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AUTOMATED ANALYSIS OF NITROGENOUS COMPOUNDS

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Total nitrogen determination

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As a preliminary to the consideration of automated, or more correctly mechanized, methods for the determination of total organic nitrogen the discussion of some general aspects of nitrogen determination is illuminating.

There are at least four recent reviews, for example those by Bradstreet (1965), Fleck & Munro (1965), Jacobs (1965), and Munro & Fleck (1969), of the determination of nitrogen from which certain general principles for the estimation of nitrogen in samples of biological materials can be derived.

An up-to-date review of analytical methods is incomplete without the mention of quality control. It is now apparent in the field of clinical biochemistry that continuous and daily checks of analytical precision and accuracy are essential for results to be reliable; the determination of nitrogen is no exception.

The two principal methods of determining organic nitrogen were first described almost a century ago, the Kjeldahl method in 1883 and the Dumas method in 1831, yet it is only in recent years that important alterations in techniques have led to considerable improvements. The advent of gas–liquid chromatography was followed by considerable refinements in the Dumas method so that it is now possible to analyse dry solid samples rapidly, only a few minutes being required for each determination of nitrogen (see Fleck & Munro, 1965).

The Kjeldahl method, however, remains the most widely used and convenient for samples of biological origin. The subsequent discussion relates only to the determination of the nitrogen content of samples of animal tissues, excreta and food. Substances such as keratin, horn, or soils may present difficulties and for such special applications it is essential to consult the recent reviews and to carry out careful recovery experiments with the technique adopted.

Kjeldahl method

The three main steps of the Kjeldahl method are: (1) digestion, (2) separation of ammonia, and (3) determination of ammonia. In some techniques the separation stage (2) is omitted and the ammonia is determined directly on the digest.

Separation of ammonia may be effected by steam distillation, aeration, or diffusion,

steam distillation being conventional. With automated procedures this separation step is invariably omitted.

The determination of ammonia may be by: (1) simple titration, (2) iodometric methods, (3) coulometric methods or (4) colorimetric methods.

Without separation of ammonia from the digest simple titration cannot be utilized. The remaining three techniques can, however, be applied directly to the digest. Iodometric and analogous methods have disadvantages (McKenzie & Wallace, 1954) and are not popular. Coulometric methods are fairly new in this field and not widely applied. Colorimetry remains as the only well-tried approach for automation.

The three popular colorimetric methods of NH_3 determination are: ninhydrin, Nessler, and the phenol-hypochlorite or Berthelot reaction.

The ninhydrin method has been successfully applied following sealed-tube digestion (Jacobs, 1965). The Nessler method, although excellent for simple aqueous ammonia solutions, is not advisable when ammonia is to be determined in complex mixtures such as in the urease method of determining blood urea (Skendzel & Muelling, 1967) or in Kjeldahl digestion mixtures (Fleck & Munro, 1965). Modifications of the Berthelot procedure are extensively applied to the determination of ammonia, for example, in blood urea estimation (Searcy, Gough, Korotzer & Bergquist, 1961) and in organic nitrogen determination (Mann, 1963) both in fully automated (Jacobs, 1968) and semi-automated methods (Fleck, 1967; Gehrke, Kaiser & Ussary, 1968).

The most important aspect of the Kjeldahl method is digestion, which may be carried out in an open tube or in a sealed tube. The critical factors are: (1) temperature, (2) catalyst, (3) time, (4) reflux, and (5) decomposition of the ammonia–catalyst complex.

The optimum temperature for sealed-tube digestion is in the region of 450° and the main advantage is that no catalyst or other additions are required. For a discussion of this technique the review by Jacobs (1965) should be consulted.

The more commonly utilized open-tube digestion requires a temperature close to 400° for adequate decomposition of nitrogenous compounds to ammonia. The evidence for this is clear (see Bradstreet, 1965; Fleck & Munro, 1965), as is the evidence that the only satisfactory means of attaining this temperature is to add the appropriate amounts of K_2SO_4. Bradstreet (1957) seems to have been responsible for the observation that when the temperature exceeds 400° the digest solidifies on cooling. This is an important practical point because temperatures in excess of 400° lead to loss of nitrogen (as well as loss of acid which leads to the solid cold digest).

With regard to the catalyst, survey of the literature indicates that mercury is the only 'safe' catalyst, with which no losses have been reported (Bradstreet, 1965; Fleck & Munro, 1965). Reports of losses with selenium or copper or mixtures of these date back to the 1930's (see Fleck & Munro, 1965) with adequate confirmation in 1948 (Hiller, Plazin & Van Slyke, 1948; Patel & Sreenivasan, 1948). The disadvantage of mercury is that it forms a mercury–ammonium complex which must be decomposed before determining ammonia. This decomposition may be achieved by using sodium thiosulphate or zinc dust. In our experience the use of zinc dust

is to be preferred for both manual and semi-automated procedures, because there is a distinct risk with sodium thiosulphate depending on the procedure adopted of releasing acid fumes in the distillation apparatus.

The use of oxidizing agents is generally considered to be hazardous (Bradstreet, 1965), for example, perchloric acid can cause loss of nitrogen (Peters & Van Slyke, 1932). For a full discussion of the digestion procedure and the effects of oxidizing agents Bradstreet (1965) should be consulted, but it appears that in some circumstances H_2O_2 may be useful. This is especially so with mixtures containing large amounts of carbon either as fat or carbohydrate. The addition of H_2O_2 just at the point when charring and consequent frothing begin prevents excess frothing (see Bradstreet, 1965) and considerably aids digestion without leading to loss of nitrogen (Fleck, 1967).

With an appropriate mixture 30 min digestion is adequate at the micro level (McKenzie & Wallace, 1954) and 1 h at the macro scale (Lake, McCutchan, Van Meter & Neel, 1951) even with refractory compounds. Such a mixture consists of

$$\left.\begin{array}{r} 3 \text{ ml conc. } H_2SO_4 \;\; 2{\cdot}4 \text{ g } K_2SO_4 \\ 100 \text{ mg HgO} \end{array}\right\}$$

The K_2SO_4 and HgO may be conveniently added as 'Special Kjeltabs' (available from Thompson & Capper Ltd, Liverpool). These quantities are suitable for the digestion of 2–12 mg N and it is important to scale up or down in relation to the amount of nitrogen to be digested.

Finally an important practical point is that during digestion the acid should reflux and not be boiled off. This requires that the angle of the tube should not be greater than 45° to the horizontal as it is with one commercially available macro-digestion set. Failure to observe the ring of condensing acid about one-third of the way up the neck of the flask indicates that acid and hence nitrogen will be lost.

The automation of the determination of organic nitrogen

Automation may be achieved with a modified Dumas procedure but with the Kjeldahl method there are two possibilities: semi-automation or full automation.

By semi-automation is meant that the digestion process is carried out manually while the colorimetric determination of NH_3 is effected using a continuous flow method of analysis—the Technicon AutoAnalyzer (Trade mark of the Technicon Company) for example.

In this system samples are loaded on to a circular plate which has a capacity of forty samples. The samples and reagents are pumped by a constant-speed pump—the relative quantities being determined by the bore of the pump tubing. Mixing of samples is prevented by the introduction of air bubbles, which is a patented process. Various operations such as dialysis and heating are possible and the final colour produced is monitored by a colorimeter and a recorder which yields a series of peaks. In the semi-automated determination of nitrogen no dialysis is required, only a heating bath containing two 40-foot coils of glass tubing in which the blue colour is developed with the phenol-hypochlorite reagents.

Full automation is achieved by the addition of a digestor which consists essentially of a rotating glass Archimedes screw, three sections of which can be heated independently. The Technicon Company also markets equipment with which it is possible to automate the complete analytical process.

With the semi-automated process it is possible to meet all the criteria for adequate digestion. The advantage of this procedure is that it is possible to determine ammonia in digests at the rate of fifty to sixty samples/h with satisfactory accuracy and precision. Obviously digestion is the limiting factor (see, for example Fleck, 1967), although in fact the most tedious aspect is the dilution of the digest and making it up accurately to volume. In this laboratory we have found this procedure satisfactory for the analysis of large numbers of specimens of food, urine and faeces, and the method has considerable potential for increased sensitivity if required.

Fully automated digestion

In contrast with the manual or semi-automated procedures outlined above there are aspects of the fully automated process which might be controversial. There are few reports in the literature in which the method has been satisfactorily applied. Indeed in one of the early papers published (Marten & Catanzaro, 1966), in which it was demonstrated that it was possible that satisfactory results could be obtained, it was nevertheless clear that nitrogen was lost during digestion. In order to check recoveries, ammonium sulphate, urea and nicotinamide standards were employed. With ammonium sulphate a continuous decline in optical density with increase of temperature occurred. With urea and nicotinamide there was an increase in optical density with increase in energy (i.e. temperature) until at temperatures greater than 350° the three curves (i.e. for $(NH_4)_2SO_4$, urea and nicotinamide) showed a parallel decline in optical density with increase in temperature. It seems that loss of nitrogen with this system is inevitable but that it can be compensated by the observation that above about 350° nitrogen is lost from all substances at approximately the same rate. Hence ammonium sulphate can serve as a standard for nicotinamide, and Marten & Catanzaro (1966) illustrate two standard curves obtained with $(NH_4)_2SO_4$ and nicotinamide which are virtually identical. It is obvious however that digestion conditions, particularly the flow rate of acid, are extremely critical and must be carefully determined for each substance to be analysed.

In summary, the problems which may arise in the use of the digestor could be related to: (1) it is an open system without true reflux; (2) the high temperature used in the absence of K_2SO_4 may lead to loss of acid; (3) the use of selenium catalyst; (4) the presence of an oxidizing agent (perchloric acid).

If a mercury catalyst was used, it would be difficult to achieve satisfactory decomposition of the mercury–ammonium complex.

In addition there is the difficulty of handling solid or semi-solid material such as food or faeces. Equipment has been described to deal with such substances which require preliminary homogenization and suspension in water (Alexander, 1969). However, in addition to these obvious difficulties such materials may give rise to flow difficulties in the segmented stream.

Summary

In this brief review a manual micro-Kjeldahl digestion procedure is outlined, which is satisfactory for most substances of biological origin. The final step of ammonia determination may be achieved by steam distillation and titration, if only one or two samples are to be processed. If up to say thirty or forty analyses/d are to be analysed then the semi-automated procedure has considerable advantages. If more than 50 and up to 100 samples must be analysed/d then the suitability of the fully automated method should be carefully considered. Depending on availability of staff and digestion equipment such large numbers could also be processed by the semi-automated procedure outlined above.

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Influence of transcription techniques on the precision of autoanalyser assays

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Most workers whose interests include methodology are aware that errors may be introduced at any stage involving operator intervention. With the development of the autoanalyser, operator errors are considerably reduced but where transcription is manual errors will always occur. The magnitude of the error in any given sample may be small and is probably difficult to measure, but there can be individual samples where the error will be appreciable. It is the intention of this communication to quantify the errors involved in various transcription techniques: the work was