

We developed a procedure by combining and — if necessary — modifying the individual operations of the methods reported in the literature. With our procedure we obtained results related to the above-listed materials that corresponded to literature values.

The procedure consisted of the following operations:

Solvent extraction

The milled product is dried at 105 ± 2 °C. The weighed sample is thoroughly rubbed in a mortar with sea sand, in order to reduce grain size and enhance extraction. It is then extracted with methanol in a Soxhlet apparatus.

Alkali treatment of the extract

The extract containing bound choline and other accompanying substances extracted by methanol is boiled with an aqueous solution of $Ba(OH)_2$. This treatment not only releases the choline base, but also effects other changes that are favourable for the determination: volatile bases that would interfere at a later stage with the determination are removed, fat-like impurities are saponified and precipitated in the form of well filterable barium soap, so that they can easily be separated from the choline base.

Solvent extraction and alkaline treatment were carried out simultaneously: methanol and aqueous $Ba(OH)_2$ were mixed in the extraction flask and extraction was carried out in the usual manner. Thus, the bound choline content of the refluxed methanolic extract was decomposed continuously and simultaneously with extraction.

pH adjustment and filtration

After extraction and alkaline treatment, the major part of methanol is evaporated. The residue is strongly alkaline as a result of $Ba(OH)_2$ present. Before precipitating the free choline base with Reinecke salt, pH is adjusted to neutral with acetic acid in the presence of thymolphthalein as indicator and the mixture is filtrated.

Precipitation, filtration and spectrophotometric measurement of the choline base

From the filtrate, choline is precipitated with a solution of Reinecke salt $[Cr(NH_3)_2(SCN)_4NH_4 \cdot H_2O]$ in methanol. The solution containing a pink precipitate is held for 2 hours at 0 to 5 °C and subsequently left standing at ambient temperature for 12 hours. Then the precipitate is collected on a G4 glass filter, washed with a small volume of ice water and with n-propanol, and dissolved in acetone. Light absorption of this solution is measured at 526 nm.

The calibration curve for the evaluation of the results is prepared from a series of aqueous solutions containing pure choline in known amounts. Choline is precipitated with a solution of Reinecke salt in methanol and treated as described above. The calibration curve is constructed by means of six values measured in the 5 to 50 μg choline per cm^3 acetone concentration range.

Results obtained with this procedure are presented in Table 1, indicating good agreement with data from the literature.

Table 1

Determination of choline in vegetable substances by spectrophotometry

Ground vegetable product	Choline content	
	reported in the literature, mg/kg	measured, mg/kg
Wheat groats	730 to 1000	790
Bran	1000 to 1100	1040
Oat groats	1000 to 1100	970
Maize groats	440 to 500	450
Alfalfa flour	900 to 1100	1340
Soy groats	2600 to 3500	2900

Determination of free choline in premixes

Since choline is introduced into premixes as choline chloride and not combined with organic molecules, treatment with alkali is unnecessary. Thus, the recovery of choline from premixes is much simpler than from seeds. It is extracted with warm water, filtered and determined.

The choline content in the tested premixes was in the 20 to 200 mg/g concentration range. The choline content of the aqueous extract can be determined both by spectrophotometry and by oxidation in alkaline medium.

Spectrophotometric method

The principle of the method agrees with that of the method described for seeds. Choline is precipitated from the aqueous solution with Reinecke salt dissolved in methanol. After standing, the precipitate is filtered on a G4 glass filter and washed with ice-water and n-propanol. Subsequently the precipitate is dissolved in acetone and the solution is measured spectrophotometrically.

Oxidation in alkaline medium

The choline content of the aqueous extract is oxidized with $KMnO_4$ to trimethylamine in the solution made alkaline with NaOH. Gaseous tri-

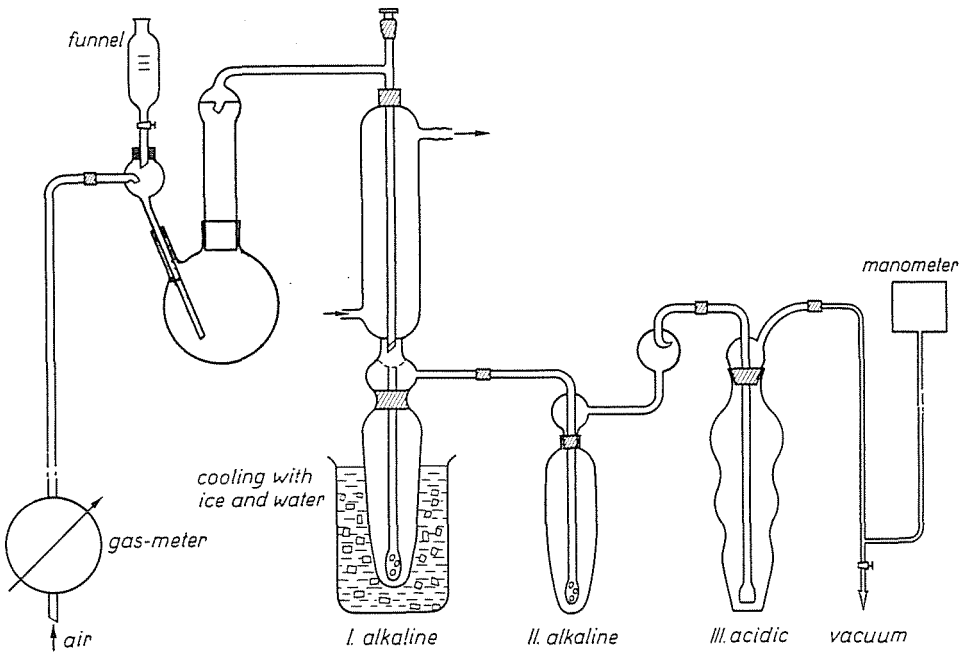

Fig. 1. Apparatus for the determination of choline and trimethylamine in premixes

methylamine is absorbed in a known excess of sulphuric acid and the excess acid is back-titrated with a standard solution of trimethylamine.

Oxidation is carried out in the apparatus shown in Fig. 1. The aqueous solution of choline and NaOH is introduced into the glass flask and heated to boiling temperature by means of the electrical heating device. Then an aqueous $KMnO_4$ solution (0.5 or 0.25%) is added dropwise from the funnel, at a rate of 1 to 4 cm^3/min. Oxidation should be completed in 15 to 30 minutes.

During oxidation, air is sucked through the apparatus at a rate of 20 l/hr which transports the released gaseous trimethylamine through the cooler and the alkaline absorbers I and II into the acid absorber III where it will combine with the acid in the form of its sulphate.

The alkaline absorbers I and II contain alkaline formaldehyde solutions. Their purpose is to hold back ammonia and primary amines eventually formed during the oxidation.

When oxidation is completed, $KMnO_4$ addition and heating is stopped and air is sucked through the system at an increased rate of 45 l/hr to transport trimethylamine dissolved in the alkaline absorbers quantitatively to the acid absorber.

The dosage rate of the $KMnO_4$ solution has a decisive effect on the accuracy of the method. At low rates, a part of the choline will be left unreacted, i.e. a negative error will arise. At too rapid dosage, the partial excess of the oxidizing agent will oxidize choline partially to ammonia. This will also give rise to a negative error, since the released ammonia will be absorbed in the alkaline absorbers and will not reach the acid absorber.

Reliable results are obtained when the amount of $KMnO_4$ added during the oxidation period is sufficient to oxidize choline, $KMnO_4$ in excess may only be present at the end of the oxidation process.

After all trimethylamine has been transported into the acid absorber containing an excess amount of 0.02 N sulphuric acid, the solution is titrated with 0.02 N trimethylamine standard solution in the presence of methyl red—methylene blue mixed indicator.

Two variants of this alkaline oxidation method were applied in our work:

(i) *Direct oxidation of choline chloride.* An aliquot part of the filtered and cooled warm-water extract of the premix sample was introduced into the flask of the apparatus shown in Fig. 1. A solution of NaOH was added, the mixture was brought to boil and oxidized with $KMnO_4$ as described above.

(ii) *Indirect oxidation (oxidation of choline reineckate).* In an aliquot part of the filtered and cooled warm-water extract of the premix, choline was precipitated with a solution of Reinecke salt in methanol. The precipitate was treated similarly as in the case of spectrophotometric determination. Its solution in acetone was transferred to the flask of the oxidation apparatus. A small volume of water was added and the glass flask was detached from the rest of the apparatus. Acetone was removed by slight heating and suction. The insoluble choline reineckate formed a suspension in the water. The apparatus was then reassembled, NaOH was added to make the suspension alkaline, the mixture was brought to boil and choline was oxidized with $KMnO_4$.

The results obtained with the three methods are presented in Table 2. A comparison of the results demonstrates that the greatest difference between the amount of choline added to the premix and the found value is around

$\pm 10\%$ in the case of the spectrophotometric method and the direct oxidation method, while the corresponding difference in the case of indirect oxidation is within $\pm 2\%$, i.e. the latter method is the most accurate of all.

Time requirement for the spectrophotometric and indirect oxidation procedures (since 12 hours are necessary to achieve complete precipitation) is one and a half to two days, while direct oxidation only takes 3 to 4 hours.

For analyzing a great number of samples, the spectrophotometric procedure is preferable, since it is simple to carry out.

Table 2

Comparison of methods applied for the determination of choline in premixes

Choline added to the premix, mg/kg	Found by spectrophotometry		Found by direct oxidation		Found by indirect oxidation	
	mg/kg	d, %	mg/kg	d, %	mg/kg	d, %
200,000	181,200	−9.4	205,600	+2.8	198,900	−1.1
100,000	107,200	+7.2	96,650	−3.4	100,450	+0.5
23,10	24,040	+4.1	20,930	−9.4	22,800	−1.3

d = Difference between added and found amount, %

Determination of trimethylamine in premixes

Choline chloride added to premixes also contains trimethylamine. The maximum admissible percentage of trimethylamine in crystalline choline chloride is 0.003%. Trimethylamine is toxic, therefore its determination in premixes is imperative.

The determination is carried out by extracting the premix with warm water and boiling the aqueous extract with $Ba(OH)_2$. This reagent releases choline from the choline chloride extracted together with trimethylamine, but does not decompose it to trimethylamine.

The treatment with $Ba(OH)_2$ is carried out in the apparatus shown in Fig. 1. The mixture is boiled and a stream of air is sucked through the apparatus. Volatile trimethylamine is transported by the air stream through the alkaline absorbers into the acid absorber. The choline base, being a quaternary amine, is not volatile. Ammonia and primary amines are absorbed in the alkaline absorbers that are identical with those used in the determination of free choline. Thus, only trimethylamine reaches the acid absorber. Since the samples analyzed contain only small amounts of trimethylamine, its volu-

metric determination is not feasible. Therefore, after the release and absorption of trimethylamine, the content of the acid absorber is neutralized with $Ba(OH)_2$ and trimethylamine is precipitated with Reinecke salt. The precipitate is dissolved in acetone and trimethylamine content is determined by spectrophotometry at 328 nm.

With this method, trimethylamine can be determined in premixes down to percentages of 0.005%.

Summary

The free choline content of premixes as well as the bound choline content of plant substances: wheat groats, bran, oat groats, maize groats, alfalfa flour and soy groats have been determined. Choline chloride was extracted from the samples, then it was precipitated in the form of reineckate. After filtration and wash the precipitate was dissolved in acetone and its choline content was determined either by spectrophotometry or by oxidation with potassium permanganate in basic solution. The toxic trimethylamine present in small amount was also determined. The extract was treated with barium hydroxide and the trimethylamine content of the premixes was also determined by spectrophotometry after dissolving the reineckate precipitated.

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