Pure & Appl. Chem., Vol. 51, pp. 1367-1373.
Pergamon Press Ltd. 1979. Printed in Great Britain.

0033-4545/79/0601-1367 $02.00/0

INTERNATIONAL UNION OF PURE
AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION

COMMISSION ON FOOD ADDITIVES

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PERGAMON PRESS
OXFORD · NEW YORK · PARIS · FRANKFURT

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A SURVEY OF METHODS FOR THE DETERMINATION OF NON-VOLATILE NITROSAMINES IN FOODS

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INTRODUCTION

At an International Agency for Research on Cancer (IARC) conference in London, in 1969, it was agreed to describe nitrosamines as volatile when they can be distilled from strong solutions of salts or caustic soda with a minimum recovery of 80%. In fact, however, this rule is not rigidly applied as, for example, N-nitrosopyrrolidine is numbered among the volatile nitrosamines even though recoveries are frequently less than 80%.

On the other hand, it is generally accepted that the term "non-volatile nitrosamine" applies to all N-nitroso compounds which are not volatile in steam. Such compounds include long-chain dialkylnitrosamines, N-nitrosoureas, N-nitrosopeptides and N-nitroso derivatives of organic bases of which N-nitrososarcosine and N-nitrosoproline are the best known examples. Thus, non-volatile nitrosamines differ widely in their chemical and physical properties. It is therefore hardly surprising that progress is slow in establishing methods for the estimation of non-volatile nitrosamines and that no method can be regarded as completely reliable. Collaborative studies organised by IARC demonstrated that several methods for the determination of volatile nitrosamines have a reliability of ± 50% at the µg/kg level. The approach used most commonly is a total determination of non-volatile nitrosamines, as few methods are sufficiently well defined to allow selective or specific estimations.

Present evidence (Ref. 1) suggests that precursors of N-nitroso compounds existing in food usually give rise to non-volatile rather than volatile nitrosamines.

METHODS FOR THE DETERMINATION OF TOTAL NON-VOLATILE NITROSAMINES

One of the first methods for the determination of non-volatile nitrosamines was published in 1970 by Walters and his co-workers (Ref. 2). It is based on the adsorption of volatile and non-volatile N-nitroso compounds from an aqueous extract on activated carbon followed by desorption with methanol. Individual nitrosamines are then determined by differential polarography. Unfortunately, lipids interfere with nitrosamine adsorption and the method can therefore not always be regarded as reliable.

The most widely accepted method for the estimation of non-volatile nitrosamines is that of Eisenbrand and Preussmann (Ref. 14). Its principal advantage is the efficient splitting of the N-NO group, although the efficiency is less for N-nitrosamides than for N-nitrosamines. The nitrite formed is determined after reaction with sulfanilamide and N-(1-naphthyl)ethyl-enediamine. Complete elimination of water from the reaction medium is essential in this method, which has the additional advantage that the amines resulting from denitrosation may be determined as the heptafluorobutyryl derivatives (Ref. 13) by GLC or as the fluorescent dansyl derivatives by TLC. This procedure was used by Eisenbrand et al. (Ref. 33) in studies on N-nitrosocarbaryl and by Ungerer et al. (Ref. 34) in studies on N-(nitrosobenzo) thiazuron. Downs (Ref. 35) used chemiluminescent products for the colorimetric procedure and claims similar performance and an improved detection limit of about 1 ng.

In 1971, Walters et al.(Refs. 3, 4) adapted Eisenbrand and Preussman's method for the estimation of volatile nitrosamines. After lyophilization of the sample, nitrosamines are extracted with methanol and separated by TLC on silica gel. A colour develops after spraying with sulfanilamide and N-(1-naphthyl)ethylenediamine, and the limit of detection is 0.5 mg. This may be reduced to about 2 µg by extraction of the compounds from the TLC plate and denitrosation with HBr in glacial acetic acid. The authors also analysed the extracts by mass spectrometry. The reaction with HBr is inconvenient in food analysis since it requires relatively large volumes of reagent which reduce the sensitivity of the method. In order to avoid the difficulty of separating and concentrating NOBr, Walters and his co-workers (Ref. 5) used thionyl chloride instead of HBr. Nascent NOCl is more volatile and can be removed easily in a stream of nitrogen and collected in a small volume (5 ml) of NaOH solution. The main problem with this method is that the efficiency of the reaction with thionyl chloride depends on the nature of the N-nitroso compound and varies between 20% and 90%. Both Eisenbrand's and Walter's methods are claimed to be reasonably specific for N-nitroso compounds, but in Walter's method N-nitrosothiol compounds may interfere.

A method for the estimation of total volatile and non-volatile nitrosamines has been developed by Dikun (Ref. 36). After distillation of the volatile nitrosamines, non-volatile ones are extracted from the residue, decomposed by UV irradiation and estimated colorimetrically by the Griess reaction. The limit of detection is 0.4 µmol/kg and the method has been used for the analysis of smoked fish.

Fan and Tannenbaum (Ref. 13) described a "universal method" for the determination of N-nitrosamines (volatile and non-volatile) based on the use of an autoanalyser the principal element of which is a reactor consisting of a quartz tube (about 2 m in length with an internal diameter of 3 mm) and a UV radiation source. While the sample passes through the reactor (about 0.5 hr), NO ions split off by photolysis are reacted with an alkaline medium to form nitrite, which is then determined colorimetrically with sulfanilamide and N-(1-naphthyl)ethylenediamine. An improved version for the analysis of non-volatile N-nitroso compounds in food in the 10-100 µg/kg range was reported by Iwaoka and Tannenbaum (Ref. 37). They use a photohydrolytic detector in combination with HPLC.

A different approach was made by Fine and Rufeh (Refs. 7-10), who constructed and used a special thermal energy analyser (TEA) taking advantage of the low energy (8 to 50 kcal/mol) of the N-N bond in N-nitroso compounds. Prolysis on a tungsten-molybdenum-chromium catalyst leads to the formation of NO which is oxidized with ozone to NO_2 in the exited state. In a vacuum excited NO_2 emits a characteristic band in the near infrared region. The method is selective for the N-nitroso group, with the result that initial clean-up procedures can be simplified. The only compounds so far reported to give a TEA response similar to that observed for N-nitroso compounds are organic and inorganic nitrites and 2,2',4,4',6,6'-hexanitrodiphenylamine (Ref. 8). Although Fine (Ref. 7) suggested that extracts of nitrosamines could be directly injected into the TEA other workers have found this approach to be unreliable. The coupling of the TEA with a gas chromatograph or a high performance liquid chromatograph (HPLC) for non-volatile nitrosamines is the preferred method of injection.

Young (Refs. 38,39) has applied TLC to the separation of nitrosamines. In one procedure, after separation of the mixture and irradiation of the plates with UV light, the resulting amines are revealed by fluorescamine reagent. Spraying directly with 2,4-dinitrophenylhydrazine and phosphomolybdic acid reagent reveals only N-phenyl and carbazolyl compounds.

METHODS FOR THE DETERMINATION OF SPECIFIC NON-VOLATILE NITROSAMINES

Kelly and Hunn (Ref. 18) have worked out a method for the selective estimation of high molecular weight aliphatic N-nitroso compounds such as N-nitrosodicycloheptylamine, N-nitrosodi-n-octylamine and N-nitroso-di-n-heptylamine giving recoveries from 70% to 90%. The method is based on the extraction of N-nitrosamines with dichloromethane, drying after freezing, vacuum distillation and cleaning-up by treatment with sulfuric acid and sodium hydroxide. After concentration, the solution is analysed by TLC using chromatoplates impregnated with 6% methylsilicone gum E-30, and GLC using a column of 1% E-30 on Chromosorb GAW DMCS with FID detection (See also Ref. 25).

Eisenbrand and co-workers (Refs. 21, 22) have recently described a method for the determination of N-nitrosoproline, N-nitrosohydroxyproline and N-nitrososarcosine which is based on silylation with MSTFA and gas chromatographic separation of the volatile derivatives using a column of 3% OV-17 on 100/120 Gas-Chrom Q. Both FID and single ion-monitoring in a gas chromatograph combined with a mass spectrometer are used for quantitative assessments, the limit of detectability being about 2 ng with GLC-MS. This approach has only been used on the pure substances. Kushnir et al. (Ref. 23) have published a method of isolation and identification of N-nitrosoproline in uncooked bacon based on the extraction of N-nitrosoproline with water, cleaning-up by chromatography with dichloromethane on silica gel, esterification with diazomethane and analysis by means of GC-MS using a column of 6% Silar 10C on 60/80 Chromosorb P. The authors also report that N-nitrosoproline may be the precursor

of N-nitrosopyrrolidine, finding N-nitrosoproline in uncooked bacon at concentrations from 0.38 to 1.18 mg/kg. Although no statistical parameters of the method are reported, losses of about 80% were encountered in the silica gel procedure. Sen (Ref. 40), made derivatives of 3-hydroxy-1-nitrosopyrrolidine to form stable volatile 3-methoxy-1-nitrosopyrrolidine, but was able to use GC-MS for trace analysis of a particular nitrosamine. Specific ion monitoring for NO^+ or the molecular ion is used for identification with a sensitivity of 1 ng. Lee et al. (Ref. 41) demonstrated the formation of 3-hydroxy-N-nitrosopyrrolidine from 4-hydroxy-N-nitrosoproline in model systems simulating the conditions for frying bacon. GC-MS and TLC were used for analysis.

Dhont and Ingen (Ref. 24) describe a method for the determination of N-nitrosoproline, N-nitrosohydroxyproline and N-nitrososarcosine in meat products by means of GLC-FID, GLC-MS and TLC. The N-nitrosaminoacids are extracted with a mixture of methanol and water. Subsequent steps are alkalinisation of the extract, separation of any precipitate by centrifugation and evaporation of the methanol from the liquid phase. The aqueous solution obtained is then saturated with sodium chloride, acidified with HCl and extracted with ethyl acetate. After evaporation of the extract to dryness the residue is dissolved in water and the solution passed through an ion-exchange column. The GLC separation of these compounds as methyl ester derivatives is carried out using a column with 10% PEG 20 M on 60/80 Chromosorb W. Because of the decomposition of N-nitrosohydroxyproline on the GLC column, a TLC technique on silica gel is also used. The recoveries obtained for N-nitrosoproline and N-nitrososarcosine are ca. 100%.

References may be found in the literature to the use of mass spectrometry for the analysis of non-volatile nitrosamines. Gough and Webb (Ref. 25) have described a method using a silver membrane separator for a GLC -MS system to analyse nitroso derivatives of higher N-alkylamines and cycloalkylamines. The GLC separation is carried out using a 15 m column packed with 5% SE-52 on 100/200 Chromosorb W DMCS. For dialkyl derivatives the intensity of the parent ion (P) is very weak (about 4%) and the ion (P – OH) is preferred for measurement. GLC-MS analysis of the products of spermidine reaction with sodium nitrite [Ferguson et al. (Ref. 26)] did not yield positive results: Hildrum et al. (Ref. 27) have shown that the principal volatile nitrosamine formed is γ-butenyl-(β-propenyl)nitrosamine. Heyns and Röper (Ref. 37) have identified the derivatives of N-nitrosoalkylurea and N-nitroso-N-alkylurethanes using MS after separation of the compounds by HPLC. They have found that only a few high-mass ions are formed, and that the occurrence of parent ions does not exceed 5% for derivatives of urethanes. For urea derivatives the level is 20%. Unlike N-nitrosodialkylamines, the compounds examined do not form the (P – NO) ions. The authors also describe the mechanism of formation of the most characteristic ions.

Despite the fact that the development of methods is still at an early stage, it seems likely that the most profitable approach to the analysis of non-volatile nitrosamines will be through the use of high pressure liquid chromatography (HPLC). Iwaoka and co-workers (Ref. 28) have devised a method for the analysis of N-nitrosomorpholine and NDMNA on a column containing Corasil 2. Nitrosamines are extracted from the sample by a buffer solution, extracted by dichloromethane and cleaned up by solvent partition using n-heptane and solutions of potassium chloride and potassium carbonate. The recovery of N-nitrosomorpholine amounts to 50%. The same authors (Ref. 35) used HPLC for resolving the conformers of N-nitrosoethyl-aminoethanol, N-nitrosoproline and N-nitrososarcosine.

Fine et al.(Ref. 30) have succeeded in interfacing the TEA with HPLC so that non-volatile nitrosamines such as nitrosocarbazole can be analysed simultaneously with volatile nitrosamines (NDMA) using Bondapak-NH_2 and iso-octane containing 15% of chloroform as the mobile phase. The limit of detection is about 10 μg/kg for N-nitrosodiphenylamine after re-extracting a sample with dichloromethane. Generally, the system HPLC-TEA is much less sensitive than GLC-TEA. The same group (Ref. 42) used HPLC combined with TEA for the determination of non-ionic, non-volatile nitrosamines in liquors, fish and meat. Meat is homogenised with acetonitrile in the presence of liquid nitrogen and then extracted with iso-octane. The extract is evaporated to dryness, taken up in iso-octane containing 5% acetone and the nitrosamines are separated by HPLC with detection by the TEA. Good recoveries have been obtained on spiked samples and the detection limit is about 10 μg/kg.

Rao and Bejnarowicz (Ref. 43) have separated N-nitrososarcosine from sarcosine and N-lauroyl sarcosinate by TLC.

Wolfram et al. (Ref. 44) compared GC-MS, HPLC and TLC as methods for the determination of N-nitrosoproline at the nanogram level.

OTHER METHODS

Preussmann and Schaper-Druckrey (Ref. 6) have developed a method for the selective estimation of N-nitrosamides. It involves reaction with N-(1-naphthyl)ethylenediamine and sulfanilic acid in an aqueous environment and at a temperature of 60°C. Under these conditions N-nitrosamines do not react. The method was applied to blood and liver with detection limits of 0.26 mg/l to 0.77 mg/l. Singer (Ref. 45) has used HPLC in conjunction with a specific nitrosamide detector based on the reaction with Griess reagent followed by colorimetry for the analysis of individual nitrosamines.

Issenberg and Tannenbaum (Ref. 20) give a general scheme for the isolation and estimation of N-nitrosoamino acids. After extraction with water, deproteination and removal of the nitrite, the sample is concentrated by lyophilisation. The authors claim that N-nitrosoamino acids can easily be separated from the food substrate, including proteins and peptides, because of their strongly acidic properties. At the final stage the authors use an automatic colorimetric analysis according to Tannenbaum's method (Ref. 13). Iwaoka et al. (Ref. 45) compared several column packings and eluants for the separation of the syn- and anti-conformers of N-nitrosoamino acids by HPLC. Liberek et al. (Ref. 31) have applied TLC for the same purpose.

Mirvish (Ref. 17), in kinetic studies on the inhibition of N-nitrosation of N-aryl- and N-alkylurea derivatives, used a method based on denitrosation and subsequent acylation with heptafluorobutyric anhydride in pyridine. The derivatives were extracted with n-hexane and analysed by GLC.

An interesting but limited method for the estimation of nitrosamines was developed by Bonnet and Holleyhead (Ref. 16) using ^{15}N nuclear magnetic resonance. This method has two advantages: a sharp band resonance with a frequency of 1 Hz and the possibility to work in aqueous solutions. However, its low sensitivity and the need for extensive calculations are disadvantages. The authors used the method to search for N-nitroso derivatives of peptides and amino acids in model systems.

Heyns and Röper (Ref. 37) have determined the conditions for resolving and identifying N-nitrosoureas and N-nitrosourethanes using HPLC with a UV detector and a column filled with Bondapak-Corasil-C_{18}. Mixtures of water and acetonitrile in the ratios of 15:1 and 10:1 were used as mobile phases. The limits of detection range from 10 to 50 ng, the compounds being identified by IR, UV, MS and NMR spectroscopy. The IR maximum of absorption for the NO group in N-nitrosoalkylurethanes is in the region of 1500 to 1510 cm^{-1} while the UV maximum of absorption lies between 238 and 242 nm. The absorption values increase with increasing length of the alkyl chain.

CONCLUSIONS

1. Many methods have now been reported for the estimation of total or individual non-volatile nitrosamines, but only few reports contain details of clean-up procedures that are adequate for the analysis of food.

2. A few methods for the estimation of N-nitrosoamino acids, nitrosoamides and nitrosoureas have been published, but so far they have not been widely used.

3. HPLC-TEA techniques are promising because they have a high sensitivity and allow the simultaneous analysis of volatile and non-volatile nitrosamines.

4. The reliability of many methods for the analysis of non-volatile nitrosamines and of the data obtained with them is still in doubt and there is a need for collaborative studies in this field similar to that organised by the IARC for volatile nitrosamines.

ACKNOWLEDGMENT

The author expresses his gratitude to Dr. E. A. Walker, IARC, Commission on Food Additives, and Dr. R. F. Coleman, Laboratory of the Government Chemist, London, for their kind assistance in preparation of this report.

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*IARC refers to the International Agency for Research on Cancer, Lyon, France