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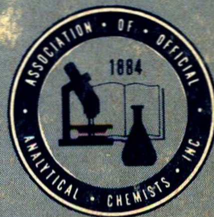

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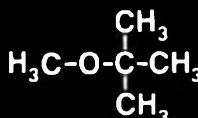

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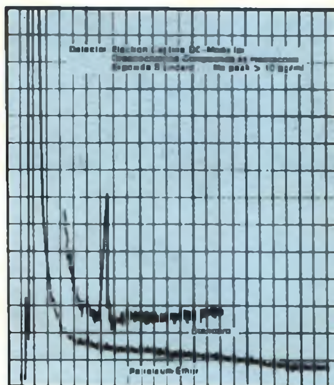

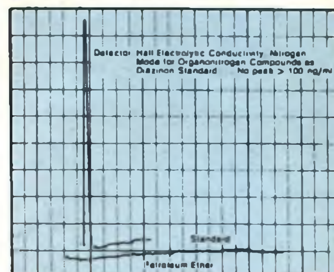

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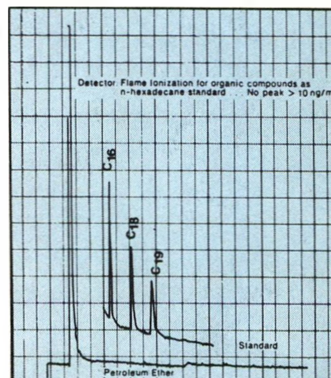

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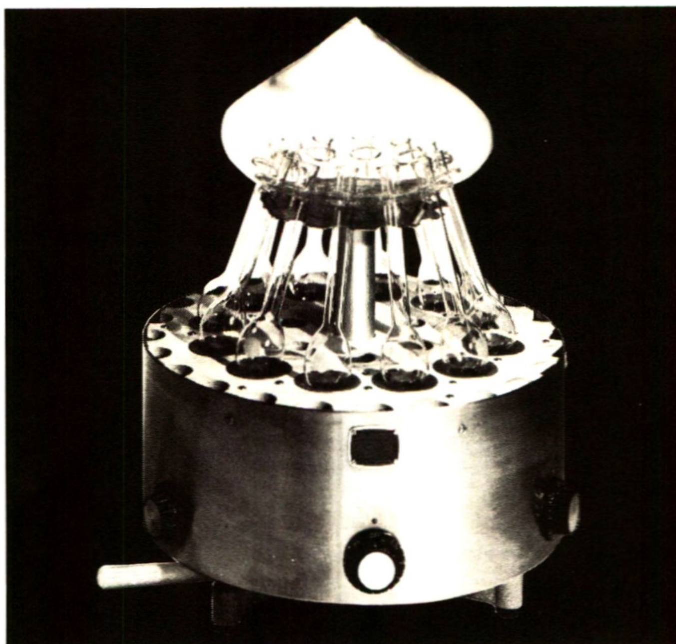

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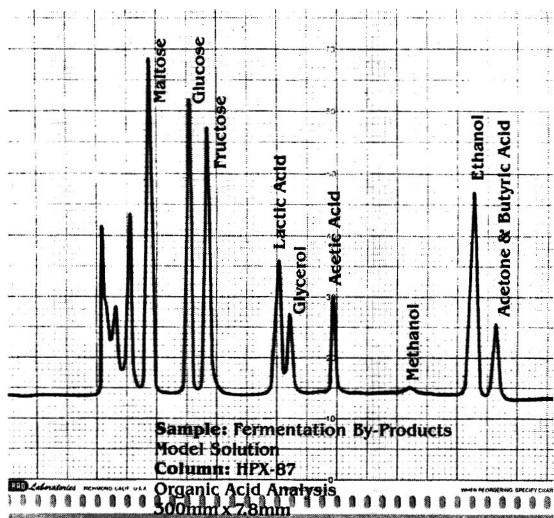

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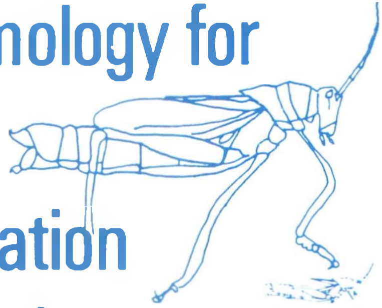

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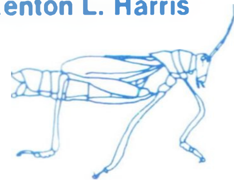

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REVIEW OF ANALYTICAL METHODS FOR *N*-NITROSAMINES IN FOODS

Analytical Methodology for Sample Preparation, Detection, Quantitation, and Confirmation of *N*-Nitrosamines in Foods

JOSEPH H. HOTCHKISS
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Significant advances have been made over the past decade in methodology for the analysis of foods and other samples for volatile *N*-nitrosamines. Procedures for the isolation, cleanup, and concentration of *N*-nitrosamines were developed and applied to a broad range of food types. Several chromatographic techniques and systems have also been developed which are capable of resolving *N*-nitrosamines in complex mixtures in a single run, and the use of highly selective detectors has decreased sample preparation time while increasing sensitivity and precision. Unequivocal confirmation of *N*-nitrosamines in foods can now be achieved by mass spectrometry at low to sub-μg/kg levels. However, further development of methodology for nonvolatile *N*-nitroso compounds is needed. This review paper discusses these and other topics related to the analysis of foods for *N*-nitrosamines.

As a class of compounds, *N*-nitrosamines[1] have biological properties which have generated a great deal of interest in the field of cancer research. The great majority of the over 100 *N*-nitroso compounds tested in laboratory animals were carcinogenic (1) and in some circumstances a single dose has produced tumors (2). In addition to being potent carcinogens, NAs are acutely toxic, mutagenic, teratogenic, and transplacental, and display a strong relationship between chemical structure and organ(s) affected (3). The biological properties (4, 5) and the chemical properties (6) of *N*-nitroso compounds have been recently reviewed. While the occurrence of these compounds is undesirable, the significance, if any, of the low levels to which humans are generally exposed is unknown (7).

Concern that human foods might contain NAs stems from the discovery that domestic animals fed nitrite-preserved herring meal developed severe liver disorders. NDMA was identified as the causal agent and it was proposed that NDMA was formed from added nitrite and di- and trimethylamines endogenous to the fishmeal (8).

It has now been well established that certain foods may contain trace amounts of one or more NAs (9). Included are not only nitrite- and nitrate-cured products but also foods to which these substances have not been intentionally added.

Humans are exposed to trace amounts of NAs through sources other than foods (10). Low level exposure has been detected in certain industrial environments (11), cigarette smoke (12), cosmetics (13), pesticides (14), and even new automobile interiors (15). In addition, we may be exposed to these compounds through in vivo nitrosation of ingested amines (16). Nitrosatable amines are found in foods (17), and several drugs can form NAs when exposed to nitrite under conditions similar to those found in the stomach (18).

Early analytical methodology for NAs in foods was in certain cases nonspecific, and some positive findings are unsubstantiated (19). Over the past decade, advances in analytical methodology for volatile NAs not only have increased the reliability of analyses but have decreased analysis time. The use of more selective detectors for chromatographic systems has simplified cleanup procedures, and improvements in mass spectrometric methods have allowed very low levels of NAs in foods to be confirmed.

The low levels at which NAs occur in foods and the heterogeneous nature of foods have complicated the analyst's task. A multistep approach has been necessary to overcome these difficulties: isolation from the food, cleanup and concentration, separation, detection, quantitation, and confirmation. Most workers have ap-

[1] Abbreviations:
NA = *N*-nitrosamine
NDMA = *N*-nitrosodimethylamine
NDPA = *N*-nitrosodipropylamine
NPYR = *N*-nitrosopyrrolidine
NDELA = *N*-nitrosodiethanolamine
NHPYR = *N*-nitroso-3-hydroxypyrrolidine
NHPIP = *N*-nitroso-4-hydroxypiperidine
NPRO = *N*-nitrosoproline
DCM = dichloromethane

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plied this general scheme but have used a variety of techniques to satisfy each step. The purpose of this review is to present selected recent approaches to each of these steps in the hope that this arrangement will be of greatest value to the analyst. The International Agency for Research on Cancer (IARC) has published a compendium of selected methods (20) and other recent review articles have dealt at least in part with the determination of NAs (9, 19, 21-23).

Sample Handling

Sampling protocols, data reliability considerations, and sample storage conditions for foods to be analyzed for NAs should be similar to those which have recently been discussed for environmental samples in general (24). NAs are relatively stable in food systems (25) but must be protected from UV irradiation, including sunlight and fluorescent indoor lighting, to prevent decomposition. Microorganisms may play a role in NA formation by reducing nitrate to nitrite or by direct catalysis of nitrosation at certain pH conditions, and samples should be stabilized against outgrowths (26). Freezing does not inhibit nitrosation and under certain conditions may increase the rate of nitrosation over room temperature rates (27).

The effects of various sample preparation methods have received little attention. Solid samples have been reduced in size by grinding (28), hand chopping (29), slurrying in water (30), and grinding to a fine powder after freezing in liquid nitrogen (31). In a study on malted barley, it was concluded that the use of liquid nitrogen during grinding had no effect on NDMA content (32). Adverse effects have not been demonstrated for any of the above techniques; however, procedures which cause excessive heating of the sample should be avoided.

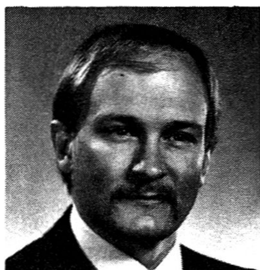
N-Nitrosamine Isolation

The 2 most common methods for removing NAs from food matrices are direct extraction and distillation. Direct extraction has been frequently used to isolate volatile NAs from aqueous samples. The NAs are usually extracted from the sample with dichloromethane (DCM), followed by concentration of the solvent. This procedure has been used to isolate volatile NAs from environmental waters (33), and both Fiddler et al. (34) and Gough et al. (35) used direct extraction with DCM to isolate NAs derived from the use of certain deionizing resins. Direct extraction has also been used to isolate both volatile and nonvolatile NAs from inlets and outlets of sewage treatment plants. Volatile NAs were extracted with DCM, and NDELA was extracted with ethyl acetate (36).

A number of workers have used direct extraction to analyze alcoholic beverages for NAs. Castegnaro et al. (37) first adjusted the alcohol content of distilled spirits to approximately 55%, saturated the solution with anhydrous magnesium perchlorate, and extracted with DCM. The magnesium perchlorate prevented co-extraction of large amounts of ethanol. These same workers expanded this technique into a general scheme for the determination of NAs in alcoholic beverages (38). Direct extraction after dilution with water and without addition of magnesium perchlorate has been used to analyze Scotch whiskies (39).

A specialized commercial apparatus has been used to liquid-liquid partition NAs from alcoholic beverages, including several beers, directly into DCM (40). Beverages with greater than 20% ethanol content were diluted to 20%, and those under 20% were analyzed undiluted. A 15 mL aliquot was loaded onto a tube containing an adsorbent, and NDMA was eluted with DCM. The recovery of NDMA from spiked beverages ranged from 45 to 62%. Workers at the Food and Drug Administration described an extraction technique based on a similar concept in which malt beverages are mixed with sufficient Celite to hold the liquid in a chromatographic column, and DCM is percolated through the column to elute NAs (41).

A number of workers have proposed direct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extraction methods for isolation of volatile NAs from solid foods. Sen (29) described a procedure in which volatile NAs are extracted by DCM from food mixed with 3N potassium hydroxide in a blender, and Eisenbrand (42) extracted wheat flour with DCM in a Soxhlet apparatus. While their recoveries from spiked wheat samples were adequate, other workers have not been successful in extracting ground and unground barley malt by Soxhlet methods. These workers (32) postulated that NDMA was bound to macromolecular components and could not be efficiently extracted by DCM. Hot water has also been used as an extracting solvent for solid foods (43) including malted barley (32). A survey of Japanese fermented foods was conducted by blending an alkaline homogenate of the food with DCM and separating the DCM by filtration through paper (44). Liquid foods were directly extracted and solids high in oil were blended frozen with cold DCM to prevent extraction of the lipid material.

Pensabene et al. (45) developed a promising direct extraction technique for NPYR in fried bacon. Ground fried bacon was mixed with anhydrous sodium sulfate and Celite. The resulting dry mixture was packed into a chromatographic column, and the lipid material was eluted with 5% DCM in pentane. The NPYR was then eluted from the column with DCM and concentrated. Analysis time was reduced while recovery efficiency was maintained. Havery et al. (46) adapted a similar procedure to nonfat dry milk.

Direct solvent extraction has found widest application in the analysis of foods for nonvolatile NAs. Several workers have used this technique to isolate NPRO and other *N*-nitroso amino acids from uncooked bacon. Kushnir et al. (47) extracted uncooked bacon with water followed by freeze drying, and Hansen et al. (48) used ion exchange to clean up a water extract of uncooked bacon. Janzowski et al. (49) analyzed cured meat products for *N*-nitroso amino acids, using acetone–water as an extracting solvent. Celite was homogenized with the meat–solvent mixture to aid in filtration. Baker and Ma (50) blended processed meats with water followed by filtration of the heated mass to remove solids. Pensabene et al. (51) first defatted the uncooked bacon by extracting with hot hexane followed by cold ether. The defatted sample was then directly extracted using 1N HCl–acetonitrile–dioxane as a solvent system. The recovery for samples spiked with an internal standard was 74%.

Nonionic nonvolatile nitrosamines have also been isolated from foods by direct extraction techniques. NHPYR was isolated from fried bacon, using water–methanol (52) and acetonitrile (53) as extracting solvents. Janzowski et al. (54) analyzed several cured meat products for NHPYR, using methanol–water as a solvent system. NHPIP was used as an internal standard and the recoveries of NAs were 58 and 45% for NHPYR and NHPIP, respectively. Fine et al. (31) extracted foods low in lipids directly with DCM and lipid-containing foods with acetonitrile to analyze for nonionic nonvolatile NAs.

Volatile NAs have been most often isolated from food matrices by distillation. Atmospheric, vacuum, and steam distillation have been used. Goodhead and Gough (55) described a steam distillation procedure which has been used for several types of foods. The sample was slurried with water and salt, and then steam distilled. Several times the sample weight in distillate was collected at atmospheric pressure. The distillate was then extracted, cleaned up, and concentrated. Recoveries from meat for 6 volatile NAs (10 μg/kg) ranged from a low of 65% for NPYR to a high of 81% for NDPA. This technique has been used to screen a broad range of food types (56), including beer (57). The procedure can be more time-consuming than recent methods but has the advantage of being adaptable to almost any food matrix.

Fine et al. (58) described a vacuum distillation procedure which has been widely accepted. A modification of this technique is currently being used by the U.S. Department of Agriculture in its bacon monitoring program (*Fed. Regist.* (1978) 43, 20992–20995). Distillation was carried out by placing a 20 g sample in a round-bottom flask along with an equal amount of mineral oil and 4 mL 0.1M sodium hydroxide. Vacuum was applied, and the contents of the flask were slowly heated to 110°C. The distillate was trapped in vapor traps immersed in liquid nitrogen. After the specified temperature was reached, the vac-

uum was discontinued, and the distillate was thawed, extracted, and concentrated. The authors reported recoveries of 71–75% for NDMA and 100% for NPYR in tuna at 5 μg/kg. Sensitivity of 10–50 ng/kg was reported but evidence was not presented to support this sensitivity.

Several modifications of the method have been applied to foods. Spiegelhalder et al. (59) used a modification of the method to survey West German foodstuffs including beer (60), and Libbey et al. (61) reported NDMA in dried dairy products with a modified vacuum mineral oil distillation. Barley malt has been surveyed by using mineral oil distillation (62), and Owens and Kinast (63) increased recovery of volatile NAs from bacon cooked-out fat by adding a water-absorbent distillation aid. A similar observation that water added to dry samples increases recovery of NDMA was reported for malted barley (32).

Havery et al. (64) compared a modified mineral oil distillation procedure with a multidetection procedure (65) for the recovery of 14 volatile NAs. Recovery averaged 75% for the multidetection method and 92% for the mineral oil distillation. In addition, 106 meat samples were analyzed by both methods. The results were in general agreement, with the mineral oil distillation giving slightly higher results. The relatively short analysis time and broad applicability of the mineral oil procedure have made it a logical first choice when protocols for new substrates are developed.

A vacuum distillation procedure based on a rotary flash evaporator has also been described (66). Samples were distilled from an alkaline slurry under reduced pressure. After distillation the condenser was rinsed with DCM which was further used to extract the distillate. In addition to fried bacon and frying vapor condensates, alcoholic beverages have been analyzed by this technique (67). These same workers recently proposed a more rapid atmospheric distillation procedure (68).

Atmospheric distillation has been limited to samples with a high water content or samples which are readily dissolved in water. A large survey of malt beverages for volatile NAs was conducted by first saturating the samples with barium hydroxide, directly distilling the mixture, and subsequently extracting the aqueous distillate (62). Sen and Seaman (68) described a similar distillation procedure in which beer samples were acidified and treated with sulfamic acid before distillation to destroy potential nitrosating agents which may be present. The sample was then made basic and distilled, followed by extraction and concentration. Recovery of added NDMA averaged 91%, and the limit of detection was 0.1 μg/kg. Both fluid and nonfat dry milks have also been surveyed for volatile *N*-nitrosamines by atmospheric distillations (69). Dry samples were dissolved in distilled water and evaporated/condensed milks were diluted 1:1 before distillation. Sensitivity for NDMA was reported to range from 0.05 to 0.3 μg/kg, depending on the product analyzed.

Fazio et al. (65) described a procedure to detect 14 volatile NAs simultaneously (70). This multidetection method included a predigestion of the food in methanolic potassium hydroxide followed by continuous liquid–liquid extraction of the digestant with DCM. Aqueous sodium hydroxide was added to DCM, and the organic phase was distilled. The remaining aqueous phase was further distilled along with the volatile NAs. This isolation method has been widely used but its use has been questioned on grounds that the alkaline digestion may lead to artifactual volatile NA formation if β-hydroxyalkylnitrosamines are present (71). However, these nonvolatile NAs have not been reported in foods to date.

Cleanup and Concentration

The lack of a detector that responds exclusively to NAs, and the low levels at which NAs occur in foods, has necessitated the use of cleanup procedures to remove interfering compounds and concentration methods to increase sensitivity. The degree of cleanup necessary and the minimum level of detection depend in large part on the selectivity and sensitivity of the detection system used. Recently, increased selectivity in NA detectors has greatly reduced the amount of cleanup necessary.

Most often, cleanup has been achieved by extraction and column chromatography. However, distillation from acidic or basic solutions has also been successful. Fiddler et al. (72) distilled aqueous extracts of fried bacon from base, acidified the resulting distillate, and extracted with DCM. Cross et al. (73) used distillation to isolate volatile NAs from bacon cooked-out fat followed by redistillation from base to effect a partial cleanup. Subsequent extraction and column chromatographic procedures were used as further cleanups. Double distillations from basic followed by acidic solutions have also been used (42). Although successful, these distillations can be time-consuming and inefficient and should be considered as a last resort.

Several workers have used extraction at some point for cleanup. In the multidetection procedure of Fazio et al. (65) the aqueous distillate was acidified before extraction with DCM, followed by extraction of the pooled DCM with aqueous base to remove basic and acidic contaminants, respectively. Variations of this procedure have been widely used. Goodhead and Gough (55) used a similar extraction scheme which has been applied to foods (74). Gray and Collins (75) cleaned up distillates of fried bacon intended for analysis by thin layer chromatography (TLC), using acid and base washes of the DCM extract.

Extraction has also been used to clean up samples high in lipids. Soybean oil was dissolved in DCM and extracted with 5N NaOH to remove interfering acids before distillation (76). The fact that the acetonitrile–heptane partition coefficient of several NAs favors acetonitrile has also been used to separate NAs from neutral lipids (77). Sen et al. (78) used this acetonitrile–heptane partition to isolate NPRO from lipids derived from uncooked bacon. The raw bacon was blended with acetonitrile, filtered, extracted with *n*-heptane, and evaporated to dryness. NPRO was then derivatized for analysis by gas chromatography (GC).

Column chromatography of both aqueous and solvent extracts, using a variety of adsorbents, has been a useful cleanup technique. Howard et al. (79) described the use of acidified Celite to remove basic compounds from extracts which were analyzed by an alkaline flame ionization detector. This procedure has been expanded by these workers to include a silica gel column cleanup in addition to the Celite column (39). The samples treated by this multiple column cleanup were intended for GC-mass spectrometric (MS) analysis. In addition to silica gel, alumina has been used to clean up solvent extracts (80, 81), and Florisil and silica gel have been used in the same column (82). *N*-Nitroso amino acids have also been cleaned up on cellulose (83) and anion-exchange resins (48).

The $\mu g/kg$ levels at which NAs occur in foods have necessitated a concentration step in the analytical procedure. This has nearly always been achieved by evaporation of a volatile solvent. Because of its favorable partition coefficients for many NAs (84) and its relatively low boiling point, DCM has been the most widely used solvent. Concentration increases of 100 to 1000× can be achieved using a Kuderna-Danish (K-D) evaporative concentrator fitted with a Snyder distilling column. Distillation is usually continued until approximately 4 mL solvent remains. The rate at which DCM is concentrated by the K-D apparatus may affect the recovery efficiency of NDMA. Distillation at a rate of 1 mL/min may be optimal (D. C. Havery, FDA, 1979, unpublished data). Final concentration to 0.25–1 mL is usually done under a stream of nitrogen although a micro Snyder procedure for final concentration to less than 1 mL has been described (68). Recoveries are better than 90% for NDMA only if care is exercised during this final concentration step. Both the ambient nitrogen procedure and the micro Snyder procedure can produce high recoveries; however, the former requires less operator supervision.

> *"Advances in analytical methodology for volatile NAs have not only increased the reliability of analysis but have decreased analysis time. Relatively few analytical procedures for nonvolatile NAs in foods have been developed to date even though there is indirect evidence that under certain conditions they may be formed."*

Separation of Nitrosamines

Chromatography has generally been used to quantitatively and qualitatively identify NAs derived from foods. The simplest but lowest resolving method of separation is thin layer chromatography. Sen and Dalpe (85) published a TLC procedure for volatile NAs in alcoholic beverages. Several beverages were analyzed, with a 25 ppb limit of detection. Young (86) presented R_f values for 24 NAs in 4 solvent systems on 2 chromatographic substrates. Wolfram et al. (87) and Young (88) independently described the determination of NPRO by TLC. Both workers produced a fluorescent derivative of the amine after cleavage of the nitroso group. Young (88) detailed a unique 2-dimensional system in which NPRO is chromatographed in one direction, the nitroso group is cleaved, and the resulting amine is chromatographed in a second direction. A quantitative procedure

> *"Because of increased sensitivity, foods such as beer and nonfat dry milk which were thought to be negative when analyzed by less sensitive methods have been shown to contain traces of volatile NAs when re-examined."*

based on densitrometry of the fluorescent derivative has been described for NAs in cooked-out bacon fat (73). This procedure was later extended to the edible portion (89). TLC systems capable of resolving hydroxyheterocyclic-*N*-nitrosamines (90) and bishydroxyalkyl-*N*-nitrosamines (91) have also been published, and the relationship between structure and R_f values has been discussed (92).

Gas chromatography has proven to be the method of choice for the separation of volatile NAs. Fazio et al. (93) analyzed smoked fish for NDMA on a glass 9 ft × 4 mm id column of 10% Carbowax 1540 plus 5% KOH, programmed from 80 to 180°C at 5°/min, and later extended this to include 14 volatile NAs (70). Stainless steel columns have also been widely used for volatile NAs. NDMA in beer was quantitated by using a stainless steel 1.8 m × 3.18 mm id column of 25% Carbowax 20M plus 2% NaOH at 170°C, isothermal (67); NDMA in nonfat dry milk was quantitated on a stainless steel 3.05 m × 3.18 mm id column of 5% OV-275 (61).

In addition to packed columns, open tubular columns have been used most often in conjunction with mass spectrometry. Gough and Sugden (94) described a system which consisted of a 1.6 m × 1.8 mm id packed column connected in series to a 30 m × 0.5 mm id support-coated open tubular (SCOT) column. Micro valving between the 2 columns allowed the solvent to be vented when large volume injections (5 μL) were made, while maintaining optimum performance of the SCOT column. A similar system in which a liquid nitrogen trap was used to collect NAs from the precolumn after the solvent was vented has been used to confirm NAs in foods (95). The trap was then warmed and the NAs were re-injected onto a 160 m × 0.5 mm id open tubular column. A procedure for trapping volatile NAs from a packed column for later GC-MS confirmation using a glass SCOT column has been detailed (96). Glass SCOT columns have also been used to confirm volatile NAs in beer and fried bacon (39).

Although Carbowax has been the most widely used liquid phase for volatile NAs, others have also been used. Fine and Rounbehler (97) used packed 15% FFAP (free fatty acid phase) columns, and Eisenbrand et al. (83) used the similar liquid phase Carbowax 20M-terephthalic acid terminated, at the same loading. Retention data for volatile NAs have been published on this phase (98). Other stationary phases have been used to a limited extent: Ucon 550-KOH (99), OV-101 (100), Chromosorb 101 (101), Chromosorb 103 (102). Derivatization of nonvolatile NAs has necessitated the use of less reactive liquid phases such as the silicone oils and rubbers (49).

Relatively few analytical procedures for nonvolatile NAs in foods have been developed to date even though there is indirect evidence that under certain conditions they may be formed (103). Difficulties in analysis of nonvolatile NAs may be overcome as analytical methods based on high pressure liquid chromatography (HPLC) are further developed. A procedure for the analysis of foods for 3 volatile NAs by HPLC and chromatographic data for 3 additional volatile NAs have been published (104). Iwaoka and Tannenbaum (105) separated syn and anti conformers of NPRO, and other workers used HPLC to analyze raw bacon for NPRO (48, 50). Both groups used reverse phase chromatography on μBondapak C_{18} columns. Fan et al. (106) described an analytical procedure involving HPLC of both volatile and nonvolatile NAs, and Fine et al. (107) separated both volatile and nonvolatile NAs on μBondapak NH_2 columns operated isocratically with different solvent systems.

The discovery that nonvolatile NAs can be contaminants in toiletries (13), cutting fluids (108), and pesticides (109) has led to the development of HPLC techniques which may be of use in food systems.

Detection and Quantitation

Detection and quantitation methods depend in part on the chromatographic procedure used to separate the NAs. Cleavage of the N—N bond of the nitroso group by UV irradiation and subsequent derivatization of the products has been used. Sen and Dalpe (85) used Griess and ninhydrin reagents to detect, respectively, nitrite and amine formed after irradiation of TLC plates. Photolytic cleavage and subsequent reaction of the nitrite formed has been used in an automatic

colorimetric device for *N*-nitroso determination (110). Hydrogen bromide–acetic acid cleavage of nitroso groups and derivatization of the nitrosylbromide with Griess reagent was given as a total *N*-nitroso group assay (111).

The use of fluorescent derivatives of the amine produced by nitroso group cleavage has been used to detect and quantitate NAs in foods. Young (88) irradiated TLC plates and used fluorescamine for visualization of *N*-nitroso amino acids. *N*-Nitroso group cleavage by HBr–acetic acid and subsequent coupling of the resulting amine with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to form the fluorescent derivative has also been described (73, 112). Wolfram et al. (87) reported the use of this derivative in TLC and HPLC systems and found the HPLC 10 times more sensitive than a GC-MS method for NPRO. A quantitative procedure for the chromophore produced when dialkyl NAs are reduced to hydrazines and then reacted with *p*-dimethylaminobenzaldehyde has been described (43).

Derivatization has been used to yield nonvolatile NAs amenable to gas chromatography. Eisenbrand et al. (113) trimethylsilylated *N*-nitroso amino acids for analysis by GC-MS and Röper and Heyns (114) methylated and silylated *N*-nitroso hydroxyamino acids. Acylation, trifluoroacylation, trimethylsilylation, and methylation have been compared for derivatization of 6 hydroxylated NAs (115). All 6 NAs were derivatized by either acylation or trimethylsilylation and the authors concluded that acylation with acetic anhydride was the most convenient procedure. All derivatives were chromatographed on silicone-based stationary phases in packed glass columns.

The flame ionization detector has found limited use in the determination of NAs in foods because of lack of specificity. Other GC detectors which exhibit increased selectivity have, however, been used. Howard et al. (79) described a modified alkali flame ionization detector (AFID) for use in the determination of volatile NAs in foods, and similar thermionic detectors have been used by other workers (116). Commercially available glass beaded detectors have overcome some of the problems associated with AFIDS. The Coulson electrolytic conductivity detector (CECD) has also been used as a selective detector for volatile NAs in foods (117). Palframan et al. (118) compared the AFID and CECD detectors and found that both had similar sensitivities; the CECD showed higher selectivity and stability. The authors concluded that the CECD was more convenient and reliable. More recently, Gough (119) similarly concluded that the CECD shows higher selectivity. A CECD was used by Goodhead and Gough (55) as a screening tool before GC-MS confirmation. About one-half of the NA-positive CECD responses could not be confirmed by GC-MS. The authors concluded, however, that the CECD detector is a useful screening tool.

The Hall modified electrolytic conductivity detector has been adapted to increase its selectivity for volatile NAs (120). This increase in NA selectivity over other nitrogen-containing compounds is achieved by replacing the nickel catalyst tube with a noncatalytic gold tube. In this noncatalytic mode, only the nitroso group is reported to be cleaved, reduced, and hence detected. While little work has been published using this detector to date, it may have certain advantages over other screening methods, especially in cost.

NAs themselves are not amenable to electron capture detection but can be derivatized to electron-capturing species. Oxidation of volatile NAs to nitramines by peroxytrifluoroacetic acid and subsequent detection by electron capture detectors (ECD) has been described (121). Telling (122) later used this oxidation technique and a ^{63}Ni ECD to detect volatile NAs at 10 μg/kg in foods; recently (123), *N*-nitroso amino acids have been detected as nitramines after esterification, oxidation, and acylation. Volatile NAs have also been directly reacted with heptafluorobutyric anhydride to form electron-capturing derivatives. Gough et al. (124) studied this reaction and found that NDMA forms 2 derivatives with greatly differing chromatographic retention times. Other volatile NAs formed single derivatives.

The thermal energy analyzer (TEA) is a commercially available detector which is highly selective for *N*-nitroso compounds. TEA has most often been used as a detector for gas chromatography (GC-TEA, 97) or more recently liquid chromatography (HPLC-TEA, 125). A similar laboratory-built detector has been described (126).

The principles of the TEA have been published in detail (127). Briefly, *N*-nitroso compounds, upon exiting a chromatographic column, enter a flash heater and catalytic pyrolysis chamber where the N—N bond, which is more easily ruptured than most other covalent bonds, is selectively cleaved. The nitric oxide produced is then swept along by an inert carrier gas through a cold trap (−150°C) where organic compounds

are frozen out. The nitric oxide is further swept to a small chamber where it is reacted with ozone to produce an energy emission in the near infrared region. This emission is detected by a photomultiplier tube, amplified, and recorded. The response is proportional to the *N*-nitroso group concentration in the original sample.

The GC-TEA system has been compared with GC-CECD, TLC, and GC-MS for the determination of volatile NAs in fishmeal and fried bacon (128). While only 3 samples were compared, all 4 methods gave similar results and the GC-TEA was at least 50 times more sensitive and required less cleanup of the sample. Because of this increase in sensitivity, foods such as beer and nonfat dry milk which were thought to be negative when analyzed by less sensitive methods have been shown to contain traces of volatile NAs when re-examined. In addition, the highly selective nature of the detector has allowed very rapid sample preparation with little or no cleanup (41). Large volatile NA surveys of the West German (59), British (129), and Japanese (44) diets have been conducted by CECD or TEA screening methods. Unfortunately, no comprehensive survey of the United States diet has been made.

The high selectivity of TEA for *N*-nitroso compounds is well established (130) but some workers have indicated that certain environmental samples contained non-*N*-nitroso compounds that can elicit a positive TEA response. Stephany and Schuller (131) showed that certain tertiary *C*-nitroso compounds are TEA positive, and Fiddler et al. (132) reported unidentified TEA positive peaks in chromatograms of tobacco smoke condensate, cheese, and fish products. They also identified 3 pyrrole compounds that could produce a response if present in very large amounts. Fan et al. (133) identified dinitroethyleneglycol as a TEA positive compound found in drinking water, and Gough and Webb (134) found an unidentified non-*N*-nitroso compound that was TEA positive and had a retention time on Carbowax 20M that coincided with NDPA. It has been suggested (135) and confirmed (136) that nitramines, in general, are TEA positive with molar responses near those of the corresponding NA. The instrument manufacturer (Thermo Electron Corp.) reported that certain organic nitrites and nitrates plus some inorganic nitrites produce a positive TEA response, particularly when the instrument is operated in the direct injection mode. More extensive lists of both positive and negative responding compounds have been published (127) and Krull et al. (137) discussed methods to discriminate between NAs and false positives. To date, no false negative *N*-nitroso compounds have been reported, but signal repression was reported when NDMA and dimethylamine were co-injected into the GC-TEA (138). Also, co-injection of ethanol and NDMA can result in erratic TEA responses (139). Effects of pyrolyzer temperature on interferences in the TEA have been investigated (140).

Early work on polarographic detection of NAs lacked specificity and was likely responsible for misidentification of NAs as products of nonenzymatic browning (141). Subsequent improvements have increased selectivity (142) and a commercial instrument has been recently promoted by the manufacturer as an HPLC detector for the analysis of NAs (143). Vohra and Harrington (144) discussed modifications of the polarographic detector and the effects of electrode potential, drop time, drop size, pH, and flow rate on the detection of NDPA chromatographed by HPLC. Although a minimum detection limit for analytical standards was 0.8 ng, data for NAs in foods were not presented. The electrochemical behavior of NPYR and some related compounds has been studied in detail (145).

Other detectors have been proposed for NA analysis but little work using these detectors on food systems has been published. Meili et al. (146) proposed use of the photo-ionization detector coupled to gas chromatography. Although the detector showed good sensitivity, the selectivity was low and, hence, would probably be of little value in complicated substrates such as foods. A photo-electro analyzer has been detailed which is capable of detecting 1 ng NDPA (147). NAs are photolytically cleaved in base, and the resulting nitrite is determined by a flow-through platinum electrode. A photochemical conductivity detector is commercially available as an HPLC detector for NAs (148). The selectivity of this detector is only moderate and its use for NAs in foods has not been established.

Several spectral methods have found use in NA chemistry but lack the specificity and/or sensitivity necessary for trace analysis. NAs have 2 UV maxima at 230–235 and 332–374 nm in water (6) and have characteristic IR absorbances. NAs produce complicated NMR spectra due to geometrical isomerism produced by the restricted rotation about the N—N bond (149).

Downes et al. (150) proposed and later modified (151) a procedure designed to detect total *N*-nitroso compound content (volatile and non-

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volatile) of foods without prior extraction. Dry samples are refluxed in solvent and denitrosated by addition of HBr–acetic acid. The resulting nitric oxide is detected by chemiluminescence when reacted with ozone. This procedure has been expanded to the determination of nitrite, total *N*-nitroso compounds, and nitrate by the sequential addition of acetic acid, HBr–acetic acid, and titanous chloride (152). Little attention has been given to isolating and identifying the compound(s) that give a response in this system, although certain potential false positives have been investigated (153). Drescher and Frank (154) proposed modification of the procedure and pointed out the large decrease in the response caused by small amounts of water. In their work, 1% water in the sample volume was sufficient to eliminate the response from the detector.

Confirmation

The potential health, regulatory, and economic significance of reports indicating the presence of NAs in human food demand that confirmation be made by the best method available. Mass spectrometry (MS) has been considered the most reliable procedure for confirming the presence and identity of NAs (155) and an NA analysis subcommittee of the International Agency for Research on Cancer (IARC) has recommended that findings which are not confirmed by MS be labeled "apparent" (156).

Standard spectra of many *N*-nitroso compounds are available. Ap Simon and Cooney (157) discussed electron impact spectra of NAs, and spectra of nearly 150 *N*-nitroso compounds are available (99, 157–159). Chemical ionization spectra have also been published (160).

While MS is generally accepted as an unequivocal method for the confirmation of NAs, a large number of specific MS techniques have been reported and many have been reviewed (161). Single ion monitoring of molecular or fragment ions by low resolution GC-MS is one of the simpler techniques and has been used to detect trimethylsilyl derivatives of *N*-nitroso amino acids (113). Several characteristic ions and their relative abundances are presented but no data are reported for these compounds in food samples. This technique is of limited value for complex samples because any fragment of the same nominal mass may produce a false positive response. Multi-ion mass fragmentography can increase specificity over single ion monitoring. Essigmann and Issenberg (95) plotted the intensity of ions at m/z 30, 42, and 74 in the GC-MS analysis of chopped ham spiked with NDMA. Tentative confirmation was made if all 3 ions maximized at the correct retention time. Gadbois et al. (117) used mass fragmentography at m/z 30, 42, and 74 and coincidence of retention time to identify NDMA in smoked fish. Others have monitored the molecular as well as fragment ions of NPYR when studying its mechanism of formation in fried bacon (162, 163).

As pointed out by Gough (161), positive results for NAs in complex media, such as foods, based on mass fragmentography must be interpreted with caution. Many compounds or combination of compounds may produce fragments of the same nominal mass as those seen in the relatively simple spectra of most NAs. Confirmation is more reliable if it is apparent that extraneous ions are absent from the NA spectrum. Mass fragmentography does have the advantage of increased sensitivity over full spectrum MS.

Full spectrum MS has been used to confirm both volatile and nonvolatile NAs in foods. Sen et al. (164) published a spectrum of NPYR found in fried bacon and later used low resolution MS to further confirm this finding (165). Low resolution MS was used to confirm NDMA, NHPIP, and NPYR in fried bacon and spice premixes at levels equal to or greater than 10 ppb (166), and Wasserman et al. (167) studied the occurrence of NDMA in frankfurters and confirmed its pres-

ence in 3 samples. Havery et al. (62) and Scanlan et al. (57) independently published spectra of NDMA isolated from malt beverages at levels of less than 10 ppb. NHPYR has been confirmed in fried bacon as the TMS derivative (52) and others (90) used low resolution MS to study the formation of NHPYR in model systems.

Some workers have regarded high resolution MS as more reliable for very complex samples such as foods (53, 100) and these and others have used high resolution MS extensively. A comprehensive survey of foods available in England was conducted by screening samples by CECD and confirming positive responses by high resolution MS (129). Iyengar et al. (168) reported the NA contents of several fish products which were confirmed by monitoring the NO^+ ion at a resolution of 5000. A 25% Carbowax 20M column was used and the lower limit of confirmation was at least 3 $\mu g/kg$. Others (169) have used high resolution MS to study the formation of NAs in the fat and lean portions of bacon. Volatile NAs were confirmed in alcoholic beverages at levels above 5 ppb by monitoring the NO^+ and molecular ions at a resolution of 9000 (38). Extracts were pooled and concentrated to 1–2 mg NA/L after screening by GC-TEA.

The degree of resolution used to confirm NAs has varied among different workers. Telling et al. (30) described a technique for monitoring NO^+ at a resolution of 15 000 which distinguished it from all species found in a pork luncheon meat extract except $C^{18}O^+$. Volatile NAs were isolated by vacuum distillation without further cleanup and with a lower limit of detection of 25 ppb. Other workers preferred to monitor individual molecular ions because of the increased sensitivity gained by using lower resolution. Sen et al. (66) monitored both the molecular and NO^+ ions at resolutions of 5000 and 10 000, respectively, to confirm NDMA and NPYR in fried bacon fumes and later used this same technique to confirm NAs in other foods. Compson et al. (170) determined that a minimum resolution of 10 000 was necessary to identify the molecular ion of NDMA in tobacco smoke condensate, and Dooley et al. (171) identified $^{29}SiMe_3$ as a potential false positive species unless sufficiently resolved from NDMA. For this reason most workers have carefully avoided the use of silicone greases or antifoam agents when determining NAs by MS. Stephany et al. (100) used retention times on 2 open tubular gas chromatographic columns and mass fragmentography of the molecular ion at a resolution of 4000 to quantitate volatile NAs in meat products. These workers concluded that this technique was more reliable than other lower resolution mass fragmentographic methods.

The maximum sensitivities reported for MS confirmation of NAs in food range over 4 orders of magnitude. Gough and Webb (172) monitored the molecular ion of NDMA at resolutions of 7000 and 12 000 and reported limits of 2 and 5 $\mu g/kg$, respectively. Others (100) have reported a detection limit of 0.1–0.2 $\mu g/kg$ at a resolution of 4000 but did not present data supporting this sensitivity. An extreme detection limit of 1 ng/kg has been reported (173). A combination of high resolution gas chromatography and peak matching MS at a resolution of 10 000 was used to quantitate volatile NAs in several environmental extracts including foods. Quantitative results were compared with GC-TEA data and it was concluded that the GC-MS method was more sensitive. The GC conditions were not equivalent for both instruments and may have favored MS.

Low resolution MS is generally somewhat less sensitive than high resolution. Volatile NAs have been confirmed at 10 $\mu g/kg$ by low resolution MS (65) and this 10 $\mu g/kg$ lower limit has been the basis for governmental regulatory action (*Fed. Regist.* (1978) 43, 20992–20995). A method for the confirmation of volatile NAs in foods at levels of 1–10 $\mu g/kg$ has recently been published (96).

Low resolution MS, high resolution MS, and GC-TEA methods of quantitating volatile NAs have been compared (174). Thirty-two samples of meats, vegetables, other foods, and urine were quantitatively compared for NDMA and NPYR content by low resolution mass fragmentography, high resolution peak matching, high resolution precise ion monitoring, and TEA analysis. All MS techniques had a detection limit of 1 $\mu g/kg$; the TEA limit was 20–40 ng/kg. The authors concluded that TEA and high resolution peak matching MS gave consistent quantitative results and that low resolution MS gave enhanced or suppressed results in 7/32 NDMA and 13/32 NPYR assays. Precise ion monitoring gave similar erroneous results. The authors point out, however, that the GC systems were not comparable, and increased efficiency may improve the results of the low resolution and precise ion monitoring MS methods.

Non-MS methods developed to support positive NA findings when TEA responses are too low for MS confirmation or when MS is unavailable have been published. Doerr and Fiddler (175) suggested that suspected NAs be ex-

posed to UV irradiation after initial TEA analysis and then re-analyzed. *N*-Nitroso compounds should be photolytically cleaved and disappear from the chromatogram when re-analyzed by TEA. A scheme has been proposed to differentiate *N*-nitroso compounds from several other TEA positive non-*N*-nitroso compounds, using both irradiation and wet chemical methods (176).

Artifacts

The possibility that analytical procedures can themselves produce erroneous results has not been sufficiently investigated for many methods. These analytical artifacts can be either false negative or false positive responses. False positive responses could be the result of NA formation during workup when the precursors are present in the sample, or a false positive response may result when a non-*N*-nitroso compound elicits a positive response. For example, dimethylnitramine is a non-*N*-nitroso compound that has a retention time on Carbowax very close to that of NDPA (136), is TEA positive, and produces a strong m/z 30 ion (177). It has also been demonstrated that the mineral oil distillation procedure can form NAs in the presence of the proper precursors if adequate nitrosation inhibitors are not added (96). In another report on the artifactual formation of NAs, Fan and Fine (178) found that NDMA was formed in the hot injection port of a gas chromatograph when an herbicide containing a high concentration of dimethylamine was injected.

Krull et al. (137) discussed both positive and negative artifacts and detailed procedures to ensure reliable analyses. Unfortunately, no data were presented verifying these recommendations. The procedures recommended by these authors to ensure that NAs are not formed during the analytical workup include use of a minimum number of analytical steps, addition of known NA precursors, addition of nitrosation inhibitors, chromatography by HPLC as well as GLC if possible, and selective denitrosation to ensure that the response is due to the presence of an *N*-nitroso compound. The authors also point out that false negative responses can result from exposure of the sample to excessive light, heat, or pH. Some of these suggestions were used to study the formation of artifacts in the analysis of rumen fluid (179). These workers found that low levels of NAs could be formed if sufficient quantities of precursors were added to the fluid. It was concluded that rapid direct extraction was the most convenient method to prevent NA formation.

Collaborative Studies

Few specific methods for determining NAs in foods have been subjected to rigorous collaborative testing. The International Agency for Research on Cancer has sponsored a series of round-robin type studies in which standards and methods of analysis were left to individual participating laboratories. Samples of spiked canned meat (180), canned spiced luncheon meat (181), and canned cheese and pesticide samples (182) have been distributed to laboratories worldwide. Analyses of the most recent data (182) revealed that methods using chemiluminescent detection of volatile NAs in cheese most accurately represented the spiking levels. Other detection systems tended to overestimate the NA content of the cheese. This is generally in agreement with the earlier studies. Studies of barley malt and malt-containing products are in progress.

A subcommittee organized by the American Society of Brewing Chemists (ASBC) has reported on a collaborative study of NDMA in beer (183). Distillation followed by extraction and direct extraction methods were compared. The methods were not significantly different. All collaborators used either the modified Hall detector or the TEA detector.

Conclusions

Without a clear understanding of the possible effects from ingestion of trace amounts of NAs, it is prudent to minimize human exposure to these compounds. A variety of methodologies exist for the isolation, cleanup, and concentration of volatile NAs. Chromatographic methods are available for separation, and highly selective detectors are capable of a high degree of specificity. Only mass spectrometry can be considered as unequivocal evidence for the presence of NAs, and advances have made confirmation possible at levels of less than 1 $\mu g/kg$. Current methods for nonvolatile NAs are inadequate and lack the systematic approach given to volatile NAs. In addition, rigorous collaborative testing of current procedures should be undertaken.

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DRUG RESIDUES IN ANIMAL TISSUES

Ultra Trace Determination of Furazolidone in Turkey Tissues by Liquid Partitioning and High Performance Liquid Chromatography

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A simple and sensitive procedure is presented for the determination of furazolidone in turkey tissues, using liquid partitioning followed by high performance liquid chromatography (HPLC). Fat, liver, kidney, skin, and muscle tissues are ground with methylene chloride in a Polytron homogenizer, followed by solvent removal, partitioning in hexane-0.01M acetic acid, and back-partitioning the 0.01M acetic acid with methylene chloride. The determination by HPLC used a reverse phase Ultrasphere-ODS 5 μm column. The method is sensitive to 0.5 ppb, with a standard deviation of 6.39% at the 2 ppb fortification level. Recovery from fortified tissues averaged 84% from samples fortified with 0.5-10 ppb furazolidone. An alternative cleanup procedure using a Sep-Pak C_{18} cartridge is also presented.

Furazolidone (*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone) is widely used as a growth promoter and as an antimicrobial feed additive to control diseases in poultry and other animals. The compound belongs to the large group of antimicrobial agents called nitrofurans, some of which are known or suspected carcinogens. Because of the continuing controversy over the carcinogenicity of furazolidone, a highly accurate, quantitative procedure is needed for its determination, which would be acceptable to regulatory agencies and capable of detecting 1 ppb or less in poultry tissues.

The procedures published for furazolidone in recent years have specified 2 primary analytical systems. A method using programmed multiple development-thin layer chromatography (1) was reported to be capable of measuring 2 ppb furazolidone in poultry, swine, and bovine tissues when the fluorodensitometer was coupled to a computing integrator. A collaborative study of this method was conducted with 5 laboratories participating. Recoveries from tissues spiked at 2 ppb were 2.2324 ng ± 20.2% (RSD). An average of 2 h per sample was required and the method could be completed in one day. Other reported procedures (2-4) used high performance liquid chromatography (HPLC) but only one procedure (2) reported an HPLC procedure for poultry tissue. This method was capable of detecting 2 ppb furazolidone in muscle and soft tissue, with no preparatory partitioning or column chromatography. However, frequent washing of the column to remove lipids and proteins was necessary when tissue samples were analyzed. Studies on skin or fat tissues were not reported and it is unlikely that they could be analyzed using this method.

The method reported here is capable of detecting as low as 0.5 ppb furazolidone in turkey tissues, including fat and skin. Approximately 1 h is required for each sample. The method is efficient, sensitive, and accurate, and can be used for routine monitoring of furazolidone in turkey tissues. The method has not been evaluated for its application to tissues of other animals.

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METHOD

Apparatus

(**a**) *Tissue homogenizer.*—Polytron®, Model PT10-35 equipped with PT20 ST generator (Brinkmann Instruments, Westbury, NY 11590).

(**b**) *Rotary flash evaporator.*—Model C5101 (California Lab Equipment Co., Oakland, CA), water bath temperature, 30°C.

(**c**) *Evaporator.*—N-Evap, Model 111 (Organomation Assoc., Inc., Shrewsbury, MA) operated with nitrogen at 30°C.

(**d**) *Liquid chromatograph.*—With Waters Associates (Milford, MA 01757) Model 6000A pump, Model U6K universal injector, Model 440 ultraviolet absorbance detector operated at 365 nm, 15 cm × 4.6 mm id 5 μm Ultrasphere-ODS column (Altex Scientific Inc., Berkeley, CA 94710). Column inlet filter 2 μm pore size Model 7302 (Rheodyne Inc., Cotati, CA 94928). Chromatograph was operated isocratically at flow rate of 1.5 mL/min, ambient temperature, 2500 psi, attenuation 0.005 AUFS.

(**e**) *Guard column.*—35 mm × 4.2 mm id, packed with 30/44 μm Vydac reverse phase.

(**f**) *Chromatographic column.*—Sep-Pak® C_{18}, part No. 51910 (Waters Associates).

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(g) *Syringe*.—Luer-Lok (Becton-Dickinson & Co., Rutherford, NJ 07070).

(h) *Needle*.—Luer-Lok, part No. 96120 (Waters Associates).

(i) *Glassware*.—All glassware was Kimax Ray-Sorb® colored glass, for use with light-sensitive materials (Kimble, Owens-Illinois, Inc., Toledo, OH 43666), or equivalent. All glassware was treated in following manner: Wash with laboratory detergent and rinse with water. Soak in H_2SO_4-sodium dichromate cleaning solution and rinse in water. Wash again with laboratory detergent, then thoroughly rinse with water to remove all traces of detergent. Dry glassware, then wet with 0.25% solution of dimethylchlorosilane (DMCS) in hexane, and let stand until dry.

(j) *Sedimentation tubes*.—6.5 mL McNaught and Mackay-Shevky-Stafford (Cat. No. 21068-002, VWR Scientific), or equivalent.

(k) *Filter paper*.—Whatman GF/F, 5.5 cm.

(l) *Filtering apparatus*.—Buchner funnel, 56 mm id (Cat. No. 30310-062, VWR Scientific); suction tube (Cat. No. 26388-022, VWR Scientific).

Reagents

(a) *Solvents*.—Methylene chloride (DCM) and hexane Nanograde® pesticide quality (Mallinckrodt, Inc., St. Louis, MO 63134); 'Photrex' methyl sulfoxide (J. T. Baker, Phillipsburg, NJ 08855); methanol, glass-distilled grade, suitable for HPLC (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442); water, distilled and stored in glass.

(b) *Mobile phase*.—Methanol-0.01M acetic acid, pH 5.0 (30 + 70). Prepare 0.01M acetic acid solution by adding 0.6 mL glacial acetic acid to 1 L water. Titrate solution with 4N NaOH to pH 5.0. Mix 300 mL methanol with 700 mL acetic acid solution and degas before use.

(c) *Standard solutions*.—Store in dark when not in use. (*1*) *Stock solution*.—0.5 mg/mL. Dissolve 100 mg furazolidone (Norwich-Eaton Pharmaceuticals, Norwich, NY 15815) in 10 mL methyl sulfoxide in 200 mL volumetric flask, and dilute to volume with methyl sulfoxide. (*2*) *Fortification standard for recovery studies*.—0.05 μg/mL. Dilute 10 μL stock solution to 100 mL with methylene chloride in 100 mL volumetric flask. (*3*) *HPLC standard working solutions*.—(*1*) 0.01 ng/μL. Dilute 10 μL stock solution to 500 mL with 0.01M acetic acid solution. (*2*) 0.005 ng/μL. Dilute 25 mL working solution 1 to 50 mL with 0.01M acetic acid solution. (*3*) 0.002 ng/μL. Dilute 20 mL working solution (*2*) to 50 mL with 0.01M acetic acid solution. (*4*) 0.001 ng/μL. Dilute 10 mL working solution (*1*) to 100 mL with 0.01M acetic acid solution.

Tissue Samples

All tissues were frozen in polypropylene jars immediately after slaughter, transported on dry ice, and stored at −27°C until use. *Caution*: Furazolidone is light sensitive. Samples must not be exposed to fluorescent or direct light. All samples were processed in subdued incandescent light throughout procedure and kept frozen when not in use.

Procedure

Extraction of furazolidone from fat, liver, kidney, muscle, and skin.—Fat from muscle, liver, and kidney is cut away at time of sampling, and sample is cut into small pieces (ca 1 cu. cm) before freezing. Transfer 2.0 g frozen tissue to 250 mL Erlenmeyer flask. Add 100 mL DCM and homogenize sample for 60 s (90 s for skin). Then filter sample under vacuum, using Whatman GF/F paper in Buchner funnel, into 250 mL round-bottom flask. Rinse generator blades and flask with 10 mL DCM, and filter through same paper into the 250 mL round-bottom flask. Clean generator blades between samples by extracting with 250 mL water, and 200 mL each of methanol, ethyl acetate, and DCM. Remove solvent in round-bottom flask under vacuum on rotary evaporator in 30°C water bath. Remove traces of solvent which remain with gentle stream of dry nitrogen.

Cleanup.—Dissolve residue containing furazolidone in round-bottom flask in 10 mL hexane, and transfer into 60 mL separatory funnel. Wash flask with one 10 mL and one 5 mL portion of hexane, and with 2 separate 12.5 mL portions of 0.01M acetic acid solution, combining washings in the 60 mL separatory funnel. Shake contents of separatory funnel and, after phases have separated, transfer lower aqueous phase containing furazolidone into 125 mL separatory funnel. (Reserve upper hexane phase.) It is important that phases are completely separated following extraction in separatory funnel. If emulsion or interphase forms, transfer contents into 50 mL round-bottom centrifuge tube, wash separatory funnel with two 2 mL washes of 0.01M acetic acid solution using a Dispo-pipette, and add washings to centrifuge tube. Centrifuge contents 5 min at 3200 rpm. If emulsions are not broken, gently swirl tube and repeat centrifugation. Wash original round-bottom flask 3 times with total of 25 mL 0.01M acetic acid solution, and add washings to 60 mL separatory funnel containing

the hexane phase. Re-extract hexane, let phases separate, and then combine with aqueous solution in the 125 mL separatory funnel. Discard hexane. Extract aqueous phase with 25 mL hexane, and transfer lower aqueous phase to another 125 mL separatory funnel containing 45 mL DCM. Extract aqueous phase, and transfer bottom phase containing DCM and furazolidone residue to 250 mL round-bottom flask. Repeat DCM extraction, making sure not to drain any aqueous phase with DCM. Flash-evaporate DCM under vacuum with rotary evaporator and 30°C water bath to ca 2 mL. Transfer DCM to 6.5 mL sedimentation tube with several 1 mL rinses of DCM, using Dispo-pipette. Evaporate to complete dryness with nitrogen, using N-Evap and 30°C water bath. After apparent dryness is reached, evaporate an additional 15 min to make sure there are no traces of DCM. Dilute sample with 1 mL 30% methyl sulfoxide–0.01M acetic acid solution, and stir vigorously.

Further cleanup of turkey tissues is generally not needed; however, use following procedure if cleaner samples are desired:

Following evaporation of DCM in 250 mL round-bottom flask to ca 2 mL, transfer 250 mL round-bottom flask to nitrogen N-Evap and continue evaporation until all traces of organic solvent are gone. Dissolve residue in 5 mL acetic acid solution. Prepare Sep-Pak by prewashing with 5 mL methanol, using Luer-Lok syringe, followed by 5 mL acetic acid solution; discard washings. Transfer sample to prewashed Sep-Pak C_{18} cartridge, followed by 1.5 mL acetic acid solution, and discard post-wash. Elute furazolidone from Sep-Pak into 60 mL separatory funnel, using 4 mL of 40% methanol–acetic acid solution. Add 7 mL acetic acid solution and 20 mL DCM to separatory funnel, shake vigorously 1 min, and let phases separate. Drain lower DCM phase into 50 mL round-bottom flask through Whatman GF/F filter paper, taking care that no aqueous phase is removed from funnel. Extract with an additional 20 mL DCM and combine in 50 mL round-bottom flask. Flash-evaporate DCM to ca 2 mL. Transfer DCM to 6.5 mL sedimentation tube with several 1 mL rinses of DCM, using disposable Pasteur-type pipet. Evaporate to complete dryness with nitrogen using N-Evap and 30°C water bath.

High performance liquid chromatography.—Prepare standard curve for furazolidone by plotting peak height vs concentration from 500 μL injections, using 1.0 mL syringe, of each of HPLC standard working solutions ranging from 0.5 to 5 ng. Inject 500 μL sample solution (dilute with 30% methyl sulfoxide–acetic acid solution for more concentrated solutions) and observe relative retention time with working standard. Calculate concentration of furazolidone in samples by reference to standard curve. Multiply by appropriate dilution factor.

Results and Discussion

Only recently has there been an HPLC procedure (2) capable of detecting furazolidone residues in animal tissues. HPLC procedures are available for animal feeds (3, 4) but these procedures are not adaptable to animal tissues primarily due to simplicity in sample handling and sensitivity required. The HPLC procedure presented here is designed for routine analysis and is capable of detecting furazolidone residues as low as 0.5 ppb in muscle, liver, kidney, fat, and skin, provided the HPLC detector is sensitive and stable at these levels. A 0.5 ng injection must give at least 4 times the noise level to achieve this sensitivity. Figure 1A is a chromatogram of 0.5 ng injection of furazolidone in 500 μL. All injections are made in the same quantity of injecting solvent, i.e., 500 μL, to obtain peak uniformity and duplication between samples. There is one unknown peak that occasionally appears near the furazolidone peak, and the intensity of this artifact may vary according to the quantity of solvent injected. At this time, we believe that it is due to the silanization treatment of the glassware. Figure 1B–D shows chromatograms of skin, liver, and fat tissue fortified with 0.5 ppb furazolidone. Figure 2B–D shows chromatograms of tissues fortified with 2.0 ppb furazolidone. Figure 2A is a chromatogram of a 2.0 ng injection of furazolidone. Fat tissues showed the highest degree of interferences.

It is very important to process the samples as rapidly as possible. After the samples are extracted, they should be analyzed within the same work day; otherwise low recoveries may result. Once the method becomes familiar, 2 analysts should be able to analyze approximately 16 samples, plus a recovery sample and a 4-point standard curve in an 8 h working day. When injecting large quantities of sample it is particularly important to frequently replace the guard column of the HPLC system or repack the top few millimeters of the HPLC column. If a guard column is not used, the top portion of the HPLC column should be repacked daily. Since this requires only a few minutes, the effort spent is well recommended when conducting routine analyses.

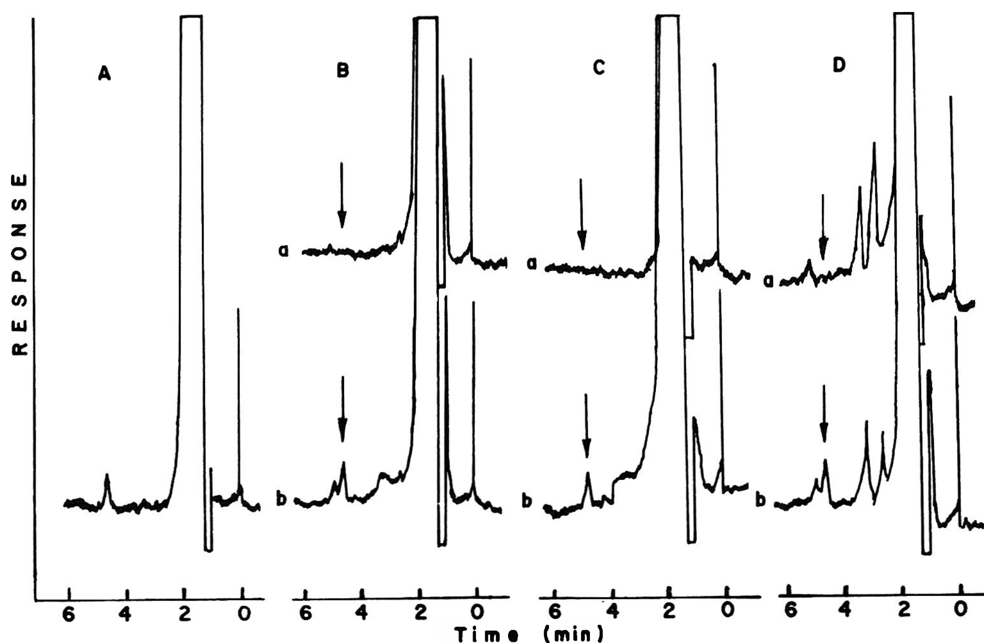

Figure 1. Liquid chromatograms of typical samples. A, 0.5 ng furazolidone standard. B, skin tissue: a, equivalent to 1 g injection of untreated tissue; b, tissue fortified at 0.5 ppb. C, liver tissue: a, equivalent to 1 g injection of untreated tissue; b, tissue fortified at 0.5 ppb. D, fat tissue: a, equivalent to 1 g injection of untreated tissue; b, tissue fortified at 0.5 ppb.

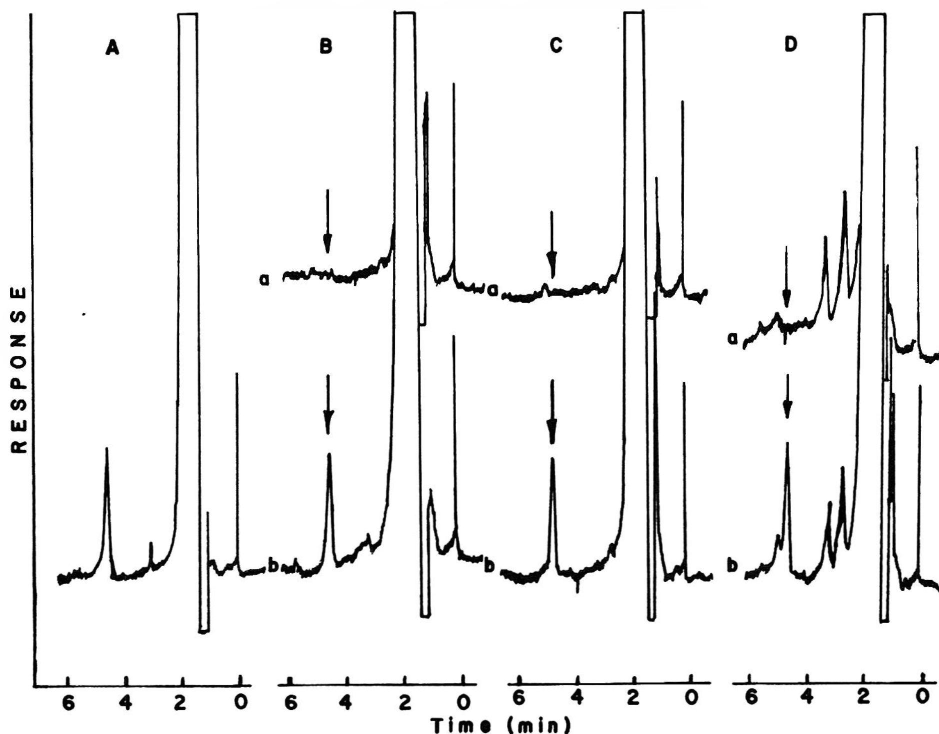

Figure 2. Liquid chromatograms of typical samples. A, 2.0 ng furazolidone standard. B, skin tissue: a, equivalent to 1 g injection of untreated tissue; b, tissue fortified at 2.0 ppb. C, liver tissue: a, equivalent to 1 g injection of untreated tissue; b, tissue fortified at 2.0 ppb. D, fat tissue: a, equivalent to 1 g injection of untreated tissue; b, tissue fortified at 2.0 ppb.

Table 1. Recovery, av. (%) ± SD, of furazolidone from fortified turkey tissue

Tissue	Fortification level, ppb 10	2.0	0.5	Tissue overall av. ± SD (n = 9)
White meat	89.9[a] ± 0.90	84.8 ± 3.92	86.6 ± 3.98	87.11 ± 3.60
Dark meat	82.5 ± 3.64	87.4 ± 5.36	83.1 ± 2.25	84.33 ± 4.14
Liver	84.8 ± 6.75	78.3 ± 7.95	75.5 ± 11.44	79.53 ± 8.78
Kidney	79.6 ± 5.58	79.9 ± 4.07	89.1 ± 10.85	82.88 ± 7.96
Fat	89.6 ± 7.09	91.9 ± 3.46	81.0 ± 27.30	87.50 ± 15.06
Skin	78.5 ± 5.25	88.8 ± 1.85	88.1 ± 7.45	85.16 ± 6.81
Level overall av. ±SD (n = 18)	84.16 ± 6.39	85.19 ± 6.39	83.90 ± 12.22	

[a] Average of 3 determinations.

The HPLC operating conditions were similar to those reported previously (2) with the only significant modification being the use of 30% methanol in the mobile phase rather than the 20% recommended originally, and a 1.5 mL/min flow rate rather than 2.0 mL/min.

Recovery studies performed in triplicate from each of the 6 tissues fortified with 0.5, 2.0, and 10 ppb furazolidone are shown in Table 1. All calculations were based on peak height found in the samples with the correct relative retention and comparison with peak height of the working standard. As might be expected, standard deviations were generally higher for lower fortification levels. The average recovery for all samples (n = 54) was 84.41% with a standard deviation of 8.63%. Quantitation is more difficult at the 0.5 ppb level because a 0.5 ng response with the detector set at 0.005 AUFS is equivalent to 8 mm peak height with a noise background of 2 mm. Therefore, unless an analysis at this level is calculated on an electronic integrator, samples should be run in duplicate or triplicate. Levels as low as 0.5 ppb may be readily determined, although the standard deviation may be greater than 15%. If all precautions are followed, endogenous materials should not interfere when conducting routine analyses because these materials do not absorb at 365 nm. However, endogenous materials can alter the chromatographic response due to a change in flow or pressure and peak dimension. Normally, Sep-Pak treatment is not necessary or recommended because this increases the amount of time required for analysis and would require additional sample handling which in turn could account for a minor but significant loss of furazolidone. However, if samples are not sufficiently clean before HPLC quantitation, Sep-Pak treatment could be used and would result in a cleaner injection and a longer-lasting guard and/or HPLC column.

The procedure described is a sensitive, rapid, convenient, and accurate method for the determination of furazolidone in turkey tissues, which may be applicable to other animal tissues following suitable studies.

Acknowledgments

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MYCOTOXINS

Thin Layer Chromatographic Determination of Aflatoxin in Corn Dust

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Methods adopted by the AOAC and the American Association of Cereal Chemists for determining aflatoxin in corn were modified, and techniques were developed for application to samples of <1 to 10 g instead of the specified 50 g samples. Analysis included chloroform extraction of dust samples or dust collected from glass fiber filters, purification of extracts on a silica gel column of appropriate size, and measurement of aflatoxin by either 1- or 2-dimensional thin layer chromatography (TLC). The solvent for 1-dimensional TLC was chloroform-acetone-water (91 + 9 + 1). Solvents for 2-dimensional TLC were, first direction, ether-methanol-water (95 + 4 + 1, lined tank) and second direction, chloroform-acetone-water (91 + 9 + 1, unlined tank), or first direction, chloroform-acetone-water (91 + 9 + 1, unlined tank) and second direction, toluene-ethyl acetate-formic acid (60 + 30 + 10, unlined tank). When samples weighed ≤0.1 g, the entire concentrated extract was applied to the TLC plate. About 0.5-1.0 ng aflatoxin B_1 could be detected on the plate, making the limit of detection about 9 ng/g for 0.1 g samples.

Two reports have discussed possible effects on humans from inhaling dusts that contained aflatoxin, but the degree of exposure was not determined nor was a cause-effect relationship established. One report was a study of workers' health in a peanut processing plant in the Netherlands. Exposures to aflatoxin from contaminated dust over a period of 13 years in different parts of the plant were estimated. The exposed group had a rate of multiple cancers and liver cancer more than 3 times that in the matched control group. The authors concluded that there was a strong indication of carcinogenic factors in the dust, but the number of workers exposed was too small to provide proof (1). The other report speculated that possible exposure of 2 scientists to aflatoxin-containing dust during preparatory thin layer chromatography (TLC) in an unventilated room could have been responsible for the carcinomas of the colon that they developed (2).

There is currently no basis for determining whether agricultural workers exposed to airborne dust during the handling of aflatoxin-containing corn are at risk. Aflatoxin contamination of corn is a recurring problem in certain regions of the United States; the severity varies from year to year (3). In the summer of 1977, conditions in the Southeast were favorable for aflatoxin formation in corn, and in one study, 16% of the samples collected had levels above 1000 ng/g (4). The incidence and levels of aflatoxin were not as high in 1978 corn as in 1977 corn (5). Because aflatoxin forms in the field as well as in storage (6), workers could be exposed to dust-borne toxin at the point of harvest and through any other operation up to the time the corn is destroyed or detoxified by ammoniation (7). There was, therefore, a need to develop analytical methods to determine aflatoxin levels in dust samples.

This paper reports on modifications of methods adopted by the AOAC and the American Association of Cereal Chemists (AACC) for aflatoxin in corn (8, 9) and the development of procedures to determine toxin in airborne corn dust samples.

Experimental

Preparation of Ground Corn Samples to Test Analytical Procedures

Three samples of ground corn containing different levels of aflatoxin were prepared by combining available naturally contaminated samples to obtain approximately the levels desired. Each of the 3 samples was partitioned on a U.S. standard No. 20 sieve. The material that did not pass through the sieve (11%) was re-

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ground in a 6 in. Raymond hammer mill equipped with a screen with 1/8 in. perforations, and resieved. Reground corn that did pass through the No. 20 sieve was combined with original sample material that passed through the sieve, and blended in a Hobart planetary mixer. Material that did not pass through the No. 20 sieve was discarded.

Collection of Dust Samples

Samples of airborne dust were collected with a high volume, total air sampler fitted with 8 × 10 in. Type A glass fiber filters, and a high volume, 4-stage Anderson sampler (Model 65-000) with perforated glass fiber type "AE" filters to sequentially retain particles. The effective cutoff diameters for the stages are 7, 3.3, 2.0, and 1.1 μm; a final backup filter collects particles in the submicron range. Samplers were positioned to collect airborne dust generated during handling of contaminated corn. The glass fiber filters were initially equilibrated in a constant temperature and humidity room and weighed before installation in the air samplers. After sample collection, the control filter and sample filters containing dusts were equilibrated in the same room 24 h before weighing to correct for moisture adsorbed on the filter. Dust samples ranged in weight from 7 mg to 11.8 g.

Extraction

Collected dust samples and corn samples prepared as described above were extracted 30 min on a wrist-action shaker. Glass fiber filters containing dust samples were extracted 3–4 min in a Waring blender by a slight modification of the AOAC–AACC method for corn (8, 9). The action of the blender shredded the glass fiber filters, ensuring contact of the dust with the solvent. Dust samples or glass fiber filter papers containing dust were extracted with 150 mL $CHCl_3$, 15 mL water, and 15 g Celite. The entire filtrate was collected from dust or glass fiber filter papers containing dust, and extracted dust and residues were thoroughly washed with $CHCl_3$. The combined extracts and washes from large samples (>1 g) were concentrated under vacuum to ca 35 mL. The concentrate was transferred quantitatively to a graduated cylinder, and diluted to 50 mL with $CHCl_3$ for transfer to the specified silica gel column. The graduated cylinder was rinsed with the hexane wash used on the column. Extracts and washes from smaller samples (<1 g) were concentrated to 2–3 mL, transferred quantitatively (Pasteur pipet) to a vial with $CHCl_3$, and dried under nitrogen for chromatography on a smaller column.

Column Chromatography

The silica gel (E. Merck, Darmstadt) silica gel 60, 0.063–0.2 mm) column (22 × 300 mm) and solvents (washes 150 mL hexane and 150 mL anhydrous ether, and elution solvent 150 mL methanol-$CHCl_3$ (3 + 97)) for samples >1 g were those specified in the AOAC-AACC method (8, 9). For samples <1 g, a 6 mm id × 21 cm column was used, with the top flared to a funnel shape to facilitate solvent addition and with a stockcock at the bottom to control solvent flow. The column was packed as a $CHCl_3$ slurry. The following were added to the column in order: glass wool plug, 1 cm anhydrous granular Na_2SO_4, 5.5–6 cm silica gel (8, 9), and 1–2 cm anhydrous granular Na_2SO_4. Residues from extraction of samples weighing ≤1 g were dissolved in 2 mL $CHCl_3$ and transferred with a Pasteur pipet to the column; vials were rinsed 3 times with 1–2 mL $CHCl_3$, and rinses were placed on the column. The column was washed with 5 mL hexane followed by 5 mL ether and then eluted with 10 mL ethanol–$CHCl_3$ (5 + 95). The entire eluates were dried under nitrogen and retained for TLC.

Thin Layer Chromatography (8, 9)

TLC plates (20 × 20 cm) were coated with 0.25 mm Adsorbosil-1 silica gel (Applied Science). For 1-dimensional preliminary plates and quantitative TLC, plates were developed with $CHCl_3$-acetone-water (91 + 9 + 1) in an unlined tank. The reference standard solution was 0.5 μg aflatoxin B_1/mL, 0.1 μg B_2/mL, 0.5 μg G_1/mL, and 0.1 μg G_2/mL in acetonitrile-benzene (98 + 2). Residues from smaller samples (0.1 to ≤1 g) from extraction and column chromatography were dissolved in 0.100–0.250 mL acetonitrile–benzene (98 + 2). If observations from the preliminary TLC plate were negative or indicated very little aflatoxin B_1 in an extract, the entire remaining extract, including washes of the vial in which it was contained, was applied to the TLC plate for 2-dimensional development.

For 2-dimensional TLC, solutions of unknowns and the diluted reference standard solution were applied to TLC plates and developed according to a spotting and development pattern similar to that shown in *Official Methods of Analysis* (10). Optional development solvents for 2-dimensional TLC were, first direction, $CHCl_3$–acetone–water (91 + 9 + 1, unlined tank) and second direction, toluene–ethyl acetate–formic acid (60 + 30 + 10, unlined tank). Afla-

Table 1. Comparison of analyses [a] of 3 corn lots for aflatoxins using 50, 10, and 1 g subsamples

		Lot 1			Lot 2			Lot 3		
Subsample size, g [b]	Aflatoxin	Mean, ng/g	Std dev.	Coeff. of var., %	Mean, ng/g	Std dev.	Coeff. of var., %	Mean, ng/g	Std dev.	Coeff. of var., %
50	B_1	2380	497	20.7	89.2	3.59	4.42	33.7	1.71	5.06
	B_2	97	24	24.5	7.5	0.58	7.70	3.75	0.96	25.6
	G_1	145	32	21.9						
	G_2	17 [c]								
10 [d]	B_1	2406	126	5.3	99.5	11.09	11.14	32.7	3.30	10.08
	B_2	102	13	12.7	7.25	2.50	34.48	3.75	0.50	13.31
	G_1	142	41	29.2						
	G_2	21	18	88.5						
1 [e]	B_1	2000	117	5.6	104	20.12	19.30	39.2	4.72	12.02
	B_2	103	20	19.1	15.2	6.60	43.42	7.75	0.96	12.34
	G_1	166	50	28.1						
	G_2	31	28	90.6						

[a] Analyzed by CB method approved for corn (8, 9).
[b] Four samples of each weight from all lots were analyzed.
[c] Only one value was obtained for G_2.
[d] No aliquot was taken of extract. Extract of entire sample was placed on standard CB column.
[e] Silica gel chromatography was carried out on 6 mm id columns

toxins were measured densitometrically on TLC plates unless extracts applied to the plate contained 0.5–2.0 ng aflatoxin B_1; then amounts were estimated by visual comparisons with standards.

Recoveries of Standard Aflatoxin B_1 and Aflatoxin B_1 in Naturally Contaminated Corn from Glass Filters

Standard aflatoxin B_1 (1.5 and 3.0 μg in 1 and 2 mL, respectively, of acetonitrile-benzene (2 + 98)) was incorporated into the 8 × 10 in. glass filters used in the high volume total sampler. Aflatoxin B_1 was extracted by blending 3–4 min with 150 mL $CHCl_3$, 15 mL water, and 15 g Celite. Extracts were dried and retained for TLC. Four portions (0.1, 0.25, 0.5, and 1.0 g) of naturally contaminated corn (2100 ng B_1/g) were weighed and spread onto 4 glass filters. Filters were extracted in a blender as above; extracts were purified for TLC on a small silica gel column (6 mm id) designed for ≤1 g samples.

Confirmatory Tests

Identity of either aflatoxin B_1 or G_1 was confirmed by the formation of water adducts with trifluoroacetic acid (TFA) (11, 12). On 2-dimensional plates to which the entire extract had been applied, TFA was applied to the developed unknown spot, and to a spot of standard aflatoxin solution applied next to the unknown spot. The plate was then developed in a third direction.

Identity of aflatoxins in one sample was confirmed by high pressure liquid chromatography (HPLC) of the toxins, using a Waters Model ALC-202 chromatograph equipped with 2 M-6000 pumps, a reverse phase column, and a fluorescence detector. Operating conditions were: column, Waters μBondapak C_{18}; flow rate, 1.5 mL/min; ambient temperature; solvent, water-methanol-acetonitrile (55 + 25 + 20); primary filter, 360 nm; secondary filter, 440 nm.

Results and Discussion

Results of analyzing 50, 10, and 1 g subsamples of the 3 samples of naturally contaminated ground corn are summarized in Table 1. The mean aflatoxin B_1 levels in the 3 samples were 2380, 89, and 34 ng/g. Extracts of 1 g portions were cleaned up on the smaller silica gel columns (6 mm id) to avoid losses encountered when the column specified in the AOAC-AACC method was used. The pooled repeatabilities ($n = 3$) for the determination of aflatoxin B_1 were 10, 9, and 12% for the 50, 10, and 1 g sample sizes, respectively, which were considerably better than the 30% anticipated from the results of collaborative studies (13). More recently, a study on the variability associated with testing corn for aflatoxin revealed that the coefficient of variation for one analysis of a subsample from finely ground, well blended meal is 26% (14).

Recoveries of 1.5 and 3.0 μg standard aflatoxin B_1 from glass filters (8 × 10 in.) were 98 and 67%, respectively. Recoveries of B_1 from naturally contaminated corn (2400 ng/g) on glass filters were 107% for the 0.1 g portion; 62%, 0.25 g; 75%, 0.5 g; and 87%, 1.0 g. Recoveries were variable,

probably because of the difficulty in obtaining a homogeneous naturally contaminated corn sample for subsampling in quantities of 0.25–1.0 g. Recoveries >98% have been reported from dry films of 25 μg aflatoxin B_1 in glass vials (15).

On the 2-dimensional TLC plate, 0.5–1.0 ng aflatoxin B_1 could be detected. When 90% of the weight of original sample was applied to the TLC plate for 2-dimensional development, the limit of detection was approximately 9.5 ng aflatoxin B_1/g for a 0.1 g sample. The limit of detection was determined by applying known amounts of B_1 in a corn extract to a TLC plate and developing the plate. The detection limit can vary depending on interferences in a given sample. The water adduct of the separated spot of aflatoxin B_1 could be prepared by treatment with TFA on the same plate, and development in a third direction to confirm its identity (11, 12). All results were confirmed by the TFA test on 1- or 2-dimensional TLC plates with one exception: It was impossible to confirm aflatoxin G_1 in one sample because of the presence of extract impurities with low R_f values. G_1 identity was confirmed by HPLC. These methods were satisfactorily applied to dust samples scraped from farm equipment and surfaces of an elevator and to airborne corn dust collected with a high volume total sampler and with Anderson 4-stage samplers. Analytical results are reported in another paper (16).

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Rapid Screening Method for Aflatoxin M_1 in Milk

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A rapid screening method for detecting aflatoxin M_1 in milk has been developed, based on minicolumn chromatography and requiring 8-10 min for each test. The minicolumn is packed with dry Florisil (100-200 mesh) on the bottom, anhydrous Na_2SO_4 as the next layer, topped with neutral alumina (70-200 mesh) to which 8% water (wet basis) has been added. A blue fluorescent band at the Florisil-Na_2SO_4 interface indicates the presence of aflatoxin M_1. The limit of detection is estimated to be about 0.2 μg/kg. Because several items are disposable, both the time to maintain glassware and the cost per determination are reduced.

Recent studies have shown that milk and other dairy products can contain aflatoxin M_1 when cows consume feed containing aflatoxin B_1 (1). A practical analytical method for the determination of aflatoxin M_1 in dairy products has already been evaluated by collaborative study and is an AOAC official method (2). This method, however, does not lend itself to low cost, rapid screening where laboratory facilities are limited or nonexistent, as is the case at many dairies.

This paper describes a rapid screening method for aflatoxin M_1 in whole milk, which has a limit of detection of about 0.2 μg/kg. It is based on the minicolumn technique and can be used at field locations. The minicolumn technique was first introduced by Holaday (3) in 1968. Since this original development, several improvements in the minicolumn have been made. Romer et al. give an excellent review of the minicolumn development (4).

Disposable items were used whenever possible to reduce the time spent in washing glassware, to keep cost per determination low, and to decrease the possibility of aflatoxin M_1 carry-over to subsequent samples.

METHOD

Apparatus

(a) *Viewing cabinet.*—Chromato-Vue, Model C-6, or equivalent, fitted with longwave UV lamps.

(b) *Vacuum source.*—Water aspirator or small vacuum pump.

(c) *Separatory funnel.*—125 mL, cylindrical, graduated.

(d) *Graduated cylinder.*—100 mL capacity, mixing type with polyethylene stopper.

(e) *Erlenmeyer flask.*—Filtering type, 250 mL capacity, graduated on the side, fitted with size 8 rubber stopper with center hole (for inserting minicolumn into flask), and rubber vacuum hose for connecting flask to vacuum source.

(f) *Funnel.*—Buchner, to accommodate 9 cm filter paper, and equipped with rubber adapter.

(g) *Culture tubes.*—125 × 16 mm, disposable, and test tube rack.

(h) *Pipets.*—5 mL, disposable.

(i) *Pipet fillers.*—rubber.

(j) *Filter discs.*—9 cm, glass fiber.

(k) *Minicolumns.*—180 mm × 5.5 mm id glass tubing packed with 19-21 mm 100-200 mesh Florisil on bottom, 19-21 mm Na_2SO_4 (anhydrous powder) as next layer, and on top, 19-21 mm 70-200 mesh neutral alumina with 8% water (wet basis) added (see Figure 1). Water was added to activity level I alumina. Alumina was thoroughly mixed and lumps were broken up while it was in a closed container to prevent moisture loss caused by heating after addition of water. Adsorbent was equilibrated 24 h in an airtight container before use in column preparation. Florisil-Na_2SO_4 interface should be as even as possible. Adsorbents were held in place with Schleicher and Schuell No. 289 paper pulp (available from most chemical suppliers). Store minicolumns in an airtight container but not in a desiccator. We have kept minicolumns for up to 3 months without any noticeable deterioration.

Reagents

(a) *Salt solution.*—Dissolve 700 g NaCl and 700 g $ZnSO_4.7H_2O$ in 1.5 L water. These amounts are in slight excess of saturation point of salts in water.

(b) *Filter aid mixture.*—Diatomaceous earth–adsorptive magnesia (1 + 1).

(c) *Toluene.*—ACS grade.

[1] Retired.
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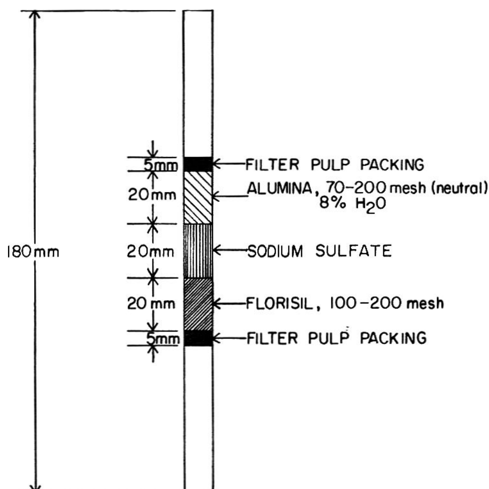

Figure 1. Preparation of minicolumn.

(d) *Toluene-methanol solution.*—Toluene-ACS grade methanol (80 + 20).

(e) *M_1 standard solution.*—M_1 standard available from L. Leistner, Federal Meat Institute, 865 Kulmbach, Germany (see reference 2 for additional sources for standards and standard solution preparation).

Procedure

Add 6-8 g filter aid mixture to 30 mL milk in graduated cylinder. If raw milk samples are to be tested, make sure that cream is thoroughly mixed with rest of milk before testing. (Although a few samples of reconstituted powdered milk were tested without difficulty, additional tests are needed to assure that the test performs satisfactorily on this product. Most tests were made on homogenized and raw milks.) Stopper cylinder and mix contents vigorously 2-3 s. Add 30 mL salt solution to mixture and again stopper cylinder and shake vigorously 2-3 s. Filter ca 45 mL of mixture on Buchner funnel. Add 40 mL of filtrate to separatory funnel together with 5 mL toluene. Mix contents by up-ending separatory funnel 3 or 4 times. Do not mix too vigorously. Let 2 layers separate. Drain lower layer and discard. Decant upper (toluene) layer into culture tube.

Insert minicolumn in 1-hole rubber stopper fitted into Erlenmeyer flask, with alumina end down (bottom-side-up). Add 2 mL toluene-methanol solution to minicolumn and pull through with aid of vacuum. Reverse column on vacuum (alumina end up) and add 3 mL of toluene extract and pull through. Then add 1 mL toluene-methanol and pull through. Remove minicolumn and place in viewing cabinet. Blue band at Florisil-Na_2SO_4 interface indicates presence of at least 0.2 μg M_1 aflatoxin/kg. Limit of detection was determined by comparing minicolumn results with thin layer (TLC) chromatographic results on several samples containing 0.2 μg M_1/kg. Some quantitation is possible by using small increments of the 3 mL toluene extract until a band becomes visible.

Results and Discussion

The minicolumn developed for aflatoxin M_1 is shown in Figure 1. Extensive testing has shown that this design works best for most applications. The back-flushing step is used to wash the adsorbents before adding the toluene extract to the minicolumn. A test can be performed in 10 min.

It is necessary to use 100-200 mesh Florisil to provide a discrete fluorescent band of aflatoxin M_1 at the Florisil-Na_2SO_4 interface. Lower mesh (60-100) Florisil is less retentive and can cause spreading of the aflatoxin M_1 band. $ZnSO_4.7H_2O$ is used to precipitate interfering materials from the extract, including the milk proteins. NaCl is needed to reduce the tendency of the extract to form an emulsion with toluene. Filter aid mixture speeds up filtration of the milk-salt mixture. Numerous tests have shown that no significant amount of aflatoxin M_1 is lost during the extract cleanup. Figure 2 shows 3 minicolumns with 0, 0.5, and 1.0 μg aflatoxin M_1/kg from left to right. Extracts of whole milk samples were used; the last 2 were naturally contaminated.

Table 1 shows results for some of the tests carried out on naturally contaminated samples

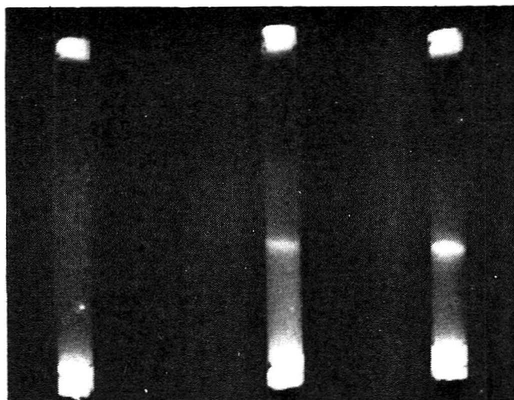

Figure 2. Minicolumns with (left to right) 0, 0.5, and 1.0 μg aflatoxin M_1/kg.

Table 1. Comparison of TLC and HPLC analyses with minicolumn analyses for aflatoxin M_1 (μg/kg) in naturally contaminated milk

Sample	TLC	Minicolumn	Sample	HPLC	Minicolumn
1	0.2	0.2–0.3	10	0.8	1.0–1.2
2	0.2	0.3–0.4	11	1.0	<0.2
3	0.2	0.3–0.4	12	0.2	0.2–0.3
4	0.3	0.4–0.5	13	0.8	0.5–0.6
5	0.3	0.4–0.5	14	0.5	0.2–0.3
6	0.3	0.3–0.4	15	1.1	0.7–1.0
7	0.5	0.5–0.6	16	0.8	<0.2
8	0.5	0.5–0.6	17	0.9	0.6–0.7
9	0.8	0.7–1.0	18	0.4	0.5–0.6

by TLC, high pressure liquid chromatographic (HPLC), and minicolumn methods. The minicolumn analysis does not agree with the HPLC analysis for Samples 11 and 16. This may be due to breakdown of the M_1 between the time the HPLC analysis was performed and that by minicolumn. Both of these samples were badly deteriorated.

Acknowledgment

The TLC analyses were performed by Donald L. Whitcomb of the Arizona Department of Health Services; HPLC analyses were performed by David Wilson of the Georgia Coastal Plains Experiment Station. The HPLC method used by Dr. Wilson was developed in his laboratory.

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Simultaneous Detection of Several *Fusarium* Mycotoxins in Cereals, Grains, and Foodstuffs

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A systematic method is described for the simultaneous determination of *Fusarium* mycotoxins (nivalenol, deoxynivalenol, fusarenon-x, diacetoxyscirpenol, neosolaniol, T-2 toxin, HT-2 toxin, butenolide, moniliformin, and zearalenone) in cereals, grains, and foodstuffs. Mycotoxins were extracted with aqueous methanol and purified by a 2-step chromatographic procedure using Amberlite XAD-4 and Florisil columns. The column eluates were concentrated and spotted on a thin layer chromatographic (TLC) plate which was then developed in $CHCL_3$-methanol (93 + 7) and toluene-acetone-methanol (5 + 3 + 2). Each mycotoxin was quantitated by gas chromatography (GC) and TLC densitometry. The minimum detectable concentrations (μg/kg) in various test materials were: nivalenol, deoxynivalenol, and fusarenon-x, 2.0; diacetoxyscirpenol, neosolaniol, T-2 toxin, and HT-2 toxin, 80; zearalenone, 10; butenolide, 30; and moniliformin, 50. Recoveries of the mycotoxins added to various cereal samples at 1.0-2.0 μg/g were greater than 71% and averaged 85%.

Mycological investigations (1-8) have shown that *Fusarium* fungi, which produce the mycotoxins nivalenol, deoxynivalenol, T-2 toxin, diacetoxyscirpenol, and zearalenone, commonly occur in cereals and feeds. Most methods for determining *Fusarium* mycotoxins are used to measure only 1 or 2 toxins (4-9). However, cereals, grains, and foodstuffs are often contaminated simultaneously with various fungal species, and certain fungi produce more than 2 or 3 mycotoxins.

Screening methods for multiple mycotoxins have been published (3, 7, 10, 11). None of these procedures, however, possess the sensitivity and utility required for our purposes.

This paper describes a systematic and simultaneous determination of 7 trichothecene mycotoxins plus butenolide, moniliformin, and zearalenone (Figure 1), which are all produced by *Fusarium* species. By this method, mycotoxins are detected by thin layer chromatography (TLC) and quantitated by gas-liquid chromatography (GLC) and TLC densitometry.

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METHOD

Apparatus

(a) *Gas chromatographs.*—(*1*) Shimazu Model GC-7A (Shimazu, Ltd, Kyoto, Japan) equipped with flame ionization detector. Operating conditions: 200 cm × 3.2 mm id glass column packed with 2% OV-17 on 80-100 mesh Gas-Chrom Q; nitrogen carrier gas 60 mL/min; temperatures (°C)—injector and detector 280, column 250. (2) Shimazu Model GC-7A equipped with ^{63}Ni electron capture detector. Operating conditions: 200 cm × 3.2 mm id glass column packed with 2% OV-17 on 80-100 mesh Gas-Chrom Q; nitrogen carrier gas 50 mL/min; temperatures (°C)—injector and detector 280, column 240.

(b) *TLC densitometer.*—Shimadzu Model CS-910 dual wavelength TLC scanner (Shimadzu Ltd, Kyoto, Japan). Operating conditions: slit width 0.3 × 8 mm; linear scanning mode; scan speed 10 mm/min; wavelength (nm) reference 700, sample 430, 480.

(c) *Data processor.*—Shimadzu Model C-R1A.

(d) *Viewer.*—Manaslu light (Manaslu Ltd, Tokyo, Japan) equipped with shortwave (254 nm) and longwave (365 nm) lights.

(e) *TLC plates.*—(*1*) 20 × 20 cm (E. Merck, Art. 5721) precoated with 0.25 mm silica gel 60 without fluorescent indicator. (2) 20 × 20 cm (E. Merck, Art. 5715) precoated with 0.25 mm silica gel 60 with fluorescent indicator.

(f) *Chromatographic column.*—Glass, 20 cm × 1.5 cm id.

Reagents

(a) *Amberlite XAD-4 for column chromatography.*—Amberlite XAD-4 (Rohm and Haas Co., Philadelphia, PA 19105). Condition resin as follows: Wash with water and 1N NaOH, and then wash with water until pH is lowered to 7.0. Discard water. Add methanol and let stand 24 h with occasional shaking. Let methanol per-

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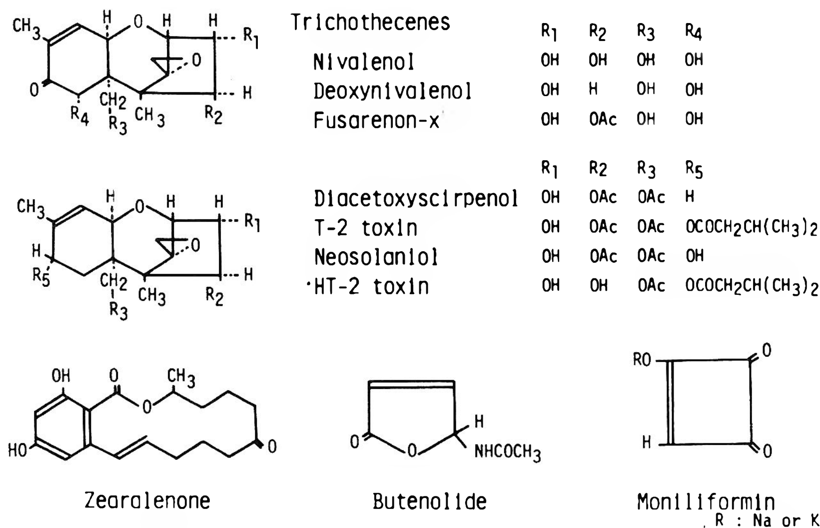

Figure 1. Mycotoxins produced by *Fusarium* species.

colate, and then wash with methanol, and discard methanol. Next, wash with water. Store resin in water.

(**b**) *Florisil for column chromatography.*—Florisil (Iwaikagaku Ltd, Tokyo, Japan), 100–200 mesh. Activate by drying 1.5 h at 105°C. Store in desiccator.

(**c**) *Aluminum chloride solution.*—Dissolve 20 g reagent grade $AlCl_3 \cdot 6H_2O$ in 100 mL 50% ethanol.

(**d**) *2,4-Dinitrophenylhydrazine solution.*—Dissolve 0.32 g reagent grade 2,4-dinitrophenylhydrazine in 100 mL 2N HCl.

(**e**) *Derivatizing reagent.*—*N*-Trimethylsilylimidazole–trimethylchlorosilane–ethyl acetate (1 + 0.2 + 9). Prepare fresh daily.

(**f**) *Sulfuric acid solution.*—H_2SO_4–water (1 + 4).

(**g**) *Mycotoxin reference standard solutions.*—Zearalenone, T-2 toxin, and diacetoxyscirpenol were obtained from Makor Chemicals Ltd, Jerusalem, Israel. Fusarenon-x, nivalenol, deoxynivalenol, neosolaniol, butenolide, and HT-2 toxin were given by Y. Ueno, T. Yoshizawa, and R. Amano. Moniliformin was prepared in our laboratory. Store all standard solutions in refrigerator in stoppered containers, but use at room temperature.

(*1*) *Nivalenol, deoxynivalenol, and fusarenon-x.*—Separate solutions each containing 50 μg mycotoxin/mL $CHCl_3$–methanol (9 + 1). (*2*) *T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol, butenolide, and zearalenone.*—Separate solutions each containing 100 μg mycotoxin/mL $CHCl_3$. (*3*) *Moniliformin.*—100 μg/mL methanol. (*4*) *Mycotoxin mixture I.*—10 μg each nivalenol, deoxynivalenol, fusarenon-x, and zearalenone, and 20 μg each T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol, butenolide, and moniliformin/mL $CHCl_3$–methanol (1 + 1). (*5*) *Mycotoxin mixture II.*—10 μg each nivalenol, deoxynivalenol, and fusarenon-x, and 20 μg each T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol, and zearalenone/mL $CHCl_3$–methanol (1 + 1). (*6*) *Mycotoxin mixture III.*—50 μg each butenolide and moniliformin/mL methanol.

(**h**) *Developing solvents.*—Use certified grade solvents, and distill before use. (*1*) $CHCl_3$–methanol (93 + 7); (*2*) toluene–acetone–methanol (5 + 3 + 2); (*3*) toluene–acetone (97 + 3); (*4*) toluene–acetone–methanol (5 + 1 + 1).

Extraction

Weigh 100 g sample, finely ground in mill, into 500 mL glass-stopper flask, add 200 mL methanol-water (95 + 5), stopper, and shake 30 min using wrist-action shaker set at fast rate. Decant through filter paper into 200 mL Erlenmeyer flask. Transfer 100 mL filtrate to 300 mL round-bottom flask, and evaporate filtrate to ca 10 mL under reduced pressure on steam bath.

Column Chromatography

Prepare Amberlite XAD-4 column as follows: Insert glass wool in bottom of chromatographic column (f) and add slurry of Amberlite XAD-4 in water so that height of settled column is ca 10 cm. Add sample to column. Rinse flask with additional small portions of methanol totaling 5 mL, add 30 mL water, transfer to column, and drain

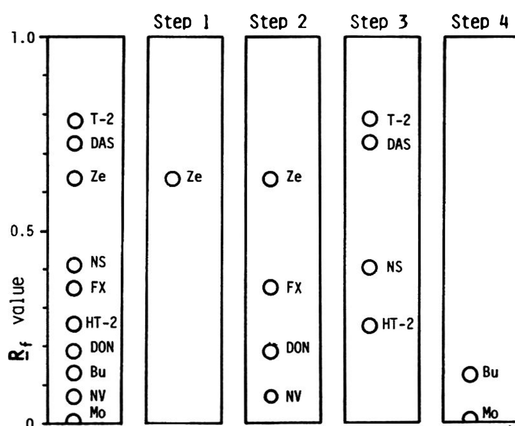

Figure 2. Thin layer chromatogram of mycotoxins: NV, nivalenol; DON, deoxynivalenol; FX, fusarenon-x; DAS, diacetoxyscirpenol; NS, neosolaniol; HT-2, HT-2 toxin; T-2, T-2 toxin; Ze, zearalenone; Bu, butenolide; Mo, moniliformin. Developing solvent: $CHCl_3$-methanol (93 + 7). Treatment: step 1, observe under UV light; step 2, spray with 20% $AlCl_3$; step 3, spray with 20% H_2SO_4; step 4, spray with 0.32% 2,4-dinitrophenylhydrazine.

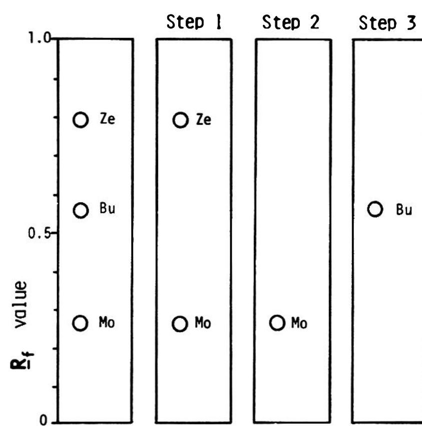

Figure 3. Thin layer chromatogram of mycotoxins: Ze, zearalenone; Bu, butenolide; Mo, moniliformin. Developing solvent: toluene-acetone-methanol (5 + 3 + 2). Treatment: step 1, observe under 254 nm UV light; step 2, spray with 0.32% 2,4-dinitrophenylhydrazine; step 3, spray with 20% H_2SO_4.

into 300 mL round-bottom flask at rate of 1.5 mL/min. Elute moniliformin with 50 mL water at rate of 3 mL/min, collecting eluate in round-bottom flask (fraction I). Next, close stopcock and add 20 mL methanol to column, release any bubbles from column with glass stirring rod, and then elute nivalenol, deoxynivalenol, fusarenon-x, neosolaniol, diacetoxyscirpenol, T-2 toxin, HT-2 toxin, butenolide, and zearalenone with 100 mL methanol at rate of 6 mL/min, collecting eluate in 300 mL round-bottom flask (fraction II).

Fraction I.—Add 20 mL ethanol to eluate, and evaporate eluate to dryness under reduced pressure on steam bath. Dissolve residue in 5 mL methanol, mix with 15 mL acetone, and filter through paper into 30 mL pear-shape flask. Evaporate filtrate to dryness under reduced pressure on steam bath, and dissolve residue in 1 mL methanol. Reserve for TLC test solution No. 1.

Fraction II.—Prepare Florisil column for cleanup of fraction II as follows: Insert glass wool in bottom of chromatographic column. Add ca 10 mL $CHCl_3$ and 5 g anhydrous Na_2SO_4, and then slurry of 10 g Florisil in $CHCl_3$-methanol (9 + 1). When gel settles, top with ca 5 g anhydrous Na_2SO_4. Drain solvent to top of Na_2SO_4. Evaporate fraction II to dryness under reduced pressure on steam bath. Dissolve residue in 10 mL $CHCl_3$-methanol (9 + 1), transfer to column, and drain into 300 mL round-bottom flask. Elute with 100 mL $CHCl_3$-methanol (9 + 1) at rate of 2 mL/min, collecting eluate in round-bottom flask. Evaporate eluate to near dryness under reduced pressure on steam bath, transfer quantitatively to 30 mL pear-shape flask, and evaporate to dryness. Dissolve residue in 1 mL methanol. Reserve for TLC test solution No. 2.

Thin Layer Chromatography

On imaginary or penciled line 3 cm from bottom of appropriate precoated TLC plate (**e**), spot 20 μL test solutions No. 1 and No. 2, and mycotoxin mixture I at 1.5 cm intervals. Dry spots with small fan.

(**a**) *Plate without fluorescent indicator.*—Develop plate in solvent 1 for 18 cm from bottom edge. After development let plate dry 10 min. Observe under 365 and 254 nm UV light for fluorescent spot (Figure 2). Zearalenone appears as blue fluorescent spot. Spray same TLC plate with 20% $AlCl_3$ solution, and heat 10 min at 110°C. Observe under 365 nm UV light. Nivalenol, deoxynivalenol, fusarenon-x, and zearalenone appear as blue fluorescent spots. Then spray with 20% H_2SO_4 solution, and heat 10 min at 110°C. Observe under 365 nm UV light. T-2 toxin, HT-2 toxin, diacetoxyscirpenol, and neosolaniol appear as blue-green fluorescent spots. Spray with 0.32% 2,4-dinitrophenylhydrazine solution, and heat 10 min at 110°C.

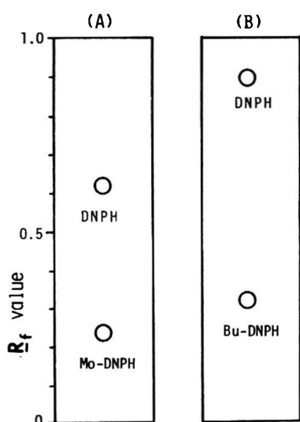

Figure 4. Thin layer chromatograms of 2,4-dinitrophenylhydrazine (DNPH) derivatives of mycotoxins: Mo, moniliformin; Bu, butenolide. Developing solvents: (A) toluene-acetone (97 + 3); (B) toluene-acetone-methanol (5 + 1 + 1).

Moniliformin appears as red-brown spot, butenolide appears as yellow spot.

(b) *Plate with fluorescent indicator.*—Develop plate in solvent 2 for 18 cm from bottom edge (Figure 3). After development, let plate dry 10 min. Observe under 254 nm UV light. Zearalenone and moniliformin appear as absorption spots. Spray with 0.32% 2,4-dinitrophenylhydrazine solution, and heat 10 min at 110°C. Moniliformin appears as red-brown spot. Spray with 20% H_2SO_4 solution, and heat 10 min at 110°C. Butenolide appears as yellow spot.

Quantitation and Confirmation by Densitometric Analysis

Moniliformin.—Transfer 0.5 mL TLC test solution No. 1 and mycotoxin mixture III to separate small screw-cap vials and evaporate to dryness in heating block under stream of nitrogen. Dissolve residue in 2 mL 2,4-dinitrophenylhydrazine solution. Seal vial with screw cap, mix contents gently by swirling, and place in 50°C water bath for ca 2 h to complete reaction. Then transfer entire 2,4-dinitrophenylhydrazine derivatives to 100 mL separatory funnel with water.

Rinse vial with additional small portions of acetone and 20 mL water. Extract aqueous layer with 10 mL $CHCl_3$ by shaking for 5 min; repeat with an additional 10 mL $CHCl_3$. Add ca 10 g anhydrous Na_2SO_4 to $CHCl_3$ phase, stir, and filter through paper into 30 mL pear-shape flask. Evaporate filtrate to dryness under reduced pressure on steam bath. Dissolve residue in 0.5 mL acetone.

At 1.5 cm intervals, spot 20 μL sample derivatives, and 5, 10, and 20 μL standard mixture derivatives on plate with fluorescent indicator. Develop plate in solvent 3 for 18 cm from bottom edge. After development, let plate dry 10 min (Figure 4). Scan on TLC densitometer at 480 nm and quantitate by data processor.

Butenolide.—Transfer 0.2 mL test solution No. 2 and mycotoxin mixture III to separate small screw-cap vials, and evaporate to dryness in heating block under stream of nitrogen. Dissolve residue in 2 mL 2,4-dinitrophenylhydrazine solution. Seal vial with screw cap, mix contents gently by swirling, and place in 50°C water bath for ca 90 min to complete reaction. Then, transfer entire 2,4-dinitrophenylhydrazine derivatives to 100 mL separatory funnel. Rinse vial with additional small portions of acetone and 20 mL water. Extract aqueous layer with 10 mL ethyl acetate by shaking 5 min; repeat with an additional 10 mL ethyl acetate. Add ca 10 g anhydrous Na_2SO_4 to ethyl acetate phase, stir, and filter through paper into 30 mL pear-shape flask. Evaporate filtrate to dryness under reduced pressure, and dissolve residue in 0.5 mL acetone.

Spot and develop as described above using solvent 4 (Figure 4). Scan on TLC densitometer at 430 nm and quantitate by data processor.

If spot of sample is too intense to match standard, dilute sample, and rechromatograph. Concentrations described above will indicate presence of moniliformin as low as 50 μg/kg, and butenolide as low as 30 μg/kg in original sample.

Quantitation and Confirmation by Gas Chromatography

Transfer 0.5 mL TLC test solution No. 2 and 1 mL mycotoxin mixture II to separate 20 mL pear-shape flasks and evaporate to dryness under reduced pressure on steam bath. Dissolve residue in 0.5 mL derivatizing reagent. Seal flask immediately with glass stopper, mix contents gently by swirling, and let stand at room temperature ca 15 min to complete reaction.

Flame ionization detection.—See Figure 5. After reaction, inject 2 μL portions of TMS derivatives directly into gas chromatograph and quantitate by data processor. Concentrations described above will indicate presence of nivalenol, deoxynivalenol, fusarenon-x, and zearalenone as low as 100 μg/kg, and T-2 toxin, HT-2 toxin, diacetoxyscirpenol, and neosolaniol as low as 200 μg/kg in original sample.

Electron capture detection.—If sample extract

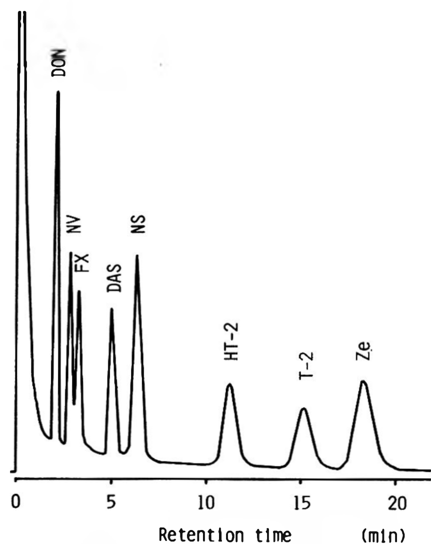

Figure 5. Gas chromatogram of TMS-derivatives of mycotoxins (see Figure 1 for identification of mycotoxins). 200 cm × 3.2 mm id column, with 2% OV-17 on 80-100 mesh Gas-Chrom Q, operated at 250°C.

derivative contains too much interfering substance on flame ionization detector gas chromatogram or if greater sensitivity is desired, electron capture detection can be used. Add 20 mL ethyl acetate to TMS derivatives for flame ionization detection, mix contents gently by swirling, inject 2 μL portions of this solution directly into gas chromatograph, and quantitate by data processor. Concentrations described above will indicate presence of nivalenol, deoxynivalenol, and fusarenon-x as low as 2.0 μg/kg, and T-2 toxin, HT-2 toxin, diacetoxyscirpenol, and neosolaniol as low as 80 μg/kg in original sample.

Recovery Experiments

Prepare samples of ground corn, rice, and barley by adding appropriate amounts of mycotoxins mixture (mycotoxin mixtures II and III) in $CHCl_3$-methanol or methanol. Seal containers with glass stoppers, mix contents by shaking for 1 min. Let stand 1 day unsealed to let solvent evaporate, and analyze by above method, using TLC and GLC to determine mycotoxins.

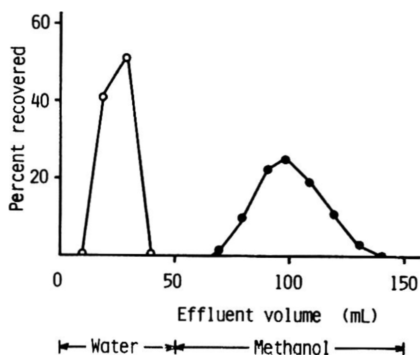

Figure 6. Elution profiles of mycotoxins from an Amberlite XAD-4 column: -O-O- moniliformin; -●-●- nivalenol, deoxynivalenol, fusarenon-x, diacetoxyscirpenol, neosolaniol, HT-2 toxin, T-2 toxin, and zearalenone.

Results and Discussion

The method described was primarily intended for the simultaneous detection of several mycotoxins that are produced by *Fusarium* species. By combining extraction and separation on Amberlite XAD-4 and Florisil columns followed by TLC with separation and quantitation by GLC and TLC densitometry, the method can be used for both qualitative and quantitative determinations in cereals, grains, and foodstuffs.

Elution profiles from the Amberlite XAD-4 column are presented in Figure 6. Moniliformin was eluted first with water. Other mycotoxins (trichothecenes, butenolide, and zearalenone) were then eluted with methanol. The fractions were collected separately for the cleanup steps.

Table 1. Limits of detection (μg/kg) of mycotoxins added to corn, barley, and wheat

	Qualitative	Quantitative		
Mycotoxin	TLC	GLC-FID	GLC-ECD	TLC
Nivalenol	20–50	100	2	20–50
Deoxynivalenol	20–50	100	2	20–50
Fusarenon-x	20–50	100	2	20–50
Diacetoxyscirpenol	100–500	200	80	100–500
Neosolaniol	100–500	200	80	100–500
T-2 toxin	100–500	200	80	100–500
HT-2 toxin	100–500	200	80	100–500
Zearalenone	10–50	100	—	10–50
Butenolide	100–500	—	—	30–50
Moniliformin	200–500	—	—	50–100

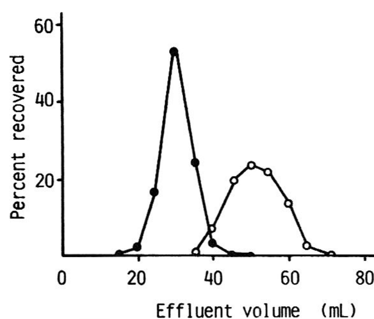

Figure 7. Elution profiles of mycotoxins from a Florisil column: —O—O— nivalenol; —●—●— deoxynivalenol, fusarenon-x, neosolaniol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, and zearalenone.

Elution profiles of mycotoxins of fraction II from the Florisil column are presented in Figure 7. Deoxynivalenol, fusarenon-x, T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol, butenolide, and zearalenone were recovered in the 15–50 mL fraction. On the other hand, nivalenol was recovered in the 35–70 mL fraction; hence an elution with 100 mL $CHCl_3$–methanol was used in the analytical procedure to complete recovery of all trichothecenes, butenolide, and zearalenone.

The mycotoxins were completely separated by ancillary TLC (Figures 2 and 3). Therefore, this procedure is useful for confirmatory, qualitative purposes. The sensitivity of the qualitative TLC was examined by subjecting various volumes of the mycotoxin standard solutions to TLC followed by sequential visualization according to the above method. Results are shown in Table 1.

Table 2. Recovery of mycotoxins added to cereals by proposed procedure [a,b]

Mycotoxin	Added, µg/g	Recovery, % Barley	Rice	Corn
Nivalenol	1.0	71	78	82
Deoxynivalenol	1.0	91	89	93
Fusarenon-x	1.0	78	86	90
Diacetoxyscirpenol	2.0	83	78	102
Neosolaniol	2.0	98	105	108
T-2 toxin	2.0	81	72	83
HT-2 toxin	2.0	89	87	92
Zearalenone	2.0	90	82	88
Butenolide	2.0	78	79	92
Moniliformin	2.0	82	71	76

[a] Each value is the mean of 3 samples.
[b] Nivalenol, deoxynivalenol, fusarenon-x, diacetoxyscirpenol, neosolaniol, T-2 toxin, HT-2 toxin, and zearalenone were measured by GLC. Butenolide and moniliformin were measured by densitometry.

Table 3. Mycotoxins (ppm) found [a] in naturally infected barley and wheat harvested in Japan

Sample	No. of samples	No. of pos.	Nivalenol	Deoxynivalenol
Wheat	18	15	tr.-7.8 (1.5)	tr.-4.7 (1.0)
Barley	25	19	tr.-36.9 (3.9)	tr.-40.4 (4.4)

[a] Range and average. Tr = trace.

The derivatization technique used in the described method is highly efficient for quantitative determination. The 2,4-dinitrophenylhydrazine derivatives of moniliformin and butenolide were prepared and subjected to densitometric analysis. The TMS derivatives of trichothecenes and zearalenone were prepared and subjected to GLC with both flame ionization and electron capture detection. The limits of detection for quantitative TLC and GLC are also shown in Table 1.

Results for recovery of 10 mycotoxins added to barley, rice, and corn at concentrations of 1 and 2 µg/g are presented in Table 2. Trichothecenes and zearalenone were measured by GLC, and butenolide and moniliformin were measured by densitometry. The recoveries were greater than 71% and averaged 85%.

The present method was applied to 43 samples

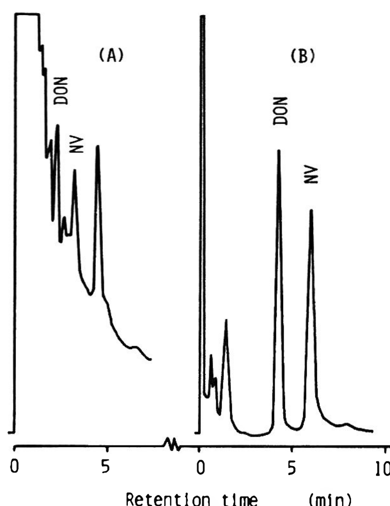

Figure 8. FID (A) and ECD (B) gas chromatograms of the silylated extracts from naturally infected barley contaminated with 1.8 µg/g deoxynivalenol (DON) and 1.4 µg/g nivalenol (NV). Conditions: carrier gas flow rate, 60 mL/min for FID and 50 mL/min for ECD; column temperatures 250°C for FID and 240°C for ECD.

of *Fusarium*-infected barley and wheat which were harvested from fields in Japan (Table 3). Both nivalenol and deoxynivalenol were detected in 30 samples, nivalenol alone was detected in 3 samples, and deoxynivalenol alone was detected in 1 sample. Figure 8 shows FID and ECD gas chromatograms of one of these samples in which nivalenol and deoxynivalenol were found at levels of 1.4 and 1.8 $\mu g/g$, respectively. The method was applied to 197 samples of commercial foodstuffs obtained from retail stores; no mycotoxins were detected.

This survey, although not statistically significant, does indicate some contamination of barley and wheat by nivalenol and deoxynivalenol, and emphasizes the need for more extensive surveys and further toxicological studies of these mycotoxins.

Acknowledgments

The authors are grateful to Y. Ueno, Microbial Chemistry, Faculty of Pharmaceutical Science, Science University of Tokyo, for samples of fusarenon-x, neosolaniol, and T-2 toxin; T. Yoshizawa, Department of Food Science, Faculty of Agriculture, Kagawa University, for samples of deoxynivalenol, HT-2 toxin, butenolide, and infected barley and wheat; and R. Amano, National Consumer Information Center, for a sample of nivalenol.

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Improved Fluorometric-Iodine Method for Determination of Aflatoxin in Corn

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Several innovations in the fluorometric-iodine (FL-I) method for determining aflatoxin in corn made the procedure faster and more convenient than the original method. Ground corn was packed into a chromatographic column and the aflatoxins were extracted with methylene chloride. Sep-Pak C_{18} cartridges were used for sample cleanup. A 5 min thin layer chromatographic slide analysis confirmed the presence of aflatoxin, which was quantitated by fluorometric determination of the iodine derivative of aflatoxin B_1.

The fluorometric-iodine (FL-I) method is more rapid, safer, and more economical than most other methods of aflatoxin assay. However, previously reported methods (1-4) have failed to distinguish qualitatively between the various aflatoxins and did not provide a direct visual confirmation of aflatoxin. This report describes several modifications permitting a simpler and more rapid extraction procedure, a more efficient cleanup procedure, and a direct visualization step which significantly improves the speed and convenience of the FL-I method. The improved procedure has been designated the FL-IM method.

Experimental

Standards

External and internal aflatoxin B_1 standards were prepared in benzene-acetonitrile (98 + 2) as described previously (1). Aflatoxin thin layer chromatographic (TLC) standards were obtained from Supelco Inc. (Bellefonte, PA 16823).

Reagents

The extraction solvent was methylene chloride (technical grade, 99%). Ten mL of 5% aqueous formic acid was stirred into each 50 g sample of ground corn before extraction. Chloroform may be used instead of methylene chloride, but is not recommended because of the health hazards involved.

Equipment

Fluorometric analyses were made with a Coleman photofluorometer Model 12 C equipped with a special filter as described previously (1). TLC analyses were made on 1 × 3 in. Whatman MK6F precoated silica gel plates or slides (Pierce Chemical Co., Rockford, IL 61105). The developing solvent was methylene chloride-acetone (95 + 5). Extraction columns were prepared by a local glass blower and were similar to Corning Pyrex 3700 butt extraction tubes, except their dimensions were 3 × 35 cm to accommodate 50 g samples of ground corn. Samples were cleaned up on Sep-Pak C_{18} cartridges (Waters Associates Inc., Milford, MA 01757).

Procedure

Corn was ground to 18 mesh using the No. 1 screen of a Model D comminuting machine (Fitzpatrick Co., Chicago, IL). Representative 50 g samples (5) were acidified with 10 mL of 5% formic acid and packed in butt tubes containing a plug of glass wool. Butt tubes were inserted through rubber stoppers into filter flasks connected to a vacuum manifold. Samples were defatted under suction using 200 mL hexane or petroleum ether. The hydrocarbon solvent was discarded or distilled and recycled. Suction was continued for several minutes until the corn meal was essentially solvent free. Aflatoxins were extracted from the corn and eluted from the column, also under suction, using 200 mL methylene chloride extraction solvent. This extract was evaporated to dryness on a steam bath under an air stream. The residue was dissolved in 2 mL warm methanol and a 5 μL aliquot was spotted on a TLC slide next to 5 μL aflatoxin standard. TLC slides were developed with 2.5 mL methylene chloride-acetone (95 + 5) for ca 3 min. Slides were examined under longwave UV light (365 nm) for the characteristic blue or green fluorescence of aflatoxin. When samples appeared to contain aflatoxin B_1, B_2, G_1, or G_2, slides were dipped into 25% aqueous sulfuric acid and re-examined for the characteristic yellow fluorescence. Other confirmation tests (6) may also be used.

All sample extracts, whether they contained aflatoxin or not, were processed further. However, in routine work, samples free of aflatoxin

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Table 1. Comparison of FL-I and modified FL-I (FL-IM) assays of corn adulterated with aflatoxin B_1

Level of adulteration, µg/kg	Aflatoxin, µg/kg[a]	
	FL-I	FL-IM
0	0	0
12.5	12	10
25	24	25
50	48	49
100	98	101

[a] Each value is the average of 3 replications.

are discarded at this point. Next, 20 mL water was added to each sample extract. The sample was applied to a Sep-Pak C_{18} cartridge attached to a 20 mL syringe fitted with a small plug of glass wool near the base of the syringe. The glass wool trapped an oily residue that sometimes entered the cartridge and interfered with the cleanup procedure. Before use, cartridges were activated by rinsing with 5 mL of 100% methanol and then washing with 10 mL water. Following sample application, the cartridge was washed twice with 10 mL water, and the wash water was discarded. The glass wool plug was removed from the syringe and aflatoxins were eluted from the cartridge with 10 mL methanol-water (80 + 20). The eluate was evaporated to dryness and the residue was taken up in 2 mL warm methanol, and then diluted to 50 mL with water. Fluorometric analysis was carried out on the iodine derivative of the sample compared with positive and negative checks as described previously (1), i.e., the fluorometer was adjusted to read directly in µg/kg (ppb) using corn adulterated with 100 ppb aflatoxin B_1 as the positive check and unadulterated corn as the zero ppb check. The aflatoxin contamination level (ppb) of iodine-treated sample extracts was then read directly from the instrument meter (1).

Results

A comparison of assays of adulterated samples, determined using the original FL-I method (1) and the Sep-Pak C_{18} method reported here, indicated that there was little or no difference in accuracy between the 2 methods (Table 1). Results of assays of 10 naturally contaminated samples as determined by both procedures were again comparable (Table 2). Also, results of direct observation of aflatoxin on TLC slides and the sulfuric acid confirmation test (data not presented) correlated perfectly with quantitative results (Table 2), except that in one instance the confirmation test showed that a blue-fluorescing spot, which suggested aflatoxin B_1, was not aflatoxin B_1.

Table 2. Assays of aflatoxin B_1 in naturally contaminated corn by the FL-I and modified FL-I (FL-IM) methods

Sample No.	Aflatoxin, µg/kg[a]	
	FL-I	FL-IM
1	16	12
2	4	5
3	8	10[b]
4	0	0
5	24	24
6	31	28
7	15	18
8	15	14
9	0	0
10	27	32

[a] Each value is the average of 3 replications.
[b] False positive detection on TLC slide; no aflatoxin B_1 present.

Discussion

The modified method (FL-IM procedure) has several advantages over the FL-I method. The extraction of ground corn packed directly into butt tubes is, in effect, a column chromatographic procedure that eliminates the need for the platform shaker, special flasks, and filtering funnels required in the FL-I procedure (1). Also, the hydrocarbon solvent used for lipid removal can be recovered, distilled, and reused, thereby reducing costs. Technical grade hexanes, petroleum ether, and methylene chloride appear to be adequate where economy is crucial. However, it would be preferable to use redistilled solvents or a better grade of solvents. Repeated checks for the presence of aflatoxin in the hydrocarbon extract were negative. It is not necessary to acidify samples of freshly harvested corn that contain >15% moisture. However, acidification is necessary in the case of corn which has been naturally or artificially dried to 13–15% moisture.

Use of a silica gel TLC slide to detect the presence of aflatoxin eliminated the false positive and false negative samples occasionally encountered in the FL-I method (1). Sep-Pak cartridges were more convenient to prepare and use than the PEI cellulose columns of the original method (1). They also provided clear aqueous 50 mL samples (as opposed to the occasional cloudy 100 mL samples of the FL-I method) making calibration of the fluorometer easier. Overall, the FL-IM method requires about 30 min per sample, is more convenient to use for large numbers of

samples, provides the same level of precision, and is more reliable than the FL-I method because it permits direct observation of aflatoxin via the 5 min TLC procedure. Results can be confirmed by spraying the developed TLC slide with 50% aqueous sulfuric acid solution in which case the fluorescence of aflatoxin changes from blue to yellow (6). The cost of equipment used in the FL-IM method is less than for the FL-I method, although the expense of materials and supplies is about the same.

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Determination of Aflatoxin B_1 in Corn, Wheat, and Peanut Butter by Enzyme-linked Immunosorbent Assay and Solid Phase Radioimmunoassay

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Determinations of aflatoxin B_1 in corn, wheat, and peanut butter by an enzyme-linked immunoassay (ELISA) and a solid-phase radioimmunoassay (RIA) were compared. Samples spiked with 2.9-43.2 ppb B_1 were subjected to AOAC extraction procedure 26.017 or 26.023. The extracts were concentrated, redissolved in methanol, diluted in phosphate-buffered saline with Tween 20, and directly analyzed for B_1 by either ELISA or RIA. At 5.8 ppb or greater, recoveries for B_1 in corn, wheat, and peanut butter samples were 80.0, 86.6, and 94.8% by ELISA and 61.0, 93.3, and 110.0% by RIA, respectively. Recoveries greater than 120% were obtained for the wheat and peanut butter samples spiked with 2.9 ppb aflatoxin B_1 by the RIA method but not by ELISA. Overall results indicated that ELISA gave more consistent data, relatively lower standard deviations, and lower coefficients of variation than did RIA. Analysis of 3 samples naturally contaminated with aflatoxins revealed that the ELISA data were comparable to those obtained by other established chemical methods.

Aflatoxins are toxic metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*, which commonly appear in such agricultural commodities as peanuts, corn, wheat, and animal feedstuffs (1-3). The toxic and carcinogenic potential of aflatoxin B_1 requires that its possible presence in the food supply be closely monitored by a rapid and sensitive technique.

Investigations in our laboratory and others have described radioimmunoassays (RIAs) for aflatoxin B_1 (4-7), aflatoxin M_1 (8), ochratoxin A (9), and T-2 toxin (10). However, only limited information exists on the feasibility of mycotoxin immunoassays in the routine surveillance of foods. Langone and Van Vunakis (4) demonstrated that a double antibody RIA could be used for the detection of B_1 in corn and peanut butter, with respective recoveries ranging from 40 to 133% and from 34 to 54%. Sun and Chu (6) found that a solid phase RIA was effective for B_1 quantitation in corn and wheat.

Recently, we have developed a microtiter plate enzyme-linked immunosorbent assay (ELISA) for aflatoxins B_1 (11) as well as M_1 (12). The methods are as sensitive as previous RIAs but are considerably faster, safer, and less expensive. When ELISA and an ammonium sulfate precipitation-type RIA were compared for the analysis of milk samples spiked with M_1, levels as low as 0.25 ppb could be directly assayed by ELISA in less than 3 h (12). However, milk samples caused a large degree of nonspecific interference in the RIA, required extensive extraction and cleanup, and allowed detection only to the 1.0 ppb level.

In light of the manifold advantages of ELISA over RIA and the lack of information concerning the efficacy of B_1 analysis in foods by immunoassay, a systematic comparison of the 2 procedures was conducted. This paper compares B_1 determination in corn, wheat, and peanut butter by ELISA and solid-phase RIA. Specific attention is directed toward those factors that are critical in the development of routine immunoassays for aflatoxin screening in food. These include extracting toxin from food, solubilizing toxin in aqueous buffer, and establishing practical limits of detection by ELISA and RIA.

Experimental

Chemicals

All inorganic chemicals and organic solvents were reagent grade or better. Bovine serum albumin (BSA, RIA grade), Tween 20, 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-Aflatoxin B_1 (13 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, CA). Disposable Quik-Sep columns were purchased from Isolab, Inc. (Akron, OH). Aflatoxin B_1 (13), aflatoxin B_1 oxime antisera (5), and aflatoxin B_1 oxime-peroxidase (11) were prepared as previously described. Peanut butter, corn meal, and whole wheat flour were purchased locally. Three samples (one peanut meal, one de-oiled peanut meal, one yellow corn) naturally contaminated with aflatoxins were provided by Marlin Friesen of the International Agency for Research on Cancer (IARC).

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Sample Extraction

Samples of peanut butter, corn, and wheat were spiked with B_1 at concentrations ranging from 2.9 to 43.2 ppb. The peanut butter, peanut meal, and de-oiled peanut meal were extracted according to AOAC method **26.023** (BF method) (14), which specifies blending the sample in methanol-water-hexane with a subsequent $CHCl_3$ extraction. An aliquot of $CHCl_3$ extract equivalent to 10 g of sample was evaporated to dryness under reduced pressure. Corn meal and whole wheat flour were extracted by AOAC method **26.017** (CB method) (14) in which samples are shaken with diatomaceous earth, water, and $CHCl_3$, and then the $CHCl_3$ phase is removed by vacuum filtration. An amount of $CHCl_3$ extract equivalent to 10 g sample is dried, and dried residues of all 5 types of food were then dissolved in 5.0 mL methanol and vortex-mixed for 30 s. An additional 5.0 mL 0.1M phosphate buffer (pH 7.5) containing 0.15M NaCl (PBS) and 0.5% (v/v) Tween 20 was added and the solution was vortex-mixed for 10 s. The samples were used in ELISA and RIA assays without further preparation.

Aflatoxin B_1 Standards

Standard B_1 concentrations were determined spectrophotometrically in methanol, using a molar absorptivity of 21 800 at 360 nm (14). Standards for immunoassay were prepared in 50% (v/v) methanol-PBS containing 0.25% (v/v) Tween 20, on the day of use.

Enzyme-Linked Immunosorbent Assay

ELISAs were conducted as described by Pestka et al. (11) with some modifications. Antisera were dissolved in PBS rather than in 1.0M saline before coating the microtiter plates. For displacement assays, the B_1 oxime-peroxidase conjugate was diluted in 1.0% BSA in PBS and 25 μL was added to the microtiter plate followed by the addition of 25 μL of either B_1 standard or food extract. Standards and samples were applied to the plates in alternating rows of eight. The plates were incubated 1 h at 37°C and the competitive displacement of the enzyme conjugate was determined as previously reported (11).

Solid-Phase Radioimmunoassay

Conditions for the preparation of IgG-Sepharose gel were the same as those previously described by Sun and Chu (6). Immediately before assay, 1.0 mL PBS solution containing 1.0% BSA and 0.5% (v/v) Tween 20 were added to 1.0 mL of either food extract solution or B_1 standard. For the solid phase RIA, 0.2 mL of an appropriate dilution of IgG-Sepharose gel in 0.1M phosphate buffer (PB, pH 7.5) was mixed with 0.1 mL ^{3}H-aflatoxin B_1 (41 000 dpm) in PB and 0.5 mL of either diluted food extract (equivalent to 0.25 g original sample) or B_1 standard (7). The assay mixture was incubated in a Quik-Sep column for 16 h at 4°C. The columns were decanted and washed with 0.2 mL PB. The combined filtrates were assayed for radioactivity and standard curves were prepared (6, 7).

Results and Discussion

Effect of Methanol on ELISA and RIA Methods

An essential requirement for the immunoassay of aflatoxin in a food extract is the complete solubilization of the toxin in an aqueous buffer. One means of achieving this is by first dissolving the extract in methanol and then diluting in phosphate-buffered saline. To test whether methanol affects the antigen-antibody reaction, the binding of B_1 oxime-peroxidase to the polystyrene-attached antibody was determined in the presence of various concentrations of methanol in PBS. Tween 20 and BSA were included to prevent nonspecific peroxidase conjugate binding (15). To avoid precipitation of the antibody at high methanol levels, the B_1 oxime-peroxidase was added to the plate before the sample. The results showed that a final well concentration of 25% (v/v) methanol caused only a 20% reduction in total bound enzyme. A competition curve was then determined for various aflatoxin B_1 levels in which the final well concentrations of methanol, Tween 20, and BSA were 25%, 0.125%, and 0.5%, respectively. The results (Figure 1) show that under these conditions the standard curve was comparable to that previously described for the B_1 ELISA (11), and thus could be used to detect B_1 at the 25 pg/assay level. Bierman and Terplan (16, 17) described a competitive microtest plate ELISA for B_1 with limits of detection approximately 10 times lower than those described here. A major difference in their procedure was the use of an aflatoxin B_1 oxime-human serum albumin immunogen prepared by a mixed anhydride method for the production of specific antisera as compared to our use of an B_1 oxime-bovine serum albumin conjugate prepared by a water-soluble carbodiimide procedure (5).

A similar experiment was carried out for the solid-phase RIA in which IgG-Sepharose gel was mixed with 0.1 mL ^{3}H-aflatoxin B_1 in PB and 0.5

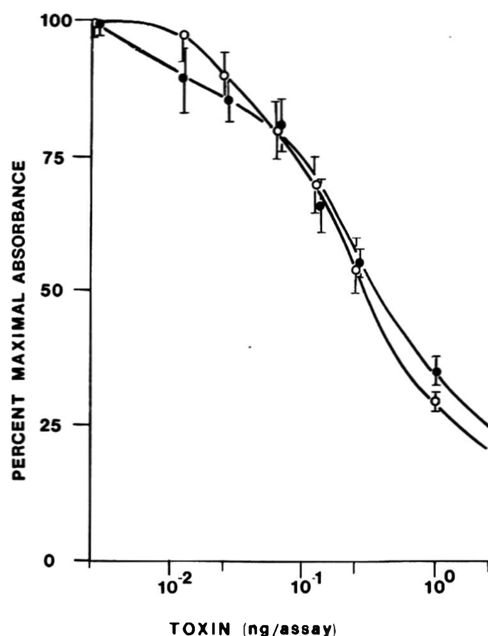

Figure 1. Competitive ELISA for aflatoxin B_1.

Standard competition curves were prepared in PBS (●) and in methanol-PBS-Tween (O) as described in the text. For both curves the response was greatest between 0.063 and 1.0 ng B_1, and the minimal detectable level was 0.025 ng because this level was significantly different from zero at the 99% confidence level. Results represent quadruplicate determinations.

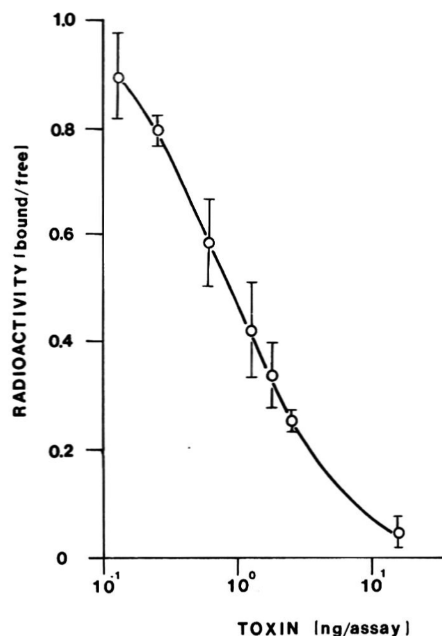

Figure 2. Radioimmunoassay of aflatoxin B_1.

The standard curve was prepared in the presence of methanol-containing buffer. Bound/free radioactivity was determined as previously described (6). Bound/free value for assay conducted in the absence of aflatoxin was 1.14 ± 0.06. The response for RIA was greatest between 0.25 and 5.0 ng/assay and the minimal detectable level was 0.25 ng because this was significantly different from zero at the 99% confidence level. Results represent triplicate determinations.

mL PBS containing various concentrations of B_1, 25% methanol, 0.5% Tween 20, and 0.5% BSA. The results (Figure 2) show that the modified solid phase RIA could detect levels of B_1 between 0.25 and 5.0 ng/assay and thus had similar detection limits to those reported previously (6, 7).

Efficacy of ELISA and RIA Methods

To test the efficacy of both the ELISA and RIA methods, samples of corn, wheat, and peanut butter were spiked with B_1 at levels of 2.9, 5.8, 14.4, 28.8, and 43.2 ppb. Extracts were prepared and subjected to analyses by ELISA and RIA. For RIA, samples had to be diluted with methanol-PBS-Tween to a concentration of <5 ng/assay so that the sample determination was made in the linear region of the standard curve (Figure 2). Extracts were assayed directly by ELISA without prior dilution. Preliminary experiments revealed that when unspiked extracts of corn, wheat, and peanut butter were prepared as described in a methanol-containing PBS-Tween buffer at a concentration of 1.0 g equivalent extracted sample/mL and subjected to RIA and ELISA, no interference in either immunoassay could be detected. Also, as previously determined with the M_1 ELISA (12), inclusion of BSA at various stages of ELISA did not interfere with the competitive assay and was actually required to prevent nonspecific binding of the peroxidase conjugate.

The results of the corn analyses showed that between 69 and 85% and 58 and 79% of added B_1 were recovered by ELISA and RIA, respectively (Table 1). However, the average percent recovery for corn samples containing 5.8 ppb B_1 or more was significantly greater at the 99% confidence level for ELISA (80.0 ± 20.5) than for RIA (61.0 ± 25.5). Table 2 shows that the average percent recoveries for wheat samples at the 5.8 ppb level or greater were not significantly different at the 95% confidence level for ELISA (86.6 ± 16.6) and RIA (93.3 ± 20.0) but that percent recoveries of B_1 from wheat at 2.9 ppb were lower

Table 1. Recovery of aflatoxin B_1 from corn by ELISA and solid phase RIA [a]

Added, ppb	ELISA		RIA	
	Rec. (ppb) ± SD	Rec. (%) ± SD	Rec. (ppb) ± SD	Rec. (%) ± SD
0[b]	0		0	
2.9	2.0 ± 1.1	69 ± 38	2.3 ± 1.4	79 ± 48
5.8	4.2 ± 1.8	73 ± 31	3.7 ± 2.4	64 ± 41
14.4	11.1 ± 1.2	77 ± 8	8.5 ± 3.7	59 ± 26
28.8	24.5 ± 5.6	85 ± 19	16.6 ± 2.2	58 ± 8
43.2	36.6 ± 10.1	85 ± 24	27.4 ± 11.8	63 ± 27
Av. rec., %	80.0		61.0	
Std dev.	20.5		25.5	
Coeff. of var., %	25.6		41.8	
No. of replicate assays	24		16	

[a] Data at 2.9 ppb level were excluded from statistical analysis.
[b] Corn extract used for zero determination.

for ELISA (62 ± 17) and higher for RIA (129 ± 40). Immunoassays of peanut butter at the 5.8 ppb level or greater as shown in Table 3 revealed excellent average percent recoveries by ELISA (94.8 ± 20.8) but these results were significantly different from the higher estimates by RIA (110 ± 29.5) at the 95% confidence level. Again, recoveries at 2.9 ppb B_1 levels were less for ELISA (72 ± 19%) and even higher for RIA (134 ± 53%).

The compiled data for the 2 B_1 immunoassays suggest that ELISA is preferable to RIA on the basis of average percent recovery, recovery estimates at low aflatoxin B_1 levels, and the coefficients of variation. In general, at the 5.8 ppb level or greater, ELISA exhibited more consistent average percent recoveries (80.0, 86.6, 94.8) compared with RIA (61.0, 93.3, 110) for corn, wheat, and peanut butter, respectively. The lower ELISA estimates for B_1 at the 2.9 ppb level can be best explained by incomplete extraction of the toxin from the sample and might be improved by a second extraction step.

The coefficients of variation for ELISA were 25.6, 19.2, and 21.9% and those for RIA were 41.8, 21.4, and 26.5% in the 3 sample types. Variability in the solid phase RIA can be attributed to problems encountered with accurately dispensing the IgG–Sepharose gel into the minicolumn and can be reduced somewhat by vortex-mixing the gel-buffer while pipetting. The coefficient of variation for ELISA in the present study was similar to that found for ELISA of aflatoxin M_1 in milk (12) and might be due to the inherent interwell variability in the adsorption properties of polystyrene microtiter plates. Kricka et al. (18) found coefficients of variation ranging from 5.2 to 29.5% for the protein adsorption characteristics of several brands of microtiter plates. Other possible causes of variation may be temperature penetration of the inner vs outer wells and errors in pipetting small (25 μL) volumes. To minimize errors, we recommend that ELISAs of standards and food samples be carried out in quadruplicate and in alternating rows of 8 across the plate. Future efforts in our laboratory will

Table 2. Recovery of aflatoxin B_1 from wheat by ELISA and solid phase RIA [a]

Added, ppb	ELISA		RIA	
	Rec. (ppb) ± SD	Rec. (%) ± SD	Rec. (ppb) ± SD	Rec. (%) ± SD
0[b]	0		0	
2.9	1.8 ± 0.5	62 ± 17	3.7 ± 1.2	129 ± 40
5.8	4.7 ± 1.1	81 ± 19	5.6 ± 1.1	97 ± 19
14.4	13 ± 2.3	90 ± 16	11.3 ± 4.7	98 ± 33
28.8	25.6 ± 4.1	89 ± 14	27.4 ± 5.0	95 ± 17
43.2	37.4 ± 7.7	87 ± 18	36.0 ± 4.8	83 ± 11
Av. rec., %	86.6		93.3	
Std dev.	16.6		20.0	
Coeff. of var., %	19.2		21.4	
No. of replicate assays	24		16	

[a] Data at 2.9 ppb level were excluded from statistical analysis.
[b] Wheat extract used for zero determination.

Table 3. Recovery of aflatoxin B_1 from peanut butter by ELISA and solid phase RIA [a]

Added, ppb	ELISA		RIA	
	Rec. (ppb) ± SD	Rec. (%) ± SD	Rec. (ppb ± SD	Rec. (%) ± SD
0 [b]	0		0	
2.9	2.1 ± 0.5	72 ± 19	3.9 ± 1.5	134 ± 53
5.8	5.6 ± 1.3	97 ± 23	6.0 ± 2.7	104 ± 47
14.4	13.6 ± 3.0	95 ± 21	15.0 ± 3.5	104 ± 24
28.8	25.2 ± 5.7	87 ± 20	33.4 ± 8.0	118 ± 28
43.2	43 ± 8.1	100 ± 19	49.2 ± 7.9	114 ± 18
Av. rec., %	94.8		110	
Std dev.	20.8		29.5	
Coeff. of var., %	21.9		26.5	
No. of replicate assays	24		16	

[a] Data at 2.9 ppb level were excluded from statistical analysis.
[b] Peanut butter extract used for zero determination.

be directed towards eliminating this high variability.

ELISA of Aflatoxin B_1 in Naturally Contaminated Samples

During the course of this study, we participated in the 1980 Aflatoxin Check Survey Programme sponsored by the Division of Environmental Carcinogenesis of IARC. Three samples naturally contaminated with aflatoxins were analyzed for B_1, using the ELISA procedures described in this report. The B_1 content of the peanut meal, de-oiled peanut meal, and yellow corn meal were 269, 35.7, and 13.8 ppb, respectively. These data were comparable to the overall mean values obtained by using other methods in which the B_1 contents (± SD) of the 3 commodities were 210.1 ± 111.2, 53.3 ± 33.7, and 15.0 ± 8.4 ppb, respectively. Although detailed statistical analysis has yet to be carried out by IARC, the preliminary results demonstrated that the ELISA method could be used for screening B_1 in naturally contaminated samples, and that the presence of other aflatoxins did not significantly interfere in the analysis. The incapability of monitoring other types of aflatoxins by the present method is the only major disadvantage of the immunochemical method. However, this disadvantage could be overcome by using another specific antibody (such as antiaflatoxin G_1) in a separate analysis.

Conclusion

Microtiter plate ELISAs offer a simple and inexpensive alternative to conventional analytical methods in both the clinical and food laboratories. Micro ELISA is the basis for a number of diagnostic kits and automated devices with computer interface possibilities, which are currently being marketed. We have recently described a microtiter plate ELISA that is effective in the direct screening of milk for aflatoxin M_1 (12), and have concurrently developed an ELISA for B_1 (11). This paper discusses for the first time the efficacy of the B_1 ELISA in the analysis of corn, wheat, and peanut butter and compares it to a previously described RIA (6, 7). The results support previous contentions that samples containing B_1 at levels above 5 ppb can be analyzed by immunoassays directly after a simple extraction without a column chromatographic step (6, 7). This represents an advantage in time and cost when compared to high pressure liquid chromatographic methods (19) which require extensive cleanup and derivatization steps to attain equivalent sensitivity.

Although Bierman and Terplan (16, 17) described ELISAs for B_1 with greater sensitivity than that described here, they found significant interference by extracts of alfalfa, the only agricultural commodity tested. However, their results do indicate the potential for further improving the detection limits of B_1 ELISA.

The ELISA described here is superior to the solid phase RIA on the basis of average recovery, recovery estimates at low aflatoxin B_1 levels, and coefficient of variation. Microtiter plate ELISA has the further advantages over RIAs of short assay time (2 h vs overnight), cost per assay, absence of radiation hazards, number of assays that can be conveniently handled, and the possibility of automation. It might be most useful to laboratories performing large scale screening of mycotoxins in food commodities.

Acknowledgments

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High Performance Liquid Chromatographic Determination of Aflatoxicol in Milk, Blood, and Liver

MARY W. TRUCKSESS and LEONARD STOLOFF
Food and Drug Administration, Division of Chemistry and Physics, Washington, DC 20204

Samples of milk were extracted with chloroform, the extract was transferred to methanol, and residual interferences were removed by liquid-liquid partition against hexane and by silica gel column chromatography. Aflatoxicol (AFL) in the purified extract was resolved on a μBondapak C_{18} column, using water - methanol - acetonitrile - tetrahydrofuran (70 + 15 + 20 + 3) as the mobile phase, and measured with a fluorescence detector (excitation 325-385 nm, emission >408 nm). Recoveries of AFL added to samples of whole pasteurized milk at levels ranging from 0.025 to 0.10 ng/mL averaged 92% (range 78-100%). The method for AFL has also been applied to the analysis of raw milk, whole beef blood, and beef liver, with recoveries of 70-88%.

Aflatoxicol (AFL) and aflatoxin M_1 (AFM) are the 2 mammalian metabolites of aflatoxin B_1 (AFB) that approximate the potency of the parent compound both in carcinogenicity and mutagenicity (1-4), the order of potency being AFB > AFL > AFM. Efforts have been made to analyze milk and meat for AFM to limit human exposure to this toxin (5, 6). However, no quantitative method for the determination of AFL has been published. Although there are reports of the production of AFL from AFB by livers of various animal species in both in vivo and in vitro studies (7-12), no information is available on the possible natural occurrence of AFL in food. To measure AFL in milk, a method has been developed that is applicable at approximately the same levels (>0.05 ng/mL) as the AFM methodology.

Development of Method

Chloroform is a good extracting solvent for aflatoxins and their metabolites; therefore, it was effectively used to extract AFL from milk. However, when chloroform is blended with milk, it is difficult to filter the resulting coagulum from the mixture. The filtration problem is avoided by placing the mixture in a separatory funnel immediately after extraction. The white foam floats to the top of the chloroform layer, which allows the chloroform to be drained off and filtered easily. Since any milk fat dissolved in the chloroform could interfere with the subsequent analysis, it is necessary to remove this component of the extract as soon as possible. Hexane has been the commonly used solvent for defatting aflatoxin-containing extracts of various commodities; usually it has been partitioned against methanol as the aflatoxin solvent, with water added to reduce the hexane solubility in the methanol. Emulsions often occur when hexane is added to the milk sample residue dissolved in aqueous methanol. However, this difficulty is not encountered when hexane is added to the sample residue dissolved in neat methanol. Since hexane and methanol are miscible to a slight degree, the methanol dissolved in the hexane is recovered by adding a small amount of methanol–aqueous sodium chloride solution. The hexane dissolved in methanol is salted out by the addition of sodium chloride solution.

The technique for separating aflatoxins B_1, B_2, G_1, and G_2 from interferences on a silica gel column (13) does not separate AFL from interferences. Both chloroform and anhydrous ethyl ether, which are used in the technique to selectively elute impurities, are capable of eluting AFL from silica gel; thus, AFL is lost. Since the major factors that determine retention by a chromatographic adsorbent are the relative polarities of the mobile phase and solute, several solvents and mixtures of solvents of different polarities were added to silica gel columns on which AFL had been adsorbed. No AFL was eluted when either methylene chloride–acetone (98 + 2) or anhydrous ethyl ether–hexane (2 + 3) was used. These solvents were then used to replace chloroform and ether, respectively, in the normal elution pattern. AFL can be eluted subsequently from the column with chloroform–acetone (98 + 2). The concentrated eluate is then suitable for quantitation by either thin layer chromatography (TLC) or high performance liquid chromatography (HPLC).

AFL fluoresces blue on a silica gel TLC plate, but with much less intensity compared with the other aflatoxins. The detection limit for AFL by

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densitometry is 2.5 ng. However, when a reverse phase HPLC column is used and the intensity of fluorescence of AFL is measured in the mobile phase with a fluorescence detector, the detection limit is 0.25 ng (signal:noise = >4). The use of HPLC with a fluorescence detector as the quantitative step thus provides a detection limit approximately 1/10 of that attainable by TLC.

METHOD

Apparatus

(a) *Chromatographic column.*—10.5 mm id × 200 mm glass, equipped with Teflon stopcock and 200 mL reservoir (Kontes K420-280).

(b) *Solvent delivery system.*—Altex Model 110A (Altex Scientific Inc., Berkeley, CA 94710).

(c) *Sample injection system.*—Syringe loading sample injection valve with 25 μL loop (Valco Instruments Co., Houston, TX 77024).

(d) *HPLC column.*—3.9 mm × 30 cm μBondapak C_{18} (Waters Associates Inc., Milford, MA 01757).

(e) *Fluorescence detector.*—DuPont Model 836 with excitation filter, range 325–385 nm (Corning CS 7-60), and emission filter cutoff below 408 nm (Corning CS 3-74) (E.I. du Pont de Nemours & Co.).

(f) *Sample vials.*—2 dram, with foil-lined screw cap.

Reagents

(a) *Solvents.*—Distilled-in-glass $CHCl_3$ (with 1% ethanol), methanol, and acetonitrile; ACS grade hexane, anhydrous ethyl ether, methylene chloride, and acetone; HPLC grade tetrahydrofuran, and Millipore (0.2 μm diameter pore size)-filtered distilled water.

(b) *Silica gel 60.*—(E. Merck No. 7734), 0.063–0.200 mm (Brinkmann Instruments Co., Westbury, NY 11590). Wash 200 g silica gel with 1 L methanol; filter and wash with 1 L $CHCl_3$. Spread in tray in hood to let solvent evaporate; activate by drying 1 h at 105°C. Add 1 mL water/100 g silica gel; seal in jar. Shake contents well to disperse moisture and store overnight before use.

(c) *HPLC mobile phase.*—Water-methanol-acetonitrile-tetrahydrofuran (70 + 15 + 20 + 3); degas in ultrasonic bath.

(d) *AFL standard solutions.*—*Stock solution.*—1 μg/mL methanol. *Working solutions.*—0.01, 0.02, and 0.04 μg/mL. Prepare by diluting stock solution to appropriate volumes with methanol. Store stock solutions in freezer and prepare working standards daily.

Extraction

Measure 100 mL milk or blood or 100 g liver into 1 L blender jar; add 400 mL $CHCl_3$, cover jar, and blend 5 min. *Immediately* pour mixture into 500 mL separatory funnel. Let solvent layers separate (ca 2 min). Filter lower layer through coarse paper into 250 mL graduated cylinder. Transfer 250 mL filtrate to 500 mL round-bottom flask and immediately evaporate to near dryness under vacuum in rotary evaporator at ca 27°C. Flush flask with stream of nitrogen to remove residual $CHCl_3$.

Liquid-Liquid Partition

Dissolve residue in 50 mL methanol; then add 150 mL hexane and mix. Transfer mixture to 250 mL separatory funnel; shake 1 min. Drain lower methanol layer into second 250 mL separatory funnel. Add 30 mL methanol–5% NaCl solution (40 + 60) to remaining hexane layer; gently mix by inverting separatory funnel ca 5 times. Drain lower layer into second separatory funnel and discard hexane layer. Add 60 ml 5% NaCl solution to aqueous methanol and mix gently. Save aqueous methanol layer; discard hexane layer. Rinse extract flask from evaporator with 50 mL additional hexane and use this hexane to wash aqueous methanol. Extract aqueous methanol layer with three 25 mL portions of $CHCl_3$; shake 1 min each time. Combine $CHCl_3$ fractions in 250 mL round-bottom flask. Evaporate $CHCl_3$ extract to dryness under vacuum at ca 27°C. Store dry extract in freezer or immediately dissolve dry extract and pass it through silica gel column.

Silica Gel Column Chromatography

Place wad of glass wool at bottom of column. Add 1 mL anhydrous Na_2SO_4. Add anhydrous ethyl ether–hexane (2 + 3) to half fill column; then add 4 mL silica gel and ca 4 mL more ether–hexane. Stir silica gel with long rod. Wash sides of column with ether–hexane and let silica gel settle. Drain ether–hexane to 2 cm above silica gel. Add 2 mL Na_2SO_4 to form layer on top of silica gel.

Quantitatively transfer sample extract to column with three 5 mL portions of ether–hexane (2 + 3). Wash column with 40 mL ether–hexane, followed by 50 mL methylene chloride–acetone (98 + 2). Elute AFL with 50 mL $CHCl_3$–acetone (98 + 2). Add each successive solvent when preceding solvent reaches top of Na_2SO_4 layer. Collect eluate in 250 mL round-bottom flask, starting when $CHCl_3$–acetone is added. Immediately evaporate eluate to dryness under vacu-

Table 1. Recovery (%) of AFL added to milk, beef blood, and beef liver

Commodity	AFL added, ng/mL or g	Av. AFL recd (individual values), % of added
Pasteurized milk	0.1	91.3 (96.2, 78.2, 93.6, 93.6, 94.9)
	0.05	89.8 (100.0, 84.6, 87.2, 94.9, 82.1)
	0.025	91.7 (87.2, 97.4, 97.4, 84.6)
	0	0 (0, 0, 0, 0, 0)
Raw milk	0.1	78.6 (78.6, 78.6)
	0.05	78.6 (77.6, 79.6)
	0.02	82.1 (82.1)
	0	0 (0)
Whole beef blood	0.1	76.4 (75.0, 69.7, 78.9, 77.8, 80.6)
	0	0 (0, 0)
Beef liver	0.5	85.8 (84.0, 87.6)
	0	0 (0, 0)

um at ca 27°C; quantitatively transfer residue with methylene chloride to small vial; evaporate to dryness in ca 27°C water bath under stream of nitrogen. Store dry extract in freezer and reserve for HPLC. This final extract represents 62.5 mL fluid milk.

This method has also been used for the determination of AFB and AFM in the same extract with AFL. AFB elutes from the silica gel column in the same fraction with AFL, and its presence can be determined by TLC, using hexane-tetrahydrofuran-ethanol (70 + 20 + 10) as the mobile phase on silica gel 60 precoated plates. AFM elutes from the silica gel column with 50 mL acetone-$CHCl_3$ (20 + 80); its presence can also be determined by TLC on silica gel 60 precoated plates using ether-methanol-water (96 + 4 + 1) as the mobile phase.

High Performance Liquid Chromatography

Stabilize instrument ca 30 min at flow rate of 1.5 mL/min. Adjust sensitivity of fluorescence detector to give 35-40% full scale recorder deflection for 1 ng AFL injected in loop volume (2 nA range). Record chromatogram at speed of 0.2 in./min until AFL elutes at ca 12 min.

Inject measured amount, >25 μL of each standard solution, into sample loop for transfer to liquid chromatograph at beginning and end of each run (25 μL loop provides constant volume transfer). Flush loop with 30 μL mobile phase after each injection has been transferred to column stream. Prepare standard curve by plotting average areas or peak heights vs quantity of AFL.

Dissolve purified sample extract in 200 μL methanol and inject measured amount >25 μL into sample loop for transfer to column stream. Flush loop with mobile phase after each sample has been transferred. Identify AFL peak from sample by comparing retention time with that of standard. If sample peak measurement is not within points on standard curve, dilute sample quantitatively with methanol or concentrate to dryness under stream of nitrogen at room temperature and redissolve in smaller volume of methanol as required for reinjection. Determine quantity of AFL (A) in each sample extract injection from standard curve, and calculate concentration of AFL in milk using following formula:

$$\text{AFL, ng/mL or g} = (A \times V/25)/S$$

where V = final sample extract volume, 25 = μL injected into column, and S = volume (mL) or weight (g) of sample represented by extract. If dilution or concentration was required, correct sample volume or weight by proportionate volume of extract removed for initial injection.

Results and Discussion

The main emphasis of the study was on the determination of AFL in milk. Pasteurized milk was used to develop the method because it is readily available. Average percentage recoveries were 91.3, 89.8, and 91.7, respectively, for AFL added to pasteurized milk samples at levels of 0.1, 0.05, and 0.025 ng/mL and refrigerated overnight before extraction (Table 1).

The method was also applied to raw milk, whole beef blood, and beef liver. Percentage recoveries from these commodities were somewhat lower than from pasteurized milk but generally greater than 75% (Table 1). Based on the limited data, the method appears to be applicable to these commodities.

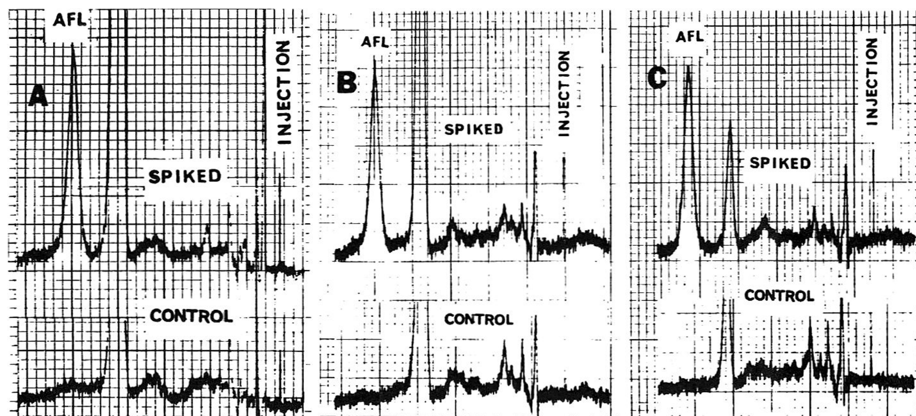

Figure 1. HPLC chromatograms of extracts with and without added AFL. A, pasteurized milk, 0.025 ng/mL added; B, beef blood, 0.1 ng/mL added; and C, beef liver, 0.5 ng/g added. A different extract dilution volume was used for each product to obtain optimum recorder response.

The HPLC chromatograms (Figure 1) of all unspiked samples showed no fluorescent eluate near the AFL area. The AFL peaks from all spiked samples appeared after all other fluorescent materials had eluted, and there were no other fluorescent compounds detected after the AFL had eluted.

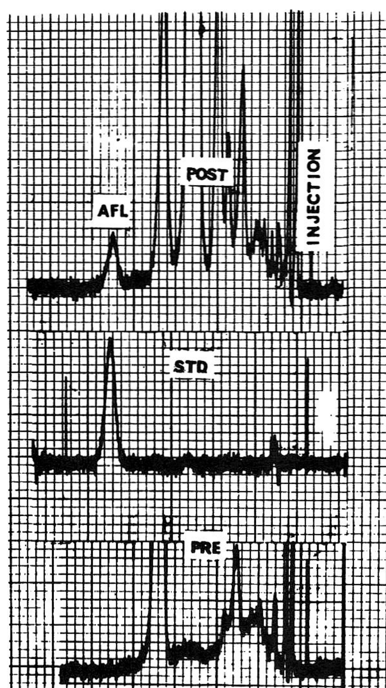

Figure 2. HPLC chromatograms of extracts of milk from a cow on clean feed (PRE) and milk from the same cow after being dosed with aflatoxin (POST) compared with the chromatogram of a standard AFL (STD).

All attempts to prepare AFL derivatives for confirmation of identity have so far been unsuccessful, and the quantities involved in the naturally contaminated samples so far encountered are too small for current mass spectrometric techniques. Confirmation of identity studies are continuing.

The method was used to analyze 20 milk samples from aflatoxin-dosed cows. A fluorescent compound eluting at the retention time of AFL was detected in all samples. Figure 2 shows a representative chromatogram. In lieu of a more suitable technique for confirmation of identity, AFL was added to one of these milk samples; it produced a fluorescent signal enhanced by the amount calculated from the addition with no aberration in the elution pattern. The data from these milks will be presented in a separate paper.

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FEEDS

Effect of Bentonites on Fluorometric Selenium Determination

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Bentonites in feeds cause error in the analysis for Se by the AOAC (3.097-3.101) fluorometric method for Se in plants. The error apparently results from the binding of the piazselenol by insoluble residue from the bentonite. This effect is avoided by diluting digests to volume after reduction with HCl, centrifuging or allowing to stand, and analyzing a portion of the clear supernatant liquid. Insoluble residues present after digestion of plant materials do not appear to cause a similar error.

The analysis of feed samples containing added bentonite by the official method for Se in plants (1, 3.074), as slightly modified (2), gives low values. The cause for these low values has been investigated, and a procedure for avoiding this error is described. In addition, studies of possible effects of insoluble residues in plant and some other perchloric acid digests are reported.

Experimental

Materials

All plant materials and mixed feed samples were finely ground with a ball mill and were analyzed on an air-dry basis.

Two powdered bentonites, Volclay sodium and FD 181 (American Colloid Co., Skokie, IL), were used in most of the studies. Bentonite is a hydrated aluminum silicate consisting principally of montmorillonite. Other materials studied are described later.

^{75}Se was used in the form of selenite, and checked for purity by ion exchange chromatography (3). Unlabeled selenite used in recovery studies was also used as a standard for Se determination.

Methods of Analysis

The modified fluorometric method for plants (2) was used for Se determinations. Variations from this method are indicated in the section on results.

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^{75}Se activity was determined with a Beckman 4000 gamma spectrometer.

Results

Bentonite Effect

Evidence for a bentonite effect had been obtained by G. M. Miller (Landmark, Inc., Columbus, OH) on the analysis of lamb feed with and without bentonite added; values for the former are much smaller. On confirming this finding in our laboratory, a study was made on the effect of adding bentonites to corn. The results in Table 1 illustrate the bentonite effect. Other data, reported later here, have shown that it occurs consistently although not always to the same degree.

Selenite Adsorption

It seemed unlikely that selenite would be adsorbed by any residue of the bentonite digestion, but the possibility was nevertheless studied. Four samples each of the 2 bentonites plus added $^{75}SeO_3^{-2}$ were digested with HNO_3–$HClO_4$ and reduced with HCl as described in the modified method (2). Two digests of each bentonite were cooled, diluted to 40.0 mL with water, and mixed. The other 2 were treated with EDTA–NH_2OH

Table 1. Effect of added bentonite on fluorometric measurement of selenium in corn

Corn sample	Bentonite additions[a]	Measured Se content,[b] µg/g
1	none	0.41 ± 0.03
	Volclay sodium 5%	0.32 ± 0.02
	FD 181 5%	0.32 ± 0.08
2	none	0.33
	Volclay sodium 1%	0.26
	2%	0.22
	3%	0.23
	4.5%	0.18
	6%	0.19

[a] Corn and corn plus bentonite samples were ball-milled 24 h before analysis.
[b] Corn sample 1 and its mixtures with bentonite were analyzed on 3 different days, and standard deviations are shown. Values are corrected to exclude contribution of Se from bentonites, based on their later analysis.

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Table 2. Adsorption of selenite by insoluble residue from bentonite digests

Sample	Added ^{75}Se found in supernatant liquid, %[a]	
	Untreated digest	pH-adjusted digest[b]
0.5 g FD 181 bentonite + 0.5 mL $^{75}SeO_3^{-2}$ solution	98.4	92.9
0.5 g Volclay sodium bentonite + 0.5 mL $^{75}SeO_3^{-2}$ solution	99.4	94.1

[a] 72 300 cpm added. Total Se <1 ng.
[b] pH about 1.5.

Table 3. Removal of piazselenol from solution by insoluble residue in bentonite digests

Sample	Added $^{75}SeO_3^{-2}$ found in supernatant liquid,[a] %	
	No DAN added	DAN added
0.5 g FD 181 bentonite + 1.0 mL $^{75}SeO_3^{-2}$ solution	90.7	6.2
0.5 g Volclay sodium bentonite + 1.0 mL $^{75}SeO_3^{-2}$ solution	89.7	4.0

[a] 88 860 cpm added. Total Se <1 ng.

solution and neutralized to about pH 1.5 according to the modified method before dilution to 40.0 mL. All solutions were allowed to stand undisturbed for 16 h, and 2.0 mL supernatant liquid was withdrawn from each for counting. The data in Table 2 show that selenite is not adsorbed on the insoluble matter that settles from the diluted digest. Treatment with EDTA-NH_2OH and adjustment of the pH to 1.5 did result in the removal of a small amount of the selenite. This effect was small, but consistent.

Piazselenol Adsorption

The adsorption of selenite from the pH-adjusted sample could not account for all of the bentonite effect. Therefore, two 0.5 g samples each of the bentonites with added $^{75}SeO_3^{-2}$ were digested, reduced, treated with EDTA-NH_2OH, and adjusted to pH 1.5 in the usual manner in a micro-Kjeldahl flask calibrated at the neck at 40.0 mL. One sample of each was heated with 2,3-diaminonaphthalene (DAN) as described in the official method and the other was heated without DAN addition. After cooling, all solutions were diluted to 40.0 mL with water, mixed, and allowed to stand undisturbed for 16 h. Aliquots of the clear supernatant liquids were then withdrawn for counting. The results in Table 3 show, again, that a small part of the selenite is adsorbed on the residue from the digested bentonites after EDTA-NH_2OH addition and pH adjustment. However, the adsorption of the piazselenol is marked, and this appears to be a major source of error.

Table 4. Effect of removal of insolubles from digests on recovery of selenium added as selenite

Sample	Se added, µg	Treatment	Se found,[a] µg	Added Se recovered, %
0.5 g Bentonite FD 181	none	insolubles not removed for analysis	0.000	
	0.400		0.055 ± 0.005	14
	none	insolubles removed by centrifugation[b]	0.135 ± 0.005	
	0.400		0.560 ± 0.015	106
0.5 g Bentonite Volclay sodium	none	insolubles not removed for analysis	0.000	
	0.400		0.040 ± 0.005	10
	none	insolubles removed by centrifugation	0.095 ± 0.020	
	0.400		0.425 ± 0.020	83
	0.200		0.310 ± 0.005	108
0.5 g Silica gel (EM Laboratories, Inc., Elmsford, NY)	none	insolubles not removed for analysis	0.000	
	0.200		0.195 ± 0.005	98
	none	insolubles removed by centrifugation	0.000	
	0.200		0.205 ± 0.010	103

[a] Results represent averages and standard deviations for 3 determinations.
[b] After digestion and reduction, sample was diluted to 25 mL and centrifuged at 10 000 × g for 10 min. A 20 mL aliquot of supernatant liquid was used for analysis, and results have been adjusted for this.

Table 5. Effect of removal of insoluble materials from digests on selenium contents

Sample	Se content,[a] µg/g	
	Insolubles not removed	Insolubles removed
Corn grain	0.41 ± 0.03	0.45 ± 0.05
Oat grain	0.44 ± 0.01	0.44 ± 0.02
Oat hay	0.53 ± 0.01	0.54 ± 0.02
Alfalfa hay	0.64 ± 0.04	0.66 ± 0.02
Bromegrass hay	1.06 ± 0.05	1.08 ± 0.05
Mixed feed (pelleted without bentonite)	0.19 ± 0.00	0.19 ± 0.00
Rock S7-394	0.38 ± 0.03	0.38 ± 0.02
Silt loam soil	0.67 ± 0.02	0.61 ± 0.03

[a] Average of 3 analyses, with standard deviation.

Avoiding the Bentonite Effect

At this point, it appeared that the removal of a portion of the diluted digest following reduction with HCl would correct the bentonite problem. Therefore, studies were made by digesting the bentonites with and without added selenite. The analyses were made with and without removal of the insoluble residue. Samples of silica gel were treated similarly as controls. This time centrifugation was used for residue removal. The results in Table 4 show that this procedure successfully avoids the problem.

In view of the studies discussed above, the following procedure was adopted for use on samples containing bentonites: After digesting, reducing, diluting to volume, and mixing in micro-Kjeldahl flasks calibrated at 40.0 mL, solutions are allowed to stand undisturbed at least 6 h. An aliquot of the clear liquid, usually 20 mL, is removed to another flask, 1.0 mL 70% perchloric acid is added, and the analysis is completed by the official method (1, **3.101**), beginning "To each flask add 5 mL NH_2OH-EDTA

Effects of Other Residues

Using the above procedure, we studied the possibility of an effect of insolubles in digests of plants and some other materials. The results are shown in Table 5. These data indicate that there should be no problem with plants. Data suggest a similar finding for rock and soils, but are too limited for any generalization.

A study on the effect of centrifuging before pH adjustment and DAN addition was made with a variety of siliceous additives or plant samples. The results are summarized in Table 6. Infusorial earth apparently does not adsorb piazselenol to any appreciable degree, a finding similar to that for silica gel (Table 4). However, kaolin and the various bentonites, which are aluminum silicates, did, although to varying degrees. Centrifugation overcame the problem for all plant samples as well as for the additives.

Table 6. Selenium recoveries from various plant materials with added bentonite or other siliceous materials and effect of removing insoluble residues after digestion

Plant material	Additive[a]	Selenium content[b]		
		Without additive (control), µg/g	With additive, % of control	
			Insolubles not removed	Insolubles removed
Wheat	infusorial earth[c]	0.35	99	104
Wheat	kaolin[d]	0.35	90	100
Wheat	bentonite, SD[e]	0.35	93	102
Wheat	bentonite, Fisher[f]	0.35	57	95
Wheat	bentonite, Sigma[g]	0.35	63	102
Wheat	bentonite, FD 181	0.35	71	102
Wheat	bentonite, Volclay sodium	0.35	67	101
Soybean meal	bentonite, Volclay sodium	0.71	73	101
Oat meal	bentonite, Volclay sodium	0.39	64	102
Alfalfa hay	bentonite, Volclay sodium	0.33	74	100
Corn silage	bentonite, Volclay sodium	0.13	59	103

[a] All additives were used at a level of 5% sample weight.
[b] Values have been corrected for selenium in additive.
[c] Source unknown. A diatomaceous earth, largely silica.
[d] Mallinckrodt, St. Louis, NF VI Colloidal. Kaolin is hydrated aluminum silicate.
[e] Raw South Dakota bentonite from Pierre formation.
[f] Fisher Scientific Co., Fair Lawn, NJ, USP.
[g] Sigma Chemical Co., St. Louis, MO.

Discussion

The bentonite effect is variable in degree, but occurs consistently. It can be easily avoided by removing the insolubles, although this does, in effect, reduce the sensitivity of the method by reducing the sample size actually used in measuring Se. The problem seems limited to bentonite, but whenever acid digests are used for the fluorometric determination of Se by this or any similar methods reported in the literature, the presence of appreciable amounts of insolubles following digestion should be reason for considering their removal.

Acknowledgments

The probability of a bentonite effect was called to the attention of the authors by J. Anderson, American Colloid Co., who also kindly furnished some materials used in the study. The assistance of T. C. Nelson in several of the studies is acknowledged.

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Automated Determination of Urea and Ammoniacal Nitrogen (NPN) in Animal Feeds

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A minor modification in the automated analytical system of the official AOAC semiautomated method for determining crude protein results in an automated method for determining urea and ammoniacal nitrogen in animal feeds and their ingredients. Urease enzyme which has high activity, yields a clear solution in water, has low ammonia impurity, and is inexpensive is used in the automated method. Weights from 1 to 2.5 g feed sample are dissolved in water, and sample solutions are analyzed at the rate of 40 samples/h. Five AAFCO feed check samples were analyzed repeatedly by the automated method, and results were compared with the grand averages from the check sample reports. The official AOAC manual urease method was used by AAFCO participants. Average recovery of urea and ammoniacal nitrogen was 100.6% by the automated method relative to the AAFCO reported averages. The range of recoveries was 98.5-102.7%. The non-protein nitrogen (NPN) concentrations, expressed as protein equivalent, ranged from 3.40 to 63.04% protein on these samples. The average relative standard deviation for the automated analyses was 0.77%, compared with 1.54% for the manual method. This method is an important adjunct to laboratories using or considering use of the semiautomated method for crude protein and needing further information on NPN.

The purpose of this work was to develop an automated method for the determination of urea and ammoniacal nitrogen in animal feeds. This is commonly referred to as non-protein nitrogen (NPN). The method will be of particular benefit to laboratories using or considering use of the official semiautomated method for crude protein and who also need to perform NPN determinations.

In response to research studies which showed that ruminants can utilize ammoniacal and urea nitrogen as partial substitutes for protein, Griem and Walker (1) reported a method in 1941 for NPN determination in feed. The method included the use of urease enzyme with distillation of ammonia from a Kjeldahl flask for the measurement of ammoniacal and urea nitrogen, exclusively.

Griem presented the results of a collaborative study on the urease method in his referee report the following year (2). The urease method was adopted official first action on the basis of the collaborative results and approved as official final action in 1944 (3).

Wall and Gehrke reported an automated method for nitrogen determinations in fertilizers (4) in which urease is used to quantitatively hydrolyze urea in a continuous flow analysis system with a reaction time of less than 5 min.

The objective of the work reported here was to include the urease hydrolysis of urea in the analytical system of the official semiautomated method for crude protein, **7.025** (5), with only minor modification in the system.

Experimental

Apparatus

Automated instrument.—The automated system (Figure 1) is the same as that described in **7.026(b)** (5) with 3 modifications: A 40/h cam with a 4:1 ratio is substituted for the 2:1 ratio cam; the original sample line is changed from 0.16 mL/min to 0.32 mL/min; and a 20T coil is added in the dilution loop in series with the 10T coil of Figure **7:01** (5).

Reagents

Reagents **7.027 (a-d)** are unchanged from the semiautomated protein method (5).

(e) *Urease solution.*—20 mg % (20 mg/100 mL) urease. Dissolve 100 mg urease powder (No. U 4002, Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178) in 500 mL 0.15M sodium citrate, add 0.5 mL Brij 35, and mix. Prepare fresh daily and maintain in ice water bath. This reagent replaces acid/NaCl reagent in dilution loop of crude protein method system.

(f) *Nitrogen standard solution.*—(*1*) *Stock solution.*—1.25 mg N/mL. Dissolve 1.4741 g $(NH_4)_2SO_4$ primary standard (dried 2 h at 105°C) in 250 mL water and mix. (*2*) *Working standard solutions.*—25, 50, 75, 100, and 125 ppm N. Prepare 5 standards by transferring 5, 10, 15, 20, and 25 mL aliquots of stock solution to individual 250

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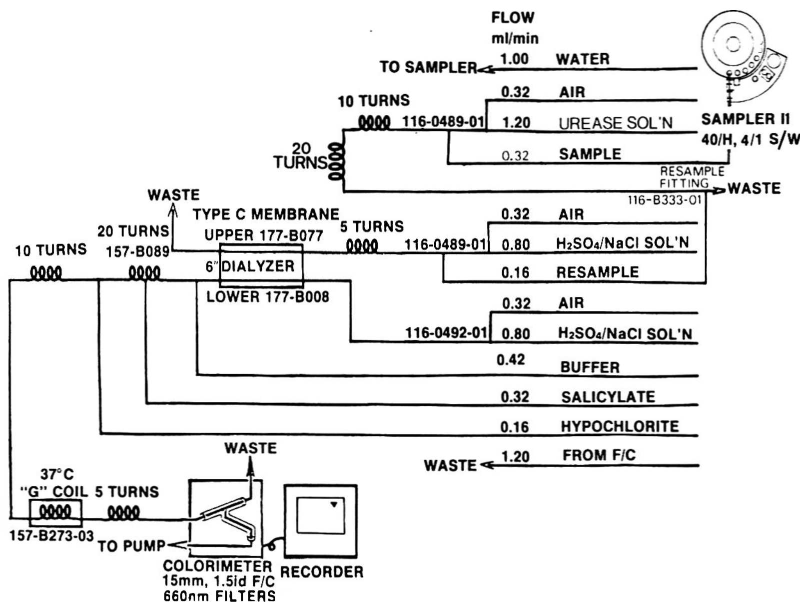

Figure 1. Flow diagram for automated determination of urea and ammoniacal nitrogen (NPN) in animal feed.

mL volumetric flasks. Dilute to volume with water and mix.

Analytical System

Refer to **7.028-7.030** (5) for description, construction, start-up, and shut-down of automated analytical system.

Determination

Weigh 1.0-2.5 g feed samples into 250, 500, or 1000 mL volumetric flasks to give final concentration between 24 and 112 ppm N (150-700 mg/L NPN protein equivalent). Add 20, 40, or 80 mL of 0.3N HCl to 250, 500, or 1000 mL flasks, respectively, swirl flasks, and let stand ≥15 min to dissolve urea. Dilute to volume with water and mix. Analyze samples by automated system at rate of 40 samples/h. The standard calibration series should be analyzed before and after each group of samples. Prepare standard curve by averaging peak heights of first and second sets of standards. Plot average peak height of standards against nitrogen concentration. Determine NPN protein equivalent concentration of unknown samples from plot.

$$\text{NPN protein equiv., \%} = \text{ppm N}/1000 \times \text{total vol. (mL)} \times 0.625 \times 1/\text{g sample}$$

Results and Discussion

A urease product was selected and the required conditions for quantitative urea hydrolysis were determined in our previous investigation (4). The urease product specified in these automated

Table 1. Comparison of non-protein nitrogen results by automated method and official AOAC manual method for urea and ammoniacal nitrogen

		Percent protein equivalent ± SD		
Sample	Description	AAFCO[a]	Automated[b]	Rec., %
1	AAFCO 7724	26.27 ± 0.91	26.20 ± 0.14	99.7
2	AAFCO 7826	63.04 ± 1.28	64.72 ± 0.41	102.7
3	AAFCO 7830	3.40 ± 0.47	3.35 ± 0.06	98.5
4	AAFCO 7926	24.62 ± 0.64	24.65 ± 0.16	100.1
5	AAFCO 7930	28.61 ± 0.73	29.21 ± 0.18	102.1
6	urea	46.65 (N, calc.)	46.83 ± 0.47	
Average (excl. urea)		29.19	29.63	100.6

[a] Analyses reported by 48-65 laboratories, using AOAC method **7.033-7.034** (5).
[b] Average of 6 determinations.

Table 2. Precision of automated method for non-protein nitrogen determination

Sample [a]	Percent protein equivalent					
	Average		Std dev.		RSD, %	
	AOAC [b]	Automated [c]	AOAC	Automated	AOAC	Automated
1	25.33	26.20	0.30	0.14	1.17	0.52
2	63.64	64.72	0.15	0.41	0.23	0.63
3	3.14	3.35	0.15	0.06	4.93	1.64
4	24.30	24.65	0.32	0.16	1.31	0.66
5	28.21	29.21	0.25	0.18	0.90	0.61
6	12.98	13.14	0.19	0.12	1.49	0.92
7	26.34	26.69	0.13	0.15	0.50	0.54
8	24.69	25.11	0.45	0.16	1.82	0.63
Average			0.26	0.17	1.54	0.77

[a] Samples 1–5 are AAFCO check feed samples, see Table 1 for identification; samples 6–8 are liquid protein supplements.
[b] Three or more independent determinations.
[c] Six determinations.

methods is critical to the success of the methods. This product has high activity, yields a clear solution in water, has low ammonia impurity, and is relatively inexpensive when used in the continuous flow analysis methods.

For the NPN application, dissolution of the urease at a concentration of 20 mg % in 0.15M sodium citrate provides optimum reaction conditions when mixed in the system with a sample having 0.024N HCl concentration. The urease reagent was substituted for the sodium chloride–sulfuric acid diluent in the dilution loop of the semiautomated protein method. The original sample line was increased for this method commensurate with the reduced nitrogen concentration range used in the method, compared with the protein method. It was necessary to add a 20-turn coil in the dilution loop of the protein manifold to allow sufficient time for quantitative urea hydrolysis.

Five AAFCO check feed samples containing non-protein nitrogen were selected to evaluate the performance of the automated method. The sample set was analyzed 3 independent times in duplicate by the proposed method. The results are shown in Table 1 and compared with results taken from AAFCO reports. The method used for the AAFCO results was AOAC **7.033-7.034** (5) for urea and ammoniacal nitrogen, the manual urease method. The automated method results were in good agreement with the results by the official method. The average recovery was 100.6% with a range from 98.5 to 102.7%.

The precision of the automated method results on these 5 samples plus 3 liquid protein samples with urea is shown in Table 2. Also shown here are the results of 3 independent runs by the manual urease method on these 8 samples. The precision of the automated urease method is excellent with an average RSD of 0.77% compared with 1.54% average RSD for the manual method in this limited study.

The 3 liquid protein supplement samples proved to be unstable over time with respect to urea and ammoniacal nitrogen. At least the results as determined by the urease method, manual or automated, were lower as the products aged. A molasses-type matrix was used for the

Table 3. Non-protein nitrogen results on liquid protein supplement at different times

Sample	Percent protein equivalent						Percent decrease
	Initial analysis			Subsequent analysis [a]			
	Manual	Auto.	Av.	Manual	Auto.	Av.	
1	20.08	—	20.08 [b]	12.98	13.14	13.06	35.0
2	29.38	29.77	29.58 [c]	26.34	26.69	26.52	10.3
3	27.44	28.27	27.88 [c]	24.69	25.11	24.90	10.7

[a] Analyzed September 1980.
[b] AAFCO report. Analyzed October 1977.
[c] Commercial product. Manufactured and analyzed July 1980.

3 samples evaluated. The results for NPN, protein equivalent, by the manual and the automated methods are shown in Table 3. We are unable to explain the cause for this reduction in measured NPN in the liquid protein supplements. We are also uncertain what significance, if any, should be attached to this finding.

In conclusion, the automated urea and ammoniacal nitrogen method presented here is simple, rapid, accurate, and precise. It complements the official semiautomated method for crude protein determinations. The recovery of NPN by the automated method was slightly greater than by the manual urease method on most of the samples tested. The results by the automated method are also more precise than the manual method results.

This method will be a valuable addition for those laboratories which are currently using the semiautomated method for crude protein determinations and which also need to perform NPN determinations.

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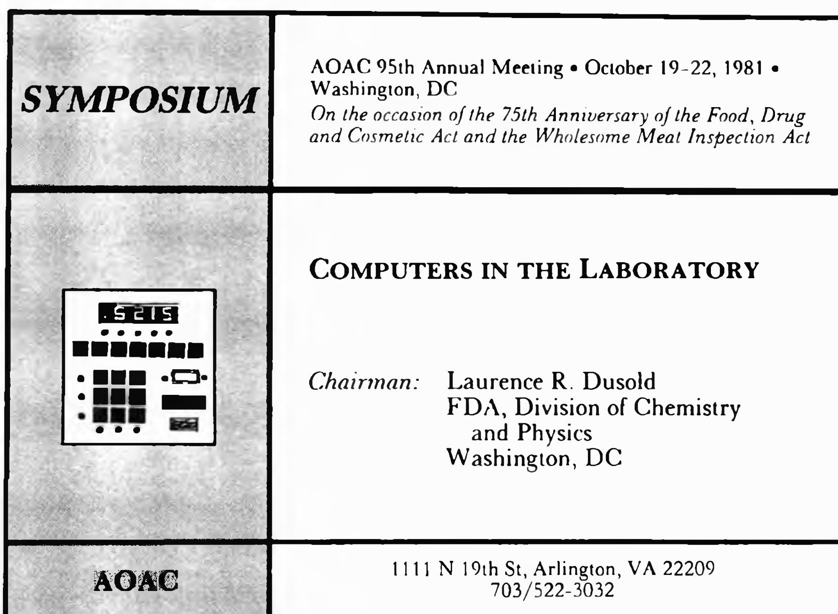

FERTILIZERS

Ammonia-Selective Electrode Determination of Nitrogen in Fertilizers

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The official AOAC magnesium oxide method (MOM), 2.065, for determining ammoniacal nitrogen in fertilizers not containing urea was compared with an alternative ammonia-selective electrode method (ASEM-1). Likewise, the official AOAC Raney powder method (RPM), 2.063-2.064, for determining total nitrogen in fertilizers, except nitric phosphates containing nonsulfate S, was compared with an ammonia-selective electrode method (ASEM-2). Each comparison included 6 samples. For ammoniacal nitrogen determination, MOM and ASEM-1 showed equivalent precision, although significant statistical differences were obtained in average values for the majority of samples. For total nitrogen determination, RPM and ASEM-2 showed equivalent precision, although significant statistical differences were obtained in 3 samples. The AOAC official methods showed better accuracy than ASEM-1 and ASEM-2. However, the accuracy and precision of ASEM-1 for ammoniacal nitrogen is suitable for routine work.

The availability of the ammonia gas-sensing electrode offers the possibility of using a rapid and inexpensive technique to simplify nitrogen determination in fertilizers and other products. The instrument has been used for nitrogen determination by the known addition technique in fertilizers and protein in feeds (1), and ammoniacal nitrogen in water and wastes (2) and sea water (3). Some studies of urea analysis have also been made using this electrode (4, 5), with good results. Woodis and Cummings (6) studied the determination of ammoniacal nitrogen in fertilizers containing urea, using this type of electrode; their results were in good agreement with those of the reduced pressure distillation method.

The Orion ammonia electrode contains a hydrophobic gas-permeable membrane which separates the test solution from the electrode internal solution. This membrane prevents a false response to dissolved ions. However, high levels of ions can affect the solubility of ammonia. Thomas and Booth (2) indicated that dissolved ammonia in test solution diffuses through the membrane until the partial pressure of ammonia is the same on both sides of the membrane. Eagan and Dubois (7) noted that the partial pressure of ammonia and its concentration are related by $P_{NH_3} = k[NH_3]$ where the constant, k, varies with dissolved species; for this reason, the ionic strength of the samples and standards must be constant.

We decided to test the applicability of the ammonia gas-sensing electrode for the determination of ammoniacal and total nitrogen in fertilizers. We compared the magnesium oxide method (MOM), which is an official AOAC method, with an alternative method using the ammonia-selective electrode (ASEM-1), in which the sample is dissolved and ammoniacal nitrogen is measured directly with the electrode. We also compared the Raney powder method (RPM), which is an official AOAC method, with the ammonia-selective electrode method (ASEM-2) that is a variant of the RPM. ASEM-2, however, consists of 2 parts: sample treatment, reduction, digestion, and distillation taken from the Raney powder method; and measurement of the distilled ammonia according to ASEM-1. A major simplification is elimination of the distillation step in determining ammoniacal nitrogen; however, preliminary work showed that it is not possible to measure the ammonia concentration directly from the solution obtained during sample digestion for total nitrogen. The electrode used in both ammonia-selective electrode methods was the same.

Magnesium Oxide Method (MOM) (8)

See 2.065.

Ammonia-Selective Electrode Method-1 (ASEM-1) (9)

Apparatus and Reagents

(**a**) *Ammonia electrode.*—Orion Model 95-10 (Orion Research Inc., 380 Putnam Ave, Cambridge, MA 02139).

(**b**) *pH meter.*—Orion Model 701, or equivalent.

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(c) *Tartaric acid solution.*—10% aqueous solution.

(d) *Sodium hydroxide solution.*—10N.

(e) *Ammoniacal nitrogen solutions.*—*Stock solution.*—1400 μg N/mL. Dissolve 5.35 g reagent grade NH_4Cl in water in 1 L volumetric flask and dilute to volume with water. *Working solutions.*—(*1*) 700 μg N/mL. Transfer 100 mL stock solution to 200 mL volumetric flask, add 20 mL tartaric acid solution, dilute to volume with water, and mix. (*2*) 140 μg N/mL. Transfer 50 mL solution 1 to 250 mL volumetric flask, add 20 mL tartaric acid solution, dilute to volume with water, and mix. (*3*) 70 μg N/mL. Transfer 100 mL solution 2 to 200 mL volumetric flask, add 10 mL tartaric acid solution, dilute to volume with water, and mix. (*4*) 14 μg N/mL. Transfer 50 mL solution 3 to 250 mL volumetric flask, add 20 mL tartaric acid solution, dilute to volume with water, and mix. (*5*) 7.0 μg N/mL. Transfer 100 mL solution 4 to 200 mL volumetric flask, add 10 mL tartaric acid solution, dilute to volume with water, and mix. (*6*) 1.4 μg N/mL. Transfer 50 mL solution 5 to 250 mL volumetric flask, add 20 mL tartaric acid solution, dilute to volume with water, and mix.

Preparation of Sample Solution

Place sample containing 10–50 mg ammoniacal N in 250 mL volumetric flask, and add 25 mL tartaric acid solution and 100 mL water. Let stand 15 min, swirling occasionally. Dilute to volume with water, and mix.

Determination

Connect ammonia electrode to pH meter, place electrode in working solution 4, and warm up pH meter. Set function switch to EXP MV. Add 1.5 mL 10N NaOH to each 100 mL solution. Set reading to 000.0 by adjusting calibration control. Use magnetic stirrer at constant rate throughout procedure. Addition of NaOH to evolve NH_3 is exothermic. A change in temperature will cause electrode response to shift and change slope. Samples and standards should be at same temperature, for convenience, room temperature. A 1°C difference in temperature will cause ca 2% measurement error (9). Place electrode in each solution and read mV of working solutions and unknown sample solutions. Plot mV readings (linear axis) against concentration (log axis) on standard 3- or 4-cycle semilogarithmic paper. Determine ammoniacal nitrogen content of unknown samples from standard curve.

After 2 readings, recalibrate electrode by using working solution 4.

Table 1. Description of fertilizer samples studied

Sample	Description	Approx. ammoniacal N content, %
1	30-0-0, Magruder 7905 B liquid check sample	8.0
2	5-10-15, Magruder 7908 solid check sample	3.0
3	11-54-0, Magruder 8003 B MAP check sample	11.0
4	18-46-0, Fertilizer grade diammonium phosphate	18.0
5	21-0-0, Ammonium sulfate, reagent grade	21.0
6	35-0-0, Ammonium nitrate, reagent grade	17.0
7	Same as Sample 1	—
8	10-34-0, Magruder 7907 B liquid check sample	—
9	9-27-6, Magruder 7807 suspension check sample	—
10	12-15-10, Magruder 7709 suspension check sample	—
11	19-19-19, Magruder 7712 solid check sample	—
12	3-9-9, Magruder 7607 solid check sample	—

Raney Powder Method (RPM) (8)

See 2.063–2.064.

Ammonia-Selective Electrode Method-2 (ASEM-2) (8, 9)

Apparatus and Reagents

See **2.063** and ASEM-1.

Determination

For sample pretreatment, reduction, digestion, and distillation, see **2.064.** For measuring ammonia in distillate, see *Determination* in ASEM-1.

Results and Discussion

Ammoniacal Nitrogen

Six samples (1–6, Table 1) were analyzed in 6 replicates for ammoniacal nitrogen by MOM and ASEM-1, respectively. However, Magruder check sample 7905 B was not analyzed by MOM because it contains urea and this method is not applicable.

Sample 7905 B was analyzed by 18 participant laboratories in the Magruder check sample program, Sample 7908 by 5 laboratories, and Sample 8003 by only one laboratory (10). The diammonium phosphate sample was analyzed by 8 laboratories in a private interlaboratory program.

Table 2. Comparison of results by official magnesium oxide and ammonia-selective electrode methods on 6 samples of fertilizers

Sample	Magruder results	Ammoniacal N, % Average MOM	Average ASEM-1	Std dev. MOM	Std dev. ASEM-1	Coeff. of var., % MOM	Coeff. of var., % ASEM-1
1	7.718 ± 0.3648	—	7.547	—	0.1084	—	1.436
2[a]	2.7040 ± 0.0430	2.717	2.593	0.0288	0.0557	1.060	2.148
3[a]	11.035 ± 0.0786	11.088	11.213	0.0821	0.0393	0.740	0.350
4[a]	18.24 ± 0.17	18.267	18.058	0.0907	0.0454	0.497	0.251
5[b,c]	21.20 (theor.)	21.138	21.063	0.0417	0.1224	0.197	0.566
6[a,d]	17.50 (theor.)	17.527	17.232	0.0698	0.0527	0.398	0.306

[a] Significant differences in averages at 95% level, *t*-test.
[b] Ammonium sulfate, reagent grade.
[c] Significant differences in standard deviation at 95% level, *F*-test.
[d] Ammonium nitrate, reagent grade.

The ammonium sulfate and ammonium nitrate samples were reagent grade.

Mean per cent nitrogen, standard deviations, and relative standard deviations (CV, %) for each of the samples by both methods are given in Table 2. No value was rejected at the 95% significance level by the Dixon test (11). When standard deviations were compared by means of the *F*-test, differences were statistically significant only for the ammonium sulfate sample, which indicates equivalent precision for both methods, although the *t*-test showed significant statistical differences in the averages for 4 of the 5 samples studied. Therefore, MOM and ASEM-1 gave comparable precision, but average values for MOM are in better agreement with reference values than are averages for ASEM-1.

Total Nitrogen

Six samples (7–12, Table 1) were analyzed in 6 replicates for total nitrogen by RPM and ASEM-2. Samples 7905 B, 7907 B, 7807, 7709, 7712, and 7607 were analyzed by 5, 5, 6, 7, 5, and 7 participant laboratories, respectively, in the Magruder check sample program (10).

In a preliminary experiment with ASEM-2, nitrogen was measured with the ammonia-selective electrode directly after digestion; however, values were very high. These high values may be due to the increased ionic strength of the solution caused by solids formed during the addition of NaOH to evolve ammonia. Such results are in accordance with observations by other authors (2, 7) who recommended that samples and standards have the same ionic strength. Further studies are necessary to consider this effect.

Mean per cent nitrogen standard deviations, and coefficients of variation for each of the samples by both methods are given in Table 3. When standard deviations were compared by means of the *F*-test, differences were statistically significant only for Sample 7709, which indicates an equivalent precision of both methods, although the *t*-test showed significant statistical differences in the averages for 3 of the 6 samples studied (50% of the total formed pairs).

Table 3. Comparison of results by official Raney powder and ammonia selective methods on 6 samples of fertilizers

Sample	Magruder results	Nitrogen, % Average RPM	Average ASEM-2	Std dev. RPM	Std dev. ASEM-2	Coeff. of var., % RPM	Coeff. of var., % ASEM-2
7[a]	29.210 ± 0.1379	29.175	29.427	0.1465	0.0962	0.502	0.327
8[a]	9.758 ± 0.0944	9.853	9.947	0.1934	0.1473	1.963	1.481
9	9.35 ± 0.07	9.367	9.530	0.1106	0.0844	1.181	0.886
10[a,b]	11.98 ± 0.09	12.000	12.098	0.0335	0.0776	0.279	0.641
11	19.28 ± 0.29	19.195	19.275	0.1349	0.1100	0.703	0.571
12	3.1929 ± 0.2615	3.175	3.277	0.1203	0.0922	3.789	2.814

[a] Significant differences in averages at 95% level, *t*-test.
[b] Significant differences in standard deviations at 95% level, *F*-test.

In general, the average values for ammoniacal and total nitrogen by MOM and RPM are in better agreement with the reference values than are the average values obtained by ASEM-1 and ASEM-2. This means that the accuracy of the magnesium oxide method and Raney powder method are better than those obtained by ammonia-selective electrode techniques. On the other hand, the average values obtained by ASEM-1 and ASEM-2 are higher than those obtained by MOM or RPM.

The accuracy and precision of the ammonia-selective electrode technique is suitable for routine determination of ammoniacal nitrogen. It simplifies the normal procedure because the distillation step is omitted. In the determination of total nitrogen with the Raney powder method, the final titration can be substituted by an electrode measurement, but with no improvement of results.

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DRUGS IN FEEDS

High Performance Liquid Chromatographic Determination of Furazolidone in Feed and Feed Premixes

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Furazolidone is separated from finished feeds by acetone-water extraction on a Goldfisch apparatus. Extracting solvent is removed, and the residue is dissolved in dimethylformamide-5% tetraethylammonium bromide (1 + 1), clarified, and chromatographed on a reverse phase C_{18} column. The mobile phase is CH_3CN-2% acetic acid (20 + 80) with detection at 365 nm. The method was tested for linearity, recovery, and ruggedness, and compared with the AOAC colorimetric assay by using field samples containing 0.0055-0.055% furazolidone. Precision data suggest a cumulative relative standard deviation of 1.43% within days and 1.78% between days. The ruggedness test predicts a between-laboratory relative standard deviation of 3.67%. Recovery was 97.5 ± 2.0% and linearity was excellent (r^2 = 0.9994) up to 0.06% furazolidone. Premixes are extracted by shaking with dimethylformamide. An aliquot of the extract is diluted (1 + 1) with 5% tetraethylammonium bromide, clarified, and chromatographed.

Furazolidone, 3-((5-nitrofurfurylidone)amino)-2-oxazolidinone, is a coccidiostat used in poultry and swine feeds. The most common level in finished feeds ranges from 0.011 to 0.033%; lower levels are not common (1). Premixes may contain 2.2, 11, or 22% furazolidone.

The official AOAC method for determining furazolidone in finished feeds involves colorimetric detection of the phenylhydrazine HCl adduct (2). Other methods involve dc polarography (3), ac polarography (4), and thin layer chromatography (5, 6). Several methods have been published recently involving high performance liquid chromatographic (HPLC) determination of furazolidone in feeds (7-11). Premixes may be determined spectrophotometrically ((1973) Hess and Clark, Inc., Ashland, OH).

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The recommendation of the Associate Referee was approved by the General Referee and Committee G and was accepted by the Association. See *J. Assoc. Off. Anal. Chem.* (1981) 64, 444.

This paper describes a thoroughly studied HPLC method for finished feeds and premixes which offers the range, selectivity, precision, accuracy, ruggedness, and ease of manipulation not possible in any one of the above methods.

METHOD

Furazolidone solutions are light-sensitive. Extracts and standards must be protected from direct sun and artificial light.

Principle and Scope

Premixes.—Unground sample is extracted with dimethylformamide (DMF), and adjusted with 5% tetraethylammonium bromide (TEAB) to ca 55 μg/mL for HPLC. For premixes containing 1-22% furazolidone.

Finished feeds.—Feed is extracted with water-acetone on continuous extraction apparatus, dissolved in DMF, and adjusted with 5% TEAB (to separate fat from extract), cooled, and clarified for HPLC. For feeds containing 0.005-0.05% furazolidone.

Apparatus

(a) *High pressure liquid chromatograph.*—Varian Model 5000 equipped with Varian Model 8055 Autosampler (Varian Associates, Palo Alto, CA 94303), or equivalent. Perkin-Elmer LC-75 variable wavelength detector (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent.

(b) *Chromatographic column.*—25 cm × 4.6 mm id, containing LiChrosorb RP-18, 10 μm (Unimetrics Corp., Anaheim, CA 92801), or equivalent. Guard column: 5 cm × 2 mm id, dry-packed with 30-40 μm Perisorb RP-18, fitted with 2 μm frits, or equivalent (optional). Chromatographic conditions: 20 μL loop; flow 1.5 mL/min; detection at 365 nm with 0.32 AUFS. Mobile phase: acetonitrile (HPLC quality)—2% acetic acid in distilled, deionized water (20 + 80) or as adjusted to give k' = 3 for furazolidone.

(c) *Continuous extraction apparatus.*—Goldfisch (Labconco Corp., Kansas City, MO 64132), or equivalent.

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(d) *Extraction thimbles.*—Whatman, single thickness, 19 × 88 mm.

(e) *Syringe.*—30 mL Plastipak disposable, ca 21 mm id (Fisher Scientific, No. 14-823-180).

(f) *Filter pads.*—Whatman GF/D microfiber filters (Fisher No. 09-874-42), 22 mm diameter.

Reagents

(a) *Extractants.*—Dimethylformamide (DMF), reagent grade for premixes. Acetone plus water for finished feeds.

(b) *Diluent.*—5% Tetraethylammonium bromide (5% TEAB) (Eastman No. 1516) in distilled, deionized water.

(c) *Furazolidone standard solutions.*—(*1*) *Stock solution.*—1.10 mg/mL. Accurately weigh ca 0.110 g (W_s) furazolidone standard (Hess and Clark, Ashland, OH 44805) into 100 mL volumetric flask. Dissolve in and dilute to volume with dimethylformamide (stable if stored in dark; sonication aids dissolution). (*2*) *Intermediate solution.*—110 μg/mL. Dilute 10 mL stock solution to 100 mL with dimethylformamide (stable if stored in dark). (*3*) *Working standard solution.*—55 μg/mL. Mix 10.0 mL intermediate solution with equal volume of 5% TEAB. Let cool to room temperature. Prepare daily. Dilution of standard (D_s) is 2000.

Finished Feed Assay

Extraction.—Determine approximate sample weight (W_u) to extract ca 550 μg furazolidone, where W_u = 0.055/% guarantee. Accurately weigh (to nearest 1 mg) the approximate weight of ground sample into extraction thimble. Press cotton plug down onto top of feed to prevent channeling. Add 50 mL acetone, 3 mL water, and 2 or 3 boiling chips to extraction beaker, and extract overnight. (Three hours extraction recovers 95–100% furazolidone, but for complete recovery in all cases overnight extraction is recommended.) Evaporate solvent on steam bath (stream of air directed into beakers or blowing across beakers, as with partially closed hood door, will hasten evaporation). If any water is evident in beaker following initial evaporation, add ca 25 mL acetone, swirl to mix, and re-evaporate. Remove when evaporation is complete. Add 5.00 mL DMF, heat on steam bath until bottom of the beaker is hot (15–30 s) and swirl, washing sides, to dissolve residue. Add 5.00 mL 5% TEAB, mix, cover with watch glass, and cool to room temperature. Transfer extract to 15 mL centrifuge tube and centrifuge 5–10 min at 2000 rpm (2500× g). Using aspirator and trap, remove fat layer floating on supernate.

Table 1. Sample sizes, dilutions, and total sample dilutions

Label claim, %	Sample wt (W_u), g	Dilns with DMF, mL	Total sample diln (D_u), mL
1.0	2.00	none	400
2.2	1.00	none	400
3.3	1.00	30/50	666.7
11.0	1.00	20/100	2000
22.0	1.00	10/100	4000

Clarification.—Insert GF/D filter disk into 30 mL syringe barrel fitted with large bore (12–16) needle. Use top of test tube, which is just smaller than syringe, to push filter pad onto shoulder at bottom of syringe barrel, taking care not to push down on center of pad. Clarify aqueous DMF extract by gravity filtration through pad into test tube. Clarified extract is ready for chromatography. Dilution (D_u) for finished feed samples is 10.

Chromatography.—Make several injections of furazolidone working standard, adjusting the mobile phase strength to give $k' \geq 3$ and peak height 60–80% full scale. Make 2 or more injections of standard to ensure that peak heights are repeatable (within 1–2%). Each 2 sample injections should be bracketed by standard injection. Average of peak heights (or peak areas) for standards (P_s) bracketing each pair of samples (P_u) is used for calculating furazolidone concentration in samples.

Premix Assay

Accurately weigh g unground sample (W_u) indicated (Table 1) into 200 mL volumetric flask. Add 100–150 mL DMF, stopper, and shake 30 min. Dilute to volume with DMF and mix. Let suspended material settle, centrifuge, or filter. Make any indicated dilutions with DMF (Table 1). Add 5.00 mL extract (or diluted extract) to 5.00 mL 5% TEAB solution, mix, and let cool to room temperature. Clarify and carry out chromatography as done for finished feeds. Total sample dilutions (D_u) for premixes are listed in Table 1.

Calculations

Furazolidone, %

$$= (P_u/P_s) \times (W_s/W_u) \times (D_u/D_s) \times 100$$

where P_u and P_s = peak heights of sample (unknown) and standard, respectively; W_u and W_s = g sample and standard, respectively; and D_u

and D_s = mL total dilutions of sample and standards, respectively. Total dilutions are determined as follows: If 1 g/200 is followed by serial dilutions of 20/100 and 5/10, then total dilution is 1 g/200 × 20/100 × 5/10 = 1 g/2000 or D_u = 2000 mL.

Results and Discussion

In preliminary studies with finished feeds, several different extraction techniques were tried; shaking, sonication, overnight soaking, heating to 50 or 100°C before shaking, and continuous extraction on a Goldfisch apparatus. Extractants tried were DMF (2), 95% DMF on a pre-wetted sample (10), acetone (6, 7, 9), tetrahydrofuran, and various amounts of water in acetone. In our preliminary methods development experiments, we attempted to assay samples by using the hot DMF extraction used in the official AOAC method (2). Injection of the clarified DMF extract resulted in precipitate forming in the mobile phase and an increase in column back pressure. Even though we tried several solvents to dilute the extract and force precipitate formation before clarification, after several days the analytical column became irreversibly plugged. The use of 95% DMF on a pre-wetted sample (10) was compared with continuous extraction on the Goldfisch apparatus with acetone on 5 samples with guarantees of 0.0175 or 0.033%. The recoveries for the 95% DMF extractant ranged from 76 to 94%. Two subsequent comparative studies gave similar results. We settled on acetone-water (93 + 7) by continuous extraction because of better recovery, the ability to concentrate the extract, and the ease of extract cleanup. Warm dimethylformamide (DMF) is a good solvent for dissolving the residue following evaporation of the extractant. The extract could be separated from fat and fat-soluble interferences by diluting clear DMF solution with an equal volume of aqueous 5% tetraethylammonium bromide (TEAB). With centrifugation, a layer of fat collects at the surface of the extract and can be removed by aspiration. Substitution of water or 1% TEAB for 5% TEAB results in a colloidal suspension that is difficult to clarify.

Clarified solutions of furazolidone are very light sensitive. A solution allowed to stand on an open laboratory bench for 20 min before injection resulted in a reduction in peak height of about 5%. There is no loss of furazolidone if the clarified extract is stored in the dark.

Furazolidone has absorption maxima at about 365 and 260 nm. The maximum at 365 is normally chosen for detection because there are fewer potential interferences at this wavelength (7, 9–11).

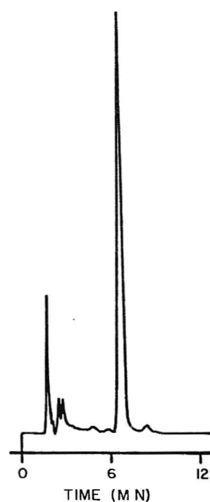

Figure 1. Chromatogram of extract from commercial feed guaranteed at 0.011% furazolidone (ca 1 μg furazolidone).

Silica (8), C_8 (9, 11), and C_{18} (7, 10) analytical HPLC columns have been used for furazolidone analysis. We tried several different C_{18} columns and found that with slight mobile phase modification, to maintain a k' of 3, all were satisfactory.

The mobile phase used is an adaptation of the one first used by Cieri (7).

In general, the chromatograms are free of extraneous peaks. The chromatogram reproduced in Figure 1 is an extract of a 0.011% level sample and contains more extraneous peaks than are normally found at that concentration of furazolidone.

The absence of interferences under the furazolidone peak was indicated by obtaining chromatograms of a standard and typical sample at 5 wavelengths in the 330–400 nm range and comparing the peak height ratios of sample to standards at each.

We studied recovery and linearity by adding varying amounts of standard furazolidone to 5 g each of hog finisher feed weighed into extraction thimbles. The concentrations studied ranged from 0.0014 to 0.06% furazolidone. In 2 experiments the recovery was 99.7 ± 1.1% and 97.5 ± 2% (mean ± standard deviation). Least squares analysis of the data showed the response to be linear with a coefficient of determination (r^2) of 0.9998 in one study and 0.9994 in the other.

Table 2. Determination of furazolidone in feeds: HPLC vs official method

Label claim, %	Found,[a] %	
	AOAC	HPLC
0.005	0.0052	0.0041
0.011	0.0097	0.0098
0.011	0.0102	0.0091
0.0165	0.0131	0.0135
0.022	0.0202	0.0209
0.033	0.0275	0.0342
0.033	0.0250	0.0255
0.05	0.0436	0.0490

[a] Averages of 2–4 determinations.

Table 3. Within-day and between-day variation of HPLC results

Label claim, %	Number of diff. samples in group[a]	Cum. rel. std dev., %	
		Within-day	Between-days
0.005	1	2.25	1.51
0.11	6	1.48	1.41
0.0165	6	1.51	2.33
0.022	3	0.86	1.72
0.033	6	1.59	1.62
0.05	2	0.92	1.42
Overall	24	1.43	1.78

[a] Duplicates assayed twice on different days.

We determined the accuracy of the method by analyzing 2–4 replicates each of 8 samples by both HPLC and the official colorimetric method (2). The furazolidone concentrations in these commercial samples ranged from 0.005 to 0.05% (Table 2). The average difference between results obtained by the 2 methods was not significantly ($P > 0.05$) different from zero by the paired Student's t-test. Hence, no bias exists between methods.

We studied the precision of the HPLC method by assaying a group of 24 commercial samples having label claims ranging from 0.005 to 0.05% furazolidone, in duplicate on 2 days (Table 3). Within-day precision was determined by averaging the relative standard deviation of sample duplicates assayed on the same day.

Between-day precision was determined by calculating the relative standard deviation between the within-day means for each sample. The results presented are the average relative standard deviations within label-claim groups. It should be noted that this method of evaluating between-day variation segregates within-day variation, thus underestimating the variance one should expect from running samples singly on different days. It does demonstrate the method's resistance to day-to-day changes.

We studied the ruggedness of the method (12) by varying: (a) the length of time the extraction beaker remained on the steam bath following evaporation of the extractant, (b) the time of warming the beaker on the steam bath to dissolve the residue in DMF, (c) the technique for swirling the warm DMF in the beaker to dissolve the residue, (d) the strength of the TEAB solution, (e) the centrifugation time, (f) the relative amount of light exposure on the crude extract, and by adding a small amount of acetone back into the extraction beaker just before adding DMF. The relative standard deviation of 3.67% should be indicative of the variation which would be expected among laboratories.

We chromatographed standard solutions of the following drugs which may be co-additives or present as contaminants in furazolidone medicated feeds: nitrofurazone, amprolium, ethopabate, lasalocid sodium, dimetridazole, sulfanitran, and sulfaquinoxaline. None of these produced peaks that interfered with the furazolidone peak.

Furazolidone premixes can be assayed spectrophotometrically after extraction with DMF ((1973) Hess and Clark, Inc., Ashland, OH). We chose shaking for 30 min as the optimum extraction after using various extraction times to assay routine regulatory samples ranging in guarantees from 0.88 to 22% furazolidone.

Extracting ground premix samples, instead of unground samples, resulted in low recoveries (86%). Recovery of spiked samples, prepared by adding furazolidone to 1.0 g portions of soybean meal at the 2.2, 11, and 22% level, was 99.7 ± 3.4%.

We assayed some low level samples by a slight modification of the HPLC method. A sample containing about 30 ppm furazolidone was assayed by extracting 11 g sample and dissolving the residue in 3 mL DMF + 3 mL 5% TEAB (instead of 5 mL DMF + 5 mL TEAB), and by using a 100 μL loop for injection. The chromatogram was satisfactory with the usual cleanup. We assayed a sample suspected of being contaminated with furazolidone by extracting 10 g sample, using a 10 μg/mL standard, a 100 μL loop, and a higher detector sensitivity than specified in the method. The sample and a spiked sample (in the DMF–TEAB soln) were passed through a 1 cm (id) column containing 3 g alumina (Fisher Basic No. A-941) before HPLC. The resulting chromatograms were quite clean. The sample contained about 7 ppm furazolidone when the

results were determined by comparison with a direct standard or by standard addition.

From our experience in using essentially the same extraction in preparing samples for ac polarography (4) and from some preliminary work with routine samples, it appears that the method can be used to assay nitrofurazone in finished feeds and premixes with no modification. We found, as reported by Cieri (7) using a similar HPLC system, that nitrofurazone and furazolidone are well separated, with nitrofurazone eluting first.

The HPLC method presented is rugged, appears to have good precision, linearity, and accuracy, and offers selectivity not afforded by the official method. Also, the HPLC method covers a wider range of furazolidone concentrations, requires less expertise to carry out, and, with an Autosampler, allows more sample throughput than the official method. The method can be used for the most common levels of finished feeds and premixes and, with minor modifications, can be used for low level samples. It also appears that the method can be used for nitrofurazone determination, although this has not been rigorously tested.

Acknowledgments

We thank Joseph L. Lewis, Smith Kline Animal Health Products, for supplying commercial samples that were outside the guarantee range available to us as routine regulatory samples.

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METALS AND OTHER ELEMENTS

Simplified Apparatus for Determination of Mercury by Atomic Absorption and Inductively Coupled Plasma Emission Spectroscopy

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A simplified apparatus has been applied to the determination of mercury by cold vapor generation. The equipment consists of a reaction flask incorporating a side arm in which a rubber septum is mounted. A sample solution is injected from a syringe through the rubber septum into the reaction flask, where it is mixed with a stannous chloride reducing solution. The elemental mercury generated is then swept with a carrier gas to the inductively coupled plasma (ICP), an absorption cell of an atomic absorption spectrometer, or a nondispersive UV monitor for determination. Detection limits were 0.009 and 0.005 μg with atomic absorption and the UV monitor, respectively, and 0.09 μg with the ICP. Repeatability of the procedure was 1.4% at 0.66 μg injected mercury with the ICP and 5.2% at 0.45 μg injected with atomic absorption. Tuna and halibut samples fortified with from 0.09 to 1.31 μg mercury/g were analyzed by the AOAC official method and the procedure described here. The average mercury recovery was 103% with the ICP, and 99% with the UV monitor and with atomic absorption. The procedure is free from interference by elements commonly present in biological material.

Numerous analytical methods have been developed for the accurate determination of mercury in foods and other samples. These methods are needed because mercury has contaminated the environment, ultimately finding its way into the food chain. The vehicle by which mercury enters the food chain and its toxicity have been adequately discussed elsewhere (1-5).

Mercury in biological material has been determined by a method in which the material is directly combusted in oxygen (6), and the mercury is temporarily collected on a gold filter, released, and determined photometrically. Pappas and Rosenberg (7) burned samples in oxygen and collected the combustion products in 1.2 N HCl. After the solutions were neutralized, mercury was collected as HgS on CdS asbestos pads. The pads were then pyrolyzed and the mercury vapor was swept to a cold vapor atomic absorption cell for determination.

Hatch and Ott (8) developed a sensitive method for determining mercury down to 1 ppb in solution. Mercury was reduced by a stannous sulfate solution and detected by flameless atomic absorption. Linstedt (9) used a tin (II) reducing solution to determine mercury in urine samples. Munns and Holland (10) carried out a collaborative study in which mercury vapor generated by a stannous chloride-hydroxylamine solution was detected by flameless atomic absorption. Overall mercury recovery was 83.5% for spiked fish samples. In another method, mercury generated by stannous chloride reduction of water samples was amalgamated onto silver wool (11). The wool was then heated to release the mercury, which was detected by a microwave-induced argon plasma emission system.

Most methods in use today rely on some type of cold vapor generation of mercury. Magos and Clarkson (12, 13) selectively determined the total and inorganic forms of mercury, respectively, by using stannous chloride or a stannous chloride-cadmium chloride reagent. Organic mercury was then calculated by difference. The mercury was detected by atomic absorption.

Use of the inductively coupled plasma (ICP) for mercury determination has generally been limited to direct solution analysis. One study (14) evaluated the 253.65 and 184.96 nm lines for mercury determination, including assessment of interferences at each line. In later work (15), 20-50 mL sample solution containing mercury was added to a reaction flask, followed by 3 mL stannous chloride reducing solution; the flask was connected to the ICP, and the mercury vapor was swept to the plasma for determination. The method suffered from poor reproducibility and required the use of background correction. In addition, peaks obtained by this method were broadened because of the time required to strip

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mercury from the relatively large volume (up to 50 mL) of sample solution.

The procedure reported in this paper uses the ICP, an atomic absorption spectrometer (AAS), or a UV monitor to detect mercury generated by reduction with a stannous chloride reducing solution. The sample solution is introduced to the reducing solution by injection from a syringe through a rubber septum in a side arm of a completely sealed reaction flask. Mercury vapor is then swept to the plasma by argon. This procedure eliminates the possibility that air will be introduced into the plasma, which might extinguish it or cause a change in background emission. With the apparatus described, as many as 20 samples can be analyzed without dismantling the equipment.

METHOD

Reagents

Use distilled, deionized (DD) water throughout. Soak all glassware overnight in 10% nitric acid; then rinse with DD water.

(a) *Diluting solution.*—Add 50 mL concentrated redistilled HNO_3 and 100 mL concentrated H_2SO_4 (spectrochemical grade) to ca 300 mL DD water, and let cool. Then add 1 mL hydrogen peroxide and 0.2 g potassium dichromate, and dilute to 1 L with DD water.

(b) *Mercury standard.*—1000 μg/mL. Dissolve 0.1354 g $HgCl_2$ in 100 mL DD water.

(c) *Reducing solution.*—Mix 50 mL H_2SO_4 with ca 300 mL DD water and cool to room temperature; then dissolve 15 g NaCl, 15 g hydroxylamine sulfate, and 25 g stannous chloride in this solution and dilute to 500 mL with DD water.

Apparatus

(a) *Reaction flask.*—See Figure 1. Flask was constructed from 30 mm od and 26 mm id pyrex tube with bulb blown out in middle to volume of ca 100 mL. Final length of flask was 19 cm. A 35/25 ball joint was mounted on top. Rubber septum (20 mm diameter) was fastened to the side arm by an aluminum seal (Alltech Associates, Inc., 202 Campus Drive, Arlington Heights, IL 60004).

(b) *Inductively coupled plasma (ICP).*—Jarrell-Ash Model 975 Plasma Atom Comp (Jarrell-Ash Co., 590 Lincoln St, Waltham, MA 02154) equipped with half-meter Ebert scanning spectrometer (f/8.6, and reciprocal linear dispersion 1.6 nm/mm). Forward power to plasma was maintained at 1100 W with reflected power less than 2 W. Argon flow carrying sample to plasma

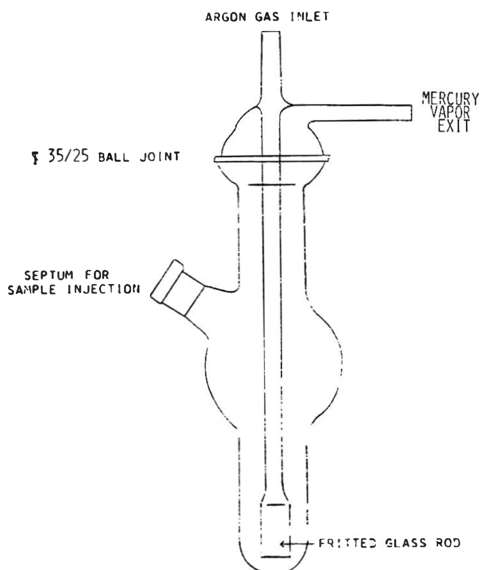

Figure 1. Reaction flask. Sample is injected, through a side arm, into the flask where it is mixed with reducing solution. Mercury vapor produced is then swept by the argon carrier gas to the plasma or absorption cell for detection.

was 0.8 L/min. Mercury was monitored by using the 253.7 nm line.

(c) *Atomic absorption spectrophotometer.*—Perkin-Elmer Model 403 equipped with mercury hollow cathode lamp and gas flow through cell as described in ref. 16. Operating conditions: wavelength 253.7 nm, slit width 1 mm.

(d) *ICP readout electronics.*—Jarrell-Ash Model 26-770 electrometer-amplifier, with Hewlett-Packard 3380A integrator for recording and integration.

Preparation of Sample

Canned, fresh, or frozen fish: If frozen, thaw first. Remove nonedible parts. Weigh fish sample and transfer to blender (5 g sample is required for each replicate). Add weight of distilled water equal to exactly 10% of weight of fish, and blend mixture at medium speed 2–3 min until homogeneous slurry is obtained. Transfer slurry to glass or plastic jar, cap tightly, and store in refrigerator until needed. If homogenized sample is frozen, thaw and rehomogenize before analyzing (5.5 g prepared sample is equivalent to 5.0 g original sample).

Sample Digestion

Weigh 5.5 g sample homogenate (equivalent to 5 g original sample) into digestion flask (125 mL flat-bottom Erlenmeyer flask) and add 20 mL

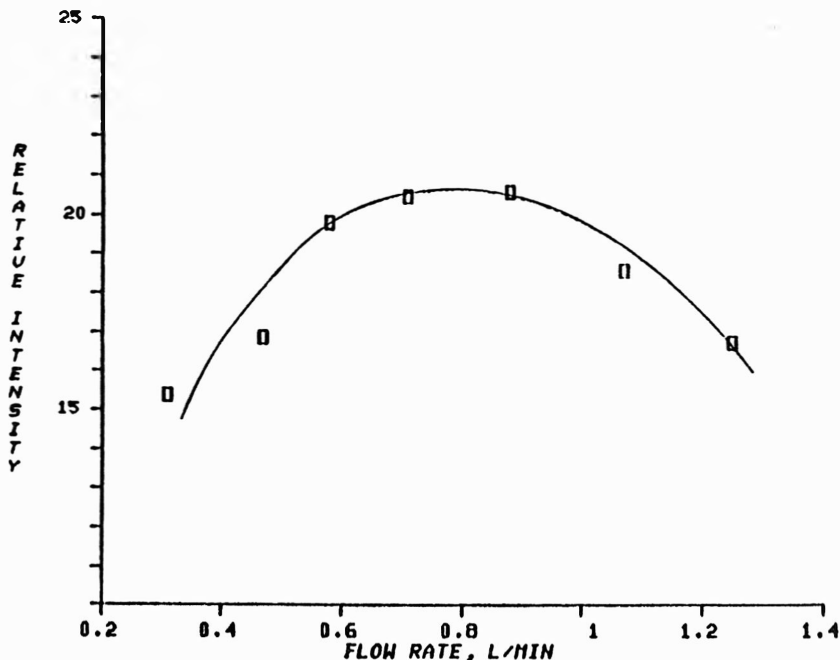

Figure 2. Variation of ICP emission intensity (integrated area of peak) for mercury vs carrier gas flow rate. Each injection was 100 μL, containing 0.66 μg mercury.

HNO_3-H_2SO_4 (2 + 1) and 10 mg vanadium pentoxide. Add 3 or 4 boiling chips and connect condenser to digestion flask. After starting water flow, apply heat to produce gentle boiling for ca 45 min. Cool to room temperature, transfer to 100 mL volumetric flask, and dilute to volume with diluting solution.

Procedure

Dilute 25 mL reducing solution with 25 mL DD water and add to reaction flask. Connect reaction flask to absorbance cell or ICP and start gas flow at 1.5 L/min (AAS) or 0.8 L/min (ICP). Construct calibration curve by injecting 0, 0.05, 0.1, 0.15, and 0.2 μg mercury and plotting ab-

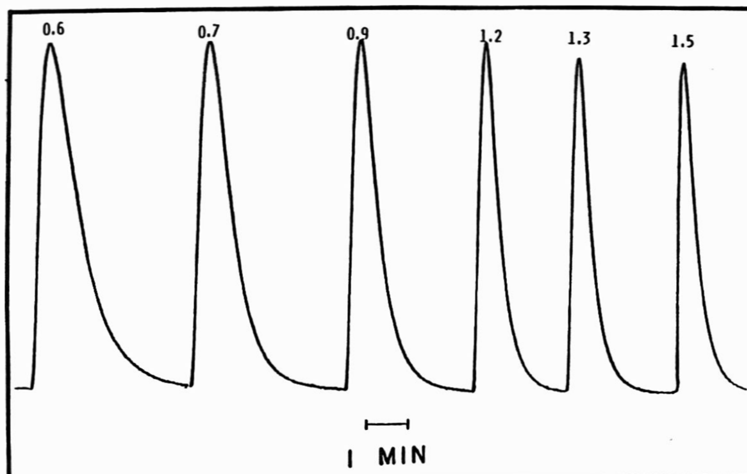

Figure 3. Influence of flow rate on peak shape obtained by atomic absorption. Gas flow (L/min) appears directly above each peak.

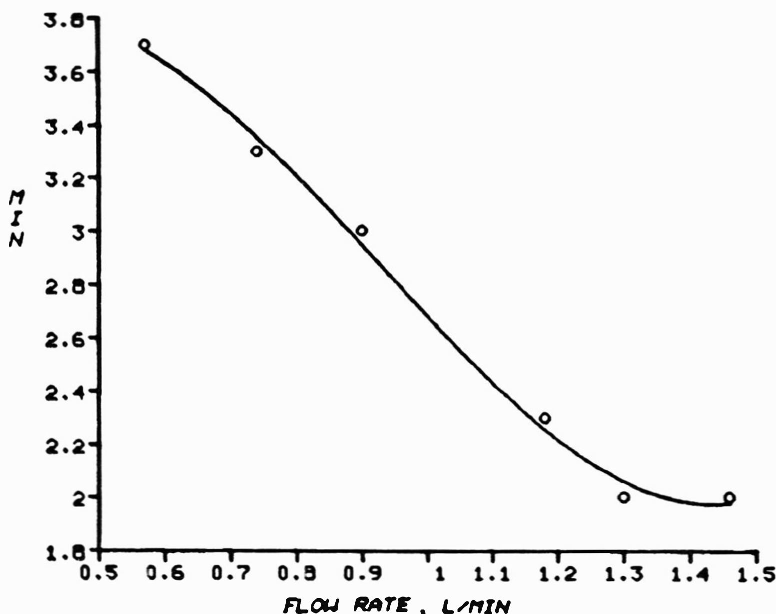

Figure 4. Influence of carrier gas flow rate on time to record one peak obtained by atomic absorption.

sorbance vs μg mercury (for AAS) or emission intensity vs μg mercury (for ICP). To determine mercury in sample, inject 1-3 mL sample solution and monitor absorbance or emission intensity; determine mercury content from calibration curve. Calculate mercury content of original sample from following equation:

$$\text{Mercury, } \mu g/g = C[D/(I_v \times SW)]$$

where D = volume to which sample digest was diluted; I_v = volume (mL) sample solution injected; SW = original sample weight; C = total μg mercury in injected sample solution.

Results and Discussion

Carrier Gas Flow Rate

The optimum argon carrier flow rate to yield a maximum mercury emission signal in the plasma was first determined. A 100 μL sample containing 0.66 μg mercury was injected into the reaction flask, and the resulting emission signal at different carrier gas flow rates was monitored. Figure 2 is a graph showing flow rate vs signal intensity. As the flow rate is increased beyond 0.3 L/min, the resulting emission intensity increases until a plateau is reached at approximately 0.8 L/min. Increasing the carrier gas flow rate beyond 0.8 L/min results in a decreasing emission signal. All subsequent ICP work reported here was carried out with a carrier gas flow rate of 0.86 L/min.

Figure 3 illustrates the effect of carrier gas flow rate on peak shape when using atomic absorption. As the flow rate is increased from 0.6 to 1.5 L/min, peak width becomes smaller, although peak height (proportional to absorbance) remains constant.

Figure 4 is a plot of flow rate vs time required to record the entire peak. At a flow rate of 0.6 L/min, approximately 3.7 min is required. The time required to record a peak decreases with increasing flow rate until a plateau is reached at about 1.5 L/min. To increase the speed of analysis, the higher flow rate of 1.5 L/min is most effective. Subsequent work performed by atomic absorption was carried out at 1.5 L/min.

Repeatability

The recorder tracings in Figure 5 illustrate the repeatability of the ICP determination. Tracing A represents 9 consecutive 40 μL sample injections, each containing 0.11 μg mercury; the coefficient of variation (CV) of the integrated areas is 5.1%. Tracing B represents 9 consecutive 100 μL sample injections, each containing 0.26 μg mercury; CV is 3.0%. Tracing C represents 9 consecutive 50 μL sample injections each containing 0.66 μg mercury; CV is 1.4%. Repeatability with this procedure is equivalent to that generally obtained with conventional ICP analysis. Linearity of the calibration curve was tested from 0.09 μg (the detection limit with ICP)

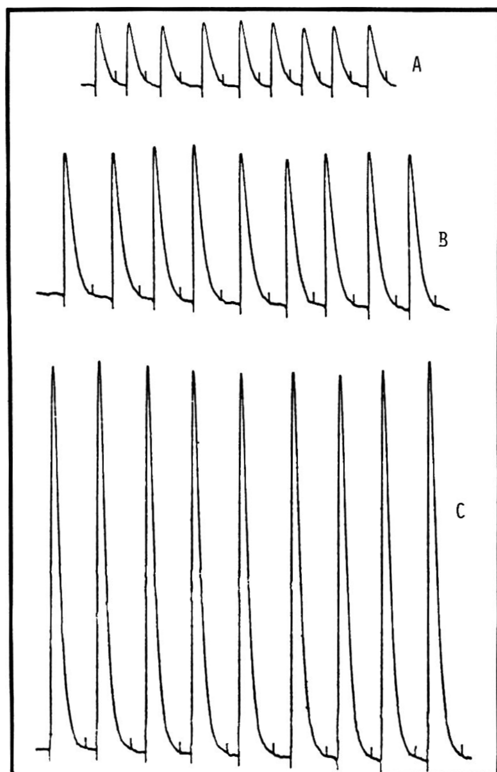

Figure 5. ICP repeatability data. Each tracing illustrates 9 consecutive mercury injections. A: 40 μL injection containing 0.11 μg mercury; coefficient of variation (integrated area), 5.1%. B: 100 μL injection containing 0.26 μg mercury; coefficient of variation (integrated area), 3.0%. C: 50 μL injection containing 0.66 μg mercury; coefficient of variation (integrated area), 1.4%.

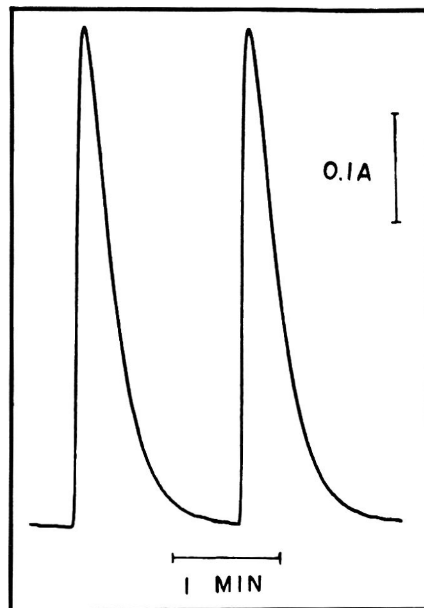

Figure 6. Repeatability of peak height with different sample volumes. Peak on left was obtained from injection of a 1.0 mL sample containing 0.82 μg mercury/mL; peak on right was obtained from injection of a 0.1 mL sample containing 8.20 μg mercury/mL.

to 1.32 μg mercury and was excellent. Linearity beyond 1.32 μg was not tested.

Repeatability with atomic absorption was approximately equivalent to that with the ICP. For nine 50 μL injections of 0.45 μg mercury CV (peak height) was 5.2%. Linearity of the atomic absorption calibration curve was excellent from 0.009 μg (detection limit with AAS) to 0.89 μg of injected mercury. The UV monitor exhibited good linearity from 0.005 μg (detection limit with UV monitor) to 0.09 μg of mercury. Linearity beyond 0.89 μg and 0.09 μg, respectively, for the AAS and the UV instruments was not tested.

Interferences

To determine if any elements commonly present in biological material affect the mercury emission or absorbance intensity, the emission or absorbance of a sample containing 1.2 μg of mercury was compared to the signal intensity of a sample containing 1.2 μg mercury in the presence of 91 μg of potentially interfering elements. Elements tested included Al, Ca, Co, Cu, Cr, Fe, Ni, Mg, Mn, P, and Sb. None of these elements affected the mercury emission or absorption signal.

Figure 6 is a recorder tracing of 2 peaks obtained by this procedure in which the same amount of mercury was injected in different sample volumes. The peak on the left represents a 1 mL injection of a 0.82 μg/mL solution of mercury; the right peak represents a 0.1 mL injection of an 8.20 μg/mL solution. The resulting absorbance for each sample is the same; thus equivalent amounts of mercury will give the same response over this range of sample volume.

To evaluate the applicability of this procedure for the analysis of "real world" samples, halibut and tuna were spiked at different mercury levels and analyzed by the procedure reported here. Results obtained by this procedure are compared in Table 1 with those obtained by the AOAC official method (16) for mercury. All samples were analyzed in duplicate. Excellent agreement was

Table 1. Comparison of methods for determination of mercury in fish

Mercury added, µg/g	Mercury found, µg/g			
	UV monitor	AAS	ICP	AOAC, ref. 16
Halibut[a]				
0.09	0.07	0.10	—[b]	0.09
0.09	0.08	0.08	—[b]	0.11
0.26	0.28	0.31	0.31	0.25
0.26	0.28	0.31	0.28	0.24
0.65	0.69	0.67	0.68	0.71
0.65	0.66	0.67	0.66	0.71
0.92	0.96	0.87	0.95	0.95
0.92	0.87	0.85	0.91	0.84
1.25	1.21	1.10	1.28	1.10
1.25	1.20	1.10	1.26	1.28
Tuna[c]				
0.09	0.12	0.08		0.10
0.09	0.11	0.07		0.10
0.26	0.25	0.23		0.30
0.26	0.26	0.23		0.30
0.45	0.43	0.42		0.48
0.45	0.44	0.48		0.58
0.87	0.82	0.83		0.88
0.87	0.84	0.85		0.95
1.31	1.27	1.38		1.58
1.31	1.31	1.49		1.44

[a] Residual Hg in halibut, 0.09 µg/g.
[b] Too low for accurate determination.
[c] Residual Hg in tuna, 0.13 µg/g. ICP determination not carried out on tuna.

obtained between the AOAC official method and the procedure reported here. Average recoveries were 106% by the AOAC method, 103% by the procedure reported here using the ICP, and 99% using the UV monitor and atomic absorption.

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VITAMINS AND OTHER NUTRIENTS

Neutron Activation Analysis of Total Diet Food Composites for Iodine

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The iodine content of Total Diet food composites was measured using neutron activation analysis. The interfering element chlorine was separated using a modified combustion and gas phase procedure. The average recovery was 94.8% (standard deviation 2.9) for the 10 matrices that were tested. In addition, iodine was measured in National Bureau of Standards Standard Reference Materials, which have no certified values for this element. Preliminary findings of iodine content of adult Total Diet market baskets collected during Fiscal Year 1980 in different regions of the United States ranged from 292 to 901 μg/day for a 2900 kcal intake.

Recently there has been great interest in evaluating the iodine content of the American diet for different age categories. Previous reports (1, 2) indicated that consumption of iodine by Americans is many times the Recommended Dietary Allowances of 40–150 μg/day (3). In addition, some studies (4, 5) have pointed out that excessive iodine intake can contribute to certain thyroid disorders in susceptible individuals.

The low iodine content of food, the complexity of the matrix, potential interferences, the possibility of contamination, and loss during analysis all are limiting factors of iodine determination. Therefore, results may vary when different analytical techniques are used (6, 7). Methods for iodine determination in foods, using colorimetry (6, 8), ion selective electrodes (6, 9), and gas chromatography (10), suffer some limitations as described above, and/or are focused on specific food matrices and chemical iodine forms.

We are concerned with the determination of iodine in composite diet samples collected as part of the Food and Drug Administration's (FDA) Total Diet Studies (11) in order to evaluate the daily intake of iodine. Each adult Total Diet market basket consists of 12 composite commodity groups: dairy products; meat, fish, and poultry; grain and cereal products; potatoes; leafy vegetables; legume vegetables; root vegetables; miscellaneous vegetables; fruits; oils, fats, and shortening; sugar and adjuncts; and beverages, collected in 4 different areas of the United States. From 1975 to the present, iodine has been determined in these Total Diet composites using a colorimetric method (12). For this study, neutron activation analysis (NAA), which is probably the most sensitive analytical technique available for determining iodine, was used. In spite of the sensitivity of NAA, a preliminary radiochemical separation is needed because of interference from high chlorine levels present in foods. Previous NAA methods for iodine separation have used chemical distillation (13) and liquid-liquid extraction (14). The combustion and gas phase separation reported by Rook (15) was of particular interest because the separation can be completed in one step. This is important since a multi-step separation procedure can lead to errors due to volatilization of iodine and decay of iodine activity. In addition, the separation of chlorine is excellent. This method was modified as described below to maximize recoveries and precision of results for food analyses.

Iodine was determined using this modified separation in National Bureau of Standards (NBS) Standard Reference Materials (SRMs), which do not have certified values for this element. Total Diet samples were then examined using this procedure, and the results of 5 market basket analyses were compared with those obtained with the colorimetric procedure (12).

METHOD

Apparatus

(a) *Separation apparatus*.—Entire apparatus was assembled with 3 quartz tubes (Figure 1): (*1*) 70 cm × 17 mm id combustion tube, (*2*) 1 cm × 9 mm id tube containing hydrated manganese dioxide (HMD), (*3*) 9 cm × 6 mm id tube containing silver-coated quartz wool. Tubes were connected with 19/38 and 10/30 ₸ joints, respectively. Combustion boat was 95 × 13 × 11 mm. Flowmeter with 0–100 mL/min range was used.

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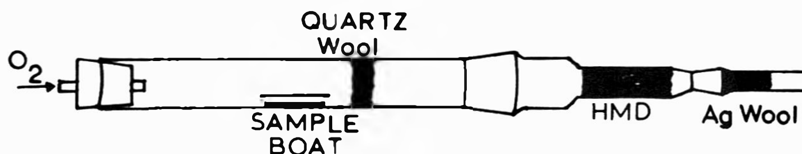

Figure 1. Quartz separation tube used for iodine determination.

(b) *Tubular furnace.*—Controllable within range 200–1200°C (Lindberg Hevi Duty, Sola Basic Industries, 304 Hart St, Watertown, WI 53094).

(c) *Neutron source.*—Samples were irradiated in 10 megawatt reactor at NBS, Gaithersburg, MD. Thermal neutron flux in irradiation tube was 5×10^{13} n/cm^2/s (16).

(d) *Radioassay equipment.*—Gamma ray detection system. Ortec Well Ge(Li) detector coupled to Nuclear Data ND6620 multichannel analyzer.

Reagents

(a) *Hydrated manganese dioxide.*—C. Erba, Via Inibouati 24, 20159 Milan, Italy. Three cm HMD columns were packed between 2 quartz wool plugs, 4 cm from the beginning of the second quartz tube.

(b) *Silver-coated quartz wool.*—Wet 10 g quartz wool lightly with ammoniacal solution of 4–5 g silver nitrate in 20–30 mL water. Add 20 mL 5% ascorbic acid in water and thoroughly mix with quartz wool to obtain uniform silver mirror. Dry in oven and burn quartz wool on Bunsen flame until all organic material is volatilized (other methods may also be suitable for silver plating). Pack 5 cm long silver-wool column in smallest tube.

(c) *Iodine carrier solution.*—80 mg/mL. Dissolve 2.64 g KI in 25 mL water.

(d) *Iodine standard solutions.*—*Stock solution.*—500 μg/mL. Accurately weigh 65.5 μg KI, dissolve in water, and dilute to 100 mL with water. Store in dark. *Working solution.*—5 μg/mL. Dilute 1 mL stock solution to 100 mL with water. Store in dark.

Preparation of Samples

Individual food items were prepared as they would be for home consumption and then composited into 12 commodity groups (17). Commodity groups were homogenized and freeze-dried. Freeze-dried samples weighing 100–300 mg each were transferred to quartz vials and sealed.

Procedure

Place quartz-encapsulated samples in polyethylene container and irradiate 15 s. After 2-min decay, cool quartz vial in liquid nitrogen, cut with glass cutter, and open. Transfer sample to combustion boat and add 75 μL (6 mg I) iodine carrier solution. Insert boat and irradiated quartz vial into combustion tube and adjust oxygen flow to 25 mL/min. Ignite sample in front of boat with oxygen-gas torch while heating HMD trap to ca 100°C with Bunsen burner flame. Heat quartz wool plug, placed ca 3 cm from boat, to burn all volatile material. After combustion of sample, place combustion tube in 1000°C furnace. When violet iodine vapor appears, convey it to HMD trap by heating exposed tube. After 4–5 min, almost all iodine is absorbed in HMD column. Thoroughly heat HMD resin with burner flame at ca 150°C until evolution of violet iodine is no longer observed, and then increase temperature to ca 300°C to effect collection of iodine in silver-wool trap. (With practice, it is possible to obtain a constant yellow-silver iodine layer and to evaluate the approximate HMD trap temperature. Trap temperature was checked with a thermocouple in preliminary experiments.) Remove tube containing iodine collectors, place in polyethylene tube, and insert into well of Ge(Li) detector. Count sample 10 min and determine iodine concentration by comparing activities of 443 keV ^{126}I photopeak in sample and standard.

Absorb 200 μL iodine working solution on filter paper disk (1 μg I), seal in quartz vial, and irradiate. Place irradiated standard in sample boat along with ca 200 μg inactive sample, and proceed as with samples. Run standard every 6–8 samples.

Results and Discussion

From some preliminary experiments carried out using Rook's procedure as written (15), it appeared that recoveries and precision, at least for foods, were matrix dependent. Therefore, in an attempt to optimize the procedure with regard to recovery and precision, we varied some

Table 1. Recovery of iodine tracer added to Total Diet composites

Composite	Range of rec., %	Mean rec., % [a]	SD
Dairy products	90.3–99.5	94.8	3.3
Meat, fish, poultry	94.7–98.5	96.4	1.7
Grain, cereal products	93.5–97.2	95.6	1.5
Potatoes	93.8–98.0	95.3	2.1
Leafy vegetables	93.3–98.8	96.5	2.3
Legume vegetables	91.3–97.6	94.6	2.4
Root vegetables	90.3–98.6	94.4	3.2
Miscellaneous vegetables	89.7–97.7	93.1	3.3
Fruits	89.7–94.0	92.4	3.7
Sugar and adjuncts	91.1–98.7	95.1	2.9
Av.		94.8	2.9
Variance test	$F = 1.17$	$F_{0.05}$ d.f. 9,40 = 2.1	

[a] Each value is the average of 5 determinations.

parameters such as HMD column length, oxygen flow, carrier quantity, and the heating temperature of the HMD trap. Oxygen flow was lowered to avoid too much buildup of pressure with some food matrices. The amount of carrier was an important factor in producing a high constant recovery. Uniform heating of the HMD resin was also an important factor. The use of a heating tape did not affect the results. Reducing the length of the HMD trap did not diminish the effectiveness of the chlorine separation.

To assess the validity of the radiochemical procedure in Total Diet composites, which vary widely in inorganic and organic composition, a tracer study with ^{126}I was carried out. A small amount of ^{126}I was added to non-irradiated matrices and the samples were processed through the separation procedure. The activity recovered was compared with the activity of the ^{126}I tracer which had not been chemically separated in the same counting geometry. Five determinations for each of the different food matrices were performed. The results are summarized in Table 1. Some activity, ranging from 2 to 6% of the total, remained trapped in the HMD resin and was not recovered by the procedure. Variance analysis ($F = 1.17$) showed that the mean recoveries were not dependent on the matrix, but rather on the counting variability. At this point, it seemed reasonable to eliminate the recovery step from the procedure.

Precision and Accuracy

Precision of the method was checked by multiple analyses of NBS SRMs, for which the iodine concentration covered a wide range of values. The results are summarized in Table 2. The differences among relative standard deviations must be ascribed mainly to counting variability. The accuracy is difficult to ascertain because SRMs have no certified values for iodine and very few data are available in the literature. Values of 2.8 μg/g for Oyster (7), 0.180 ± 0.012 (15) and 0.28 μg/g (7) for Bovine Liver, and 1.1

Table 2. Concentration of iodine (μg/g wet wt) in NBS Standard Reference Materials (SRM)

Sample No.	Spinach SRM 1570	Oyster SRM 1566	Rice Flour SRM 1568	Bovine Liver [a] SRM 1577
1	1.218	2.935	0.0121	0.238
2	1.330	3.102	0.0102	0.255
3	1.235	3.199	0.0124	0.253
4	1.321	3.158	0.0110	0.256
5	1.233	2.919	0.0101	0.251
Mean, μg/g (wet weight)	1.267	3.062	0.0111	0.246
SD	0.054	0.128	0.0010	0.011
Mean, μg/g (dry weight)	1.325	3.209	0.0121	0.249
SD	0.055	0.134	0.0010	0.012
RSD, %	4.2	4.2	9.0	4.5

[a] NBS value = 0.18 ppm (dry weight basis).

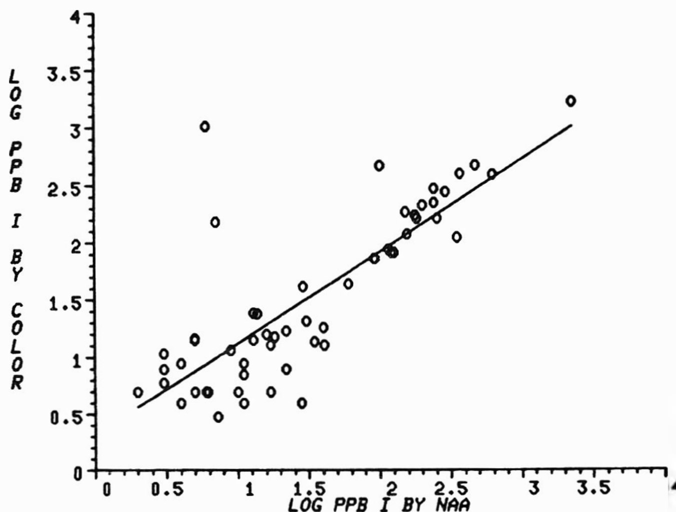

Figure 2. Comparison of iodine methods for the analysis of Total Diet composites.

± 0.2 μg/g (18) for Spinach have been reported. No values for Rice Flour were found in the literature.

Interference

The main interference before separation in the food composite was chlorine, present at high levels in dairy products, meats, cereals, and legume vegetables (17). After separation, no important interfering chlorine peaks were observed and most of the chlorine activity was trapped in the HMD resin. Under the described irradiation conditions, an estimated lower concentration of 1 ng iodine/g in Total Diet samples can be determined.

Total Diet Survey

Five Fiscal Year 1980 adult Total Diet market baskets were freeze-dried and analyzed for iodine content as part of a more comprehensive survey. The results were compared with those obtained by the Total Diet Laboratory on the same samples using an alkaline dry ash-colorimetric method (12). Figure 2 shows the log-log regression line for the 2 methods. Except for 2 or 3 data points, there was good agreement between most of the results of various iodine concentration levels. From the slope of the line it appears that the NAA data had a moderate positive bias, while some colorimetric data were below the detection limit and were not included

Table 3. Market basket survey—Total Diet analysis for iodine (μg/g) using NAA

Composite	Collection location[a] 1	2	3	4	5	Range of daily iodine intake,[b] μg/day
Dairy products	0.347	0.619	0.360	0.472	0.246	192–490
Meat, fish, poultry	0.240	0.178	0.184	0.196	0.152	39–63
Grain, cereal products	0.092	0.102	0.121	0.251	2.248	39–958
Potatoes	0.006	0.009	0.006	0.022	0.017	0.8–3.5
Leafy vegetables	0.003	0.004	0.003	0.005	0.010	0.2–0.5
Legume vegetables	0.095	0.005	0.007	0.005	0.004	0.3–6.5
Root vegetables	0.002	0.014	0.003	0.006	0.007	0.1–0.5
Miscellaneous vegetables	0.011	0.016	0.013	0.011	0.011	0.8–1.1
Fruits	0.035	0.017	0.041	0.030	0.022	3.6–8.9
Oils, fats, shortening	0.026	0.013	0.060	0.040	0.015	0.9–4.1
Sugar and adjuncts	0.155	0.292	0.126	0.029	0.114	2.3–23.3
Beverages	0.003	0.018	0.023	0.028	0.003	1.8–20.5

[a] 1, Rochester, NY; 2, Springfield, IL; 3, San Jose, CA; 4, Baltimore, MD; 5, Binghamton, NY.
[b] Total daily intake/3900 kcal (μg/day) 393–1212; total daily intake/2900 kcal (μg/day) 292–901 (range of daily intake for 5 market baskets).

in the plot. More study is needed to determine the causes of these significant differences.

Considering the variety of foods analyzed and the possible inhomogeneity of samples, these results are an improvement compared with those previously reported (7). NAA results are presented in Table 3. Daily intake was calculated using the concentration of iodine in the market basket composites as determined by NAA in conjunction with food consumption data based on the 1965 U.S. Department of Agriculture Household Food Consumption Survey (19). This diet provides 3900 kcal. More realistic male adult diets would provide 2900 kcal (20). These preliminary findings confirm high levels of iodine consumption in the United States (2–6 times the U.S. Recommended Daily Allowances for adults) (21). Food groups contributing the greatest amounts are dairy products and grain and cereal products. This may indicate the presence of an additive in some foods in these commodity groups.

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MICROBIOLOGICAL METHODS

Interlaboratory Evaluation of the AOAC Method and the A-1 Procedure for Recovery of Fecal Coliforms from Foods

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An interlaboratory evaluation was made of the 96 h AOAC method and the 24 h A-1 procedure for the enumeration of fecal coliforms in samples of yellow corn meal, rye flour, mung beans, raw ground beef, and raw oyster homogenate. Results indicated that the efficiency of the A-1 procedure, measured in terms of recovery of fecal coliforms, and the reproducibility of that recovery were dependent on the particular food being analyzed. Accordingly, until its efficiency can be more fully demonstrated, the A-1 procedure is recommended only as a screening procedure for fecal coliforms in foods.

The enumeration of indicator coliform microorganisms is widely used to indicate the bacteriological safety of any given food. Currently, up to 4 days are required to enumerate fecal coliforms in foods examined by the AOAC method (1). Successful reduction of this time has been reported by analysts using cultural (1-7), enzymatic (8), and radiometric (9) techniques. In 1972 Andrews and Presnell (10) introduced a cultural method, using a medium that required 24 h for the enumeration of fecal coliforms in estuarine water. This medium, now known as A-1 medium, had originally been formulated by Presnell (unpublished data) to monitor the depuration of fecal coliforms in shellfish. Its usefulness was subsequently evaluated by other investigators on shrimp (11), crabmeat (12), and shellfish-growing waters from diverse geographical areas (13). After its introduction, the original A-1 procedure was modified (James Redman, New York State Department of Conservation Laboratory, private communication, July 1974) to include a 3 h resuscitation period at 35°C before incubation at 44.5°C to provide more favorable conditions for the maximal recovery of injured fecal coliforms. This modified procedure is referred to here as the A-1 procedure.

Recently, the A-1 procedure received official sanction (1) as an alternative method for enumerating fecal coliforms in shellfish-growing waters. Accordingly, the A-1 procedure was subjected to an interlaboratory study to determine whether this rapid procedure could also be successfully substituted for the lengthier AOAC method for the enumeration of fecal coliforms in foods.

Interlaboratory Study

Each of 13 participants analyzed 10 samples of each of 5 foods, except where indicated. The samples were analyzed for fecal coliforms using both the AOAC and the A-1 methods. Three of these foods (yellow corn meal, rye flour, and mung beans) consisted of 2 different brands, each brand being a manually mixed composite of several retail-size packages. Four of the 5 samples of each brand were inoculated with 1 mL aliquots of a series of 10-fold dilutions of a washed cell suspension of *Escherichia coli;* the fifth sample of each brand served as the uninoculated control. Cultures used for inoculating the samples were stock culture strains of *E. coli* that had been grown overnight in trypticase soy (TS) broth. Samples were taken from raw ground beef portions weighing about 2 lb, which had been purchased at local retail outlets. All samples were naturally contaminated, and no attempt was made to further homogenize them. The first 6 in each series of 10 samples of raw oyster homogenate furnished to each laboratory were artificially contaminated; the seventh sample was the uninoculated control. The artificially contaminated raw oyster homogenate samples were prepared as follows: From 2 gallons of oysters purchased at the local retail market, 200 g amounts were blended for 1 min with an equal weight of Butterfield's phosphate buffer (pH 6.8-7.2). Four blended homogenate portions were combined to form 1600 mL pools, each of which was inoculated with one of a series of 10-fold dilutions of an overnight broth culture of *E. coli* washed in Butterfield's phosphate buffer. Oyster samples 8, 9, and 10 were naturally contaminated and were furnished to each par-

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ticipant by the Food and Drug Administration (FDA), Gulf Coast Technical Services Unit, Dauphin Island, AL. Samples of all 5 foods were provided in sterile stomacher bags, and each sample was coded with a singular, randomized 5-digit number. All artificially inoculated samples were spiked 9-15 days before planned initiation of analyses.

Samples of raw ground beef and raw oyster homogenate were frozen before shipment by the originating laboratory, and participants were asked to store them at −20°C until initiation of analyses. A follow-up questionnaire to participants, however, showed that actual storage temperatures ranged from −65°C to 0°C. Before shipping the mung bean samples, the originating laboratory found that the level of inoculated fecal coliforms was higher than desired. The inoculated mung bean samples were transferred to another sterile stomacher bag to reduce these levels. Although this transfer may have resulted in a less-than-uniform distribution of the inoculated organisms, the actual levels of fecal coliforms were not considered critical. Each sample was homogenized by blending so that, essentially, the same sample was analyzed by both the AOAC and A-1 methods.

From the 55 g samples of yellow corn meal, rye flour, and mung beans, and the samples of thawed raw ground beef weighing about 60 g, participants weighed a 50 g portion from each sample into a sterile blender jar to which 450 mL Butterfield's phosphate buffer was added. Samples of about 120 mL of the frozen raw oyster homogenate that were furnished to participants were thawed and 100 mL (containing 50 g of oyster sample) was pipetted into a sterile blender jar to which 400 mL buffer was added. Final ratio of oyster sample to phosphate buffer diluent was 1:9. Because oyster samples had been partially blended previously, the participants blended these samples for only 1 min. Although most laboratories began their analyses on schedule, some initiated sample analyses one or more days late.

For the AOAC method, 3-tube MPN determination was performed by inoculating the blended food homogenate and dilutions of the homogenate into tubes of lauryl tryptose (LST) broth (double strength for tubes receiving 10 mL of inocula) containing inverted fermentation vials. The inoculated tubes were incubated at 35 ± 2°C and examined at 24 and 48 ± 2 h intervals. A flame-sterilized loop was used for transfers from gassing LST tubes to tubes of EC medium with inverted fermentation vials. In the analysis of samples of yellow corn meal, rye flour, mung beans, and raw ground beef, the tubes of EC medium were incubated in a 45.5 ± 0.2°C water bath and examined for gas at 24 and 48 ± 2 h intervals. For the samples of raw oyster homogenate, the tubes of EC medium were incubated at 44.5 ± 0.2°C for 24 ± 2 h and examined for gas production (14). The fecal coliform MPN was calculated from the positive combination of gassing tubes.

For the A-1 procedure, a 3-tube MPN determination was performed by inoculating the sample homogenate and serial 10-fold dilutions of the homogenate into tubes of A-1 medium (10) (double strength for tubes receiving 10 mL of inocula) containing inverted fermentation vials. To achieve approximately the same level of A-1 medium and inoculum in all tubes, participants were asked to dispense 10 mL portions of single strength broth into tubes measuring about 18 × 150 mm and containing inverted fermentation vials and to dispense 10 mL portions of double strength broth into tubes measuring about 22 × 175 mm and containing inverted fermentation vials. The designation "approx." was used in the instructions to allow some tolerance in the actual size of test tubes; no such designation appears in the method (1) for the official use of A-1 medium in the examination of shellfish-growing waters. At least one participant used 20 × 150 mm tubes for dispensing both single and double strength A-1 medium; however, this discrepancy was not regarded as serious, and data from this analyst were not excluded. The inoculated tubes of A-1 medium were incubated at 35 ± 2°C for 3 h before being placed in a 44.5 ± 2°C water bath for 21 ± 2 h. The fecal coliform MPN was calculated from the positive combination of gassing tubes.

In the calculation of logarithmic and geometric means, indeterminantly low MPN values, i.e., <0.3, were given a value of 0.1. Indeterminantly high values were expressed as the value itself. For example, an MPN value of ≥110 000 was given a value of 110 000. Samples in which both the AOAC and A-1 methods gave indeterminantly low or high MPN values were included in all statistical analyses. The significance of the difference between the mean log MPN values for the 2 methods was determined by using the t-test technique for paired samples (15). The significance of differences of the fecal coliform MPN values on an individual sample basis was determined by techniques recommended by Cochran (16). Significance of differences was based on the following formula:

$$Z = (\log d_1 - \log d_2) / 0.58 \sqrt{(\log a_1/N_1 + (\log a_2/N_2)}$$

where $\log d_1$ and d_2 are common logarithms of paired MPN values, a_1 and a_2 are dilution ratios, and N_1 and N_2 represent the number of samples per dilution. The factor 0.58 is a constant for calculating the standard error of the difference between 2 MPN values. A Z value of ≥ 1.96 is significant at the 95% confidence level.

Results and Discussion

The enumeration of fecal coliforms by the AOAC and A-1 methods is shown in Table 1. For each of the 2 methods there is a calculation at each inoculum level of the logarithmic mean, geometric mean, standard deviation (SD), and the coefficient of variation (CV). In this study, the SD and CV values in part reflect the reproducibility of the AOAC and A-1 methods, which is based on the variation among log MPN values, each obtained by a different laboratory. Table 1 shows that for a given food type the CV values are inversely related to inoculum level. In most cases CV values are highest at the low inocula levels. These high CV values indicate a low degree of reproducibility for a particular method, which may be due in part to analyst variability as well as to uneven distribution of the fecal coliforms in a given food type. Accordingly, rather than emphasizing the magnitude of the CV values obtained with any one method, it may be more significant to compare the CV values obtained with the AOAC and A-1 methods for each of the food types studied.

For 4 of the 10 levels of fecal coliforms in corn meal, the logarithmic mean values were significantly higher with the A-1 procedure than with the AOAC method, whereas for the remaining 6 levels there was no significant difference. Although 5 of the CV values were higher with the AOAC method, 5 were higher with the A-1 procedure, indicating that the 2 methods were relatively comparable in the degree of reproducibility or precision of enumerating fecal coliforms in corn meal.

With samples of rye flour, the logarithmic mean values were significantly higher with the AOAC and A-1 methods for 2 and 1 of the 10 inocula levels, respectively, whereas for the other 7 inocula levels, there was no significant difference. The A-1 procedure, however, was somewhat more variable than the AOAC method; CV values obtained with the A-1 procedure were higher than those with the AOAC method for 6 of the 10 inocula levels.

The mung bean data show that the logarithmic mean values were significantly higher with the AOAC and A-1 methods for 1 and 2 of the 10 inocula levels, respectively. No significant difference in logarithmic means obtained with the 2 methods was observed for the other 7 inocula levels. The AOAC method, however, was relatively more variable than the A-1 procedure in enumerating fecal coliforms; CV values were higher with the AOAC method for 6 of the 10 inocula levels. However, as noted earlier, the inoculated mung bean samples had been transferred from the original stomacher bag to another sterile stomacher bag in an attempt to reduce the fecal coliform counts, which were higher than desired. A disproportionate adherence of the inocula to the inner surface of the original stomacher bags, therefore, might have resulted in a non-uniform distribution of the fecal coliforms in the samples.

All samples of the frozen raw ground beef were naturally contaminated (Table 1). No sequential reduction in logarithmic and geometric mean values, as seen with the preceding 3 foods, was observed. For 2 of these 10 inocula levels, the logarithmic mean values were significantly higher with the A-1 procedure than with the AOAC method. No significant difference in paired logarithmic mean values was observed between the 2 methods for the other 8 levels of fecal coliforms. Four and 6 of the paired CV values were higher with the AOAC and A-1 methods, respectively, indicating a relatively higher degree of variability with the A-1 procedure for enumerating fecal coliforms.

For the frozen raw oyster homogenate, the first 6 levels of Brand A were artificially inoculated with sequential 10-fold dilutions of *E. coli*; the seventh level was the uninoculated control. Brands B, C, and D contained naturally contaminated oyster samples. None of the first 7 levels indicated any significant differences in paired logarithmic mean values obtained with 2 methods. However, 5 of 7 CV values were higher with the A-1 procedure than with the AOAC method, indicating a relatively higher degree of variability with the A-1 procedure. Of the 3 levels of fecal coliforms naturally occurring in the oyster samples, the logarithmic mean value was significantly higher with the AOAC method for 1 level, whereas there was no significant difference in paired logarithmic means for the other 2 levels. CV values were higher for the AOAC and A-1 methods for 1 and 2 of the 3 levels of naturally occurring fecal coliforms, respectively.

Table 1. Enumeration of fecal coliforms in selected foods using AOAC and A-1 methods

			AOAC				A-1			
Food	Brand	Inoculum level[a]	Log mean (MPN/g)	Geometric mean (MPN/g)	SD	CV, %	Log mean (MPN/g)	Geometric mean (MPN/g)	SD	CV, %
Yellow corn meal	A	1	2.90	794.32	0.79	27.24	3.31	2 041.74	0.58	17.52
		2[b]	2.12	131.83	0.66	31.13	2.41	257.04	0.55	22.82
		3[b]	1.02	10.47	0.34	33.33	1.42[c]	26.30	0.51	35.92
		4	0.27	1.86	0.92	340.74	0.54	3.47	0.89	164.82
		None	−0.57	0.27	0.94	164.91	0.87[c]	7.41	1.06	121.84
	B	1	3.00	1 000.00	0.38	12.67	3.02	1 047.13	0.43	14.24
		2	1.99	97.72	0.31	15.58	2.11	128.82	0.39	18.48
		3	0.64	4.37	0.36	56.25	0.97	9.33	0.38	39.18
		4	−0.36	0.44	0.32	88.89	0.11[c]	1.29	0.57	518.18
		None	−0.70	0.20	0.84	120.00	−0.16[c]	0.69	1.00	625.00
Rye flour	A	1	3.64	4 365.16	0.34	9.34	3.73	5 370.32	0.45	12.06
		2	2.88	758.58	0.52	18.06	2.72	524.81	0.46	16.91
		3	1.75[c]	56.23	0.42	24.00	1.33	21.38	0.43	32.33
		4	0.63[c]	4.27	0.48	76.19	0.15	1.41	0.73	486.67
		None	−0.82	0.15	0.66	80.49	−0.79	0.16	0.51	64.56
	B	1	4.03	10 715.19	0.65	16.13	4.04	10 964.78	0.72	17.82
		2	2.72	524.81	0.45	16.54	2.81	645.65	0.59	21.00
		3	1.72	52.48	0.88	51.16	1.83	67.61	0.99	54.10
		4	1.25	17.78	1.19	95.20	1.10	12.59	1.02	92.73
		None	−0.12	0.76	1.49	1 241.67	0.56[c]	3.63	1.51	269.64
Mung beans	A	1	3.53	3 388.44	1.20	33.99	3.84[c]	6 918.31	1.24	32.29
		2	2.44	275.42	1.14	46.72	2.39	245.47	1.40	58.58
		3	1.60	39.81	1.20	75.00	1.64	43.65	1.20	73.17
		4	0.52	3.31	1.23	236.54	0.71	5.13	1.05	147.89
		None	−0.79	0.16	0.51	64.56	−0.63[c]	0.23	0.77	122.22
	B	1	3.83	6 760.83	1.68	43.86	3.76	5 754.40	1.76	46.81
		2	2.66	457.09	1.35	50.75	3.01	1 023.29	1.25	41.53
		3	2.04	109.65	1.58	77.45	2.26	181.97	1.26	55.75
		4	0.76	5.75	1.88	247.37	0.56	3.63	1.50	267.86
		None	0.49[c]	3.09	1.23	251.02	−0.65	0.22	0.79	121.54
Raw ground beef	A	NC[d]	1.42	26.30	0.80	56.34	1.53	33.88	0.73	47.71
	B	NC	1.86	72.44	0.74	39.79	1.70	50.12	0.53	31.18
	C	NC	3.52	3 311.31	0.46	13.07	3.39	2 454.71	0.52	15.34
	D	NC	2.24	173.78	1.09	48.66	2.22	165.96	1.13	50.90
	E	NC	2.42	263.03	0.47	19.42	2.59	389.05	0.80	30.89
	F	NC	3.15	1 412.54	0.82	26.03	3.44	2 754.23	0.99	28.78
	G	NC	3.42	2 630.27	0.64	18.71	3.35	2 238.72	0.50	14.93
	H	NC	2.47	295.12	0.37	14.98	2.77[c]	588.84	0.55	19.86
	I	NC	2.60	398.11	0.69	26.54	3.02[c]	1 047.13	0.58	19.21
	J	NC	1.54	34.67	0.67	43.51	1.29	19.50	0.85	65.89
Raw oyster homogenate[e]	A	1	4.06	11 561.12	0.59	14.53	3.77	5 821.03	0.57	15.12
		2	2.95	881.05	0.58	19.66	2.73	534.56	0.49	17.95
		3	1.78	60.67	0.51	28.65	1.77	59.02	0.64	36.16
		4	0.68	4.73	0.67	98.53	0.76	5.70	0.89	117.11
		5	−0.34	0.45	0.75	220.59	−0.40	0.40	0.77	192.50
		6	−1.00	0.10	0.00	0.00	−0.84	0.15	0.42	50.00
		None	−0.95	0.11	0.17	17.90	−0.89	0.13	0.39	43.82
	B	NC	−0.28	0.53	0.41	146.53	−0.10	0.79	0.81	810.00
	C	NC	0.64[c]	4.35	0.28	43.75	0.38	2.37	0.50	131.58
	D	NC	1.18	15.14	0.42	35.59	0.98	9.53	0.30	30.61

[a] Some sample analyses were initiated one or more days late.
[b] One collaborator was inadvertently furnished 2 samples from inoculum level 2, rather than a sample from inoculum level 3, for Brand A.
[c] Logarithmic mean values are significantly higher at $P<0.05$.
[d] Naturally contaminated.
[e] Data from one collaborator were excluded because EC medium was incubated at 45.5°C rather than at 44.5°C.

Overall, a total of 50 levels of fecal coliforms, either artificially inoculated or naturally occurring, were enumerated by the AOAC and A-1 methods. The logarithmic mean values were significantly higher for 4 (8%) and 9 (18%) of these levels by the AOAC method and the A-1 procedure, respectively. For 37 (74%) levels of fecal coliforms, there was no significant differ-

Table 2. Statistical comparison of paired fecal coliform most probable number values obtained with AOAC and A-1 methods

			No. of samples[b]		
Food	Brand	Inoculum level[a]	AOAC[c] >A-1	AOAC[c] <A-1	NSD[d,e]
Yellow corn meal	A	1	0	3	8
		2[f]	2	5	6
		3[f]	0	2	11
		4	3	6	4
		None	0	11	2 (1)
	B	1	1	1	10
		2	0	1	12
		3	1	4	8
		4	1	4	8
		None	1	5	7 (5)
		Total	9	42	76
		Percent	7	33	60
Rye flour	A	1	0	0	13 (1)
		2	2	0	11
		3	4	0	9
		4	6	0	7
		None	1	1	11 (11)
	B	1	1	1	11 (1)
		2	1	0	12
		3	1	2	10
		4	2	1	10
		None	0	6	7 (3)
		Total	18	11	101
		Percent[g]	14	9	78
Mung beans	A	1	0	4	9
		2	1	0	12
		3	1	2	10
		4	2	2	9 (3)
		None	2	3	8 (8)
	B	1	0	0	12 (2)
		2	0	2	10
		3	1	1	11 (1)
		4	4	4	5 (3)
		None	2	2	9 (8)
		Total	13	20	95
		Percent	10	16	74
Raw ground beef	A	NC[h]	1	2	10
	B	NC	1	0	12
	C	NC	1	1	10
	D	NC	1	1	11 (1)
	E	NC	1	1	11
	F	NC	1	3	8
	G	NC	2	2	8
	H	NC	0	2	10
	I	NC	0	4	9
	J	NC	3	0	10
		Total	11	16	99
		Percent[g]	9	13	79
Raw oyster homogenate[i]	A	1	4	1	7
		2	0	1	11
		3	1	1	10
		4	3	2	7
		5	1	1	10 (3)
		6	0	1	11 (9)
		None	0	1	11 (10)
	B	NC	1	2	9 (2)
	C	NC	1	0	11
	D	NC	1	0	11
		Total	12	10	98
		Percent	10	8	82

[a] Some sample analyses were initiated one or more days late.

[b] For samples of yellow corn meal, rye flour, mung beans, and raw ground beef, occurrence of fewer than 13 samples/inoculum level was due to exclusion of samples with positive tube combination too improbable for MPN calculation.

[c] Differences are statistically significant at $P<0.05$.

[d] No significant difference at $P>0.05$.

[e] Numbers in parentheses indicate number of samples in which fecal coliform MPN values were indeterminantly low or high with both methods.

[f] One collaborator was inadvertently furnished with 2 samples from inoculum level 2, rather than a sample from level 3, for Brand A.

[g] Because percentages were rounded off to the nearest whole number, total percentage was 101% in some instances.

[h] Naturally contaminated.

[i] Data were excluded from one participant, who incubated EC medium at 45.5°C rather than 44.5°C.

ence in the logarithmic mean values by the 2 methods. CV values were higher for 22 and 28 levels with the AOAC method and the A-1 procedure, respectively, indicating a somewhat greater degree of variability with the A-1 procedure for enumerating fecal coliforms in the 5 foods examined.

A statistical comparison of individually paired fecal coliform MPN values obtained with the AOAC and A-1 methods is shown in Table 2. Of the 5 foods examined, corn meal showed the largest percentage (40%) of samples with a significant difference between MPN values when analyzed by the 2 methods. Conversely, only 60% of the paired sample MPN values showed no significant differences when analyzed by the AOAC and A-1 methods. For the other 4 foods, the combined percentages of samples showing a significant difference between paired sample MPN values were as follows: rye flour (23%), mung beans (26%), raw ground beef (22%), and raw oyster homogenate (18%).

Tables 1 and 2 show the recovery and the reproducibility of that recovery of fecal coliforms from 5 foods by the AOAC and A-1 methods. The bacteriological standards established by the FDA are based on the AOAC method; therefore, any rapid or other alternative method would have to demonstrate comparability to the official method to be acceptable. In terms of actual recovery of fecal coliforms and the precision of that recovery, the A-1 procedure was shown in this study to be dependent on the particular food being analyzed.

The following alternatives for the A-1 procedure are therefore suggested: (*1*) The A-1 procedure could be recommended only for those specific food types for which its efficiency has

been demonstrated. This alternative would limit the usefulness of the A-1 procedure in laboratories that monitor the bacteriological quality of a wide variety of foods, and analysts would have to foretell its usefulness for examining foods similar to but not identical with those used in this evaluation. Such conjecturing would be undesirable from both a regulatory and a microbiological point of view. (2) The bacteriological standards would be revised for those foods which are not comparably analyzed by the 2 methods. Although such a decision may be possible for agencies or establishments responsible for regulating or monitoring the bacteriological quality and safety of one or more foods, the costs involved in such an undertaking would be prohibitive in laboratories such as those of FDA, which judge the quality and safety of a wide variety of foods according to established standards. (3) The A-1 procedure would be used as an unofficial screening technique for the individual foods for which it has demonstrated its efficiency. Of the 3 alternatives, this recommendation seems to be the most scientifically sound, with the greatest applicability in quality control laboratories which monitor the bacteriological quality of one or more foods.

The National Shellfish Sanitation Program (NSSP), a cooperative consisting of representatives from the shellfish industry, FDA, and state shellfish regulatory agencies, is charged with guaranteeing the safety of shellfish-harvesting waters. The NSSP has adopted this third alternative but has gone further by using the A-1 procedure as an official method, as opposed to an unofficial screening technique, to judge the bacteriological safety of shellfish-growing waters. Such official sanction, however, came only after the completion of a comprehensive collaborative study (13) which demonstrated no significant difference between the AOAC and A-1 methods for enumerating fecal coliforms in shellfish-growing waters. It can only be within such a framework that the A-1 procedure can and should receive official sanction.

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INDUSTRIAL CHEMICALS

Headspace Sampling and Gas Chromatographic Determination of Styrene Migration from Food-Contact Polystyrene Cups into Beverages and Food Simulants

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Migration studies using coffee, tea, water, and 8% ethanol were conducted with various types of food-contact polystyrene cups. Study conditions simulated filling and storage at room temperature and hot-filling or pasteurization above 150°F (65.6°C). The quantity of styrene migrating was determined by headspace sampling and gas chromatography (GC). The GC column was 6% Carbowax 20M on Chromosorb 101 with quantitation via a flame ionization detector. Detection limits ranged from 3 to 10 ppb in various liquids. The method and results of the migration studies are presented.

Residual monomers in food-contact polymers are known to migrate into foods, and there is growing interest in the effects of some of these chemicals on human health. It has, therefore, become increasingly important to gather data on the extractability of such substances from packaging materials.

Styrene is the second most widely used monomer in food-contact packaging polymers. Excluding housewares, 336 million lb of polystyrene and its copolymers were used in 1978, primarily in packaging for fresh red meat and poultry, cottage cheese, fresh fruits and vegetables, cookies, and delicatessen food (1).

Several adverse health effects are attributed to styrene. At exposure levels above 100 ppm, humans experience acute mucous membrane irritation with eyes, nose, and throat particularly affected (2–4); long-term exposure may cause obstructive alterations (2, 5) of the upper respiratory tract. In workers occupationally exposed to styrene, liver enlargement and functional changes were noted with definite metabolic impairments observed in the liver cells (2, 6–8). Toxic hepatitis was diagnosed in employees who had worked in a polystyrene plant for more than 5 years (9). Exposures of 375 ppm styrene caused fatigue, irritation, feelings of inebriation, decreases in concentration ability, and impairment of balance (3, 4, 10). Visuomotor accuracy and psychomotor performance were lower in styrene-exposed workers than in controls (11–13). The toxic effects on the central nervous system were further substantiated by abnormal electroencephalograms (11, 14–16). An increase in the rate of chromosomal aberrations was observed in human subjects exposed to styrene (17). Further, there was weak evidence of carcinogenicity found in mice which had been given the monomer throughout their lifetimes (18). In a recent study conducted by the National Cancer Institute (19), however, no conclusive evidence for the carcinogenicty of styrene was found.

Levels of styrene in the 60–2250 ppm range have been detected in polystyrene food containers (20).

Because of these relatively high residual concentrations, the information on toxicity, and the extensive usage of styrene in food packaging, we believe that migration studies are of value. Work is being conducted by Arthur D. Little, Inc. (Cambridge, MA) under closed-system conditions to determine the total migration of styrene into various food substances from polystyrene food packaging. To complement these studies, experimentation simulating actual usage conditions was initiated in this laboratory. Various types of polystyrene cups, including foam, impact, and crystal, were investigated. Cups, marketed as housewares, accounted for 27% or 284 of the 1058 million lb of polystyrene used in plastics packaging in 1978. The amounts of polystyrene used in manufacturing foam, impact, and crystal polystyrene cups were 90, 144, and 50 million lb, respectively (21).

Several tests from the *Code of Federal Regulations* (CFR) (22) were adapted to determine the extractability of styrene under the most severe conditions of use. These tests, as conducted, simulated filling and storage at room temperature, and hot-filling or pasteurization above 150°F (65.6°C). Initial experiments were carried out with food-simulating solvents, which per-

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mitted rapid estimation of styrene migration. Several beverages were also studied to obtain data which more closely approximated actual usage conditions. The 3 types of polystyrene cups selected for testing had been shown by previous liquid chromatographic (LC) analyses (20) to contain residual styrene levels in the 60–2250 ppm range. The styrene concentrations in the test containers were confirmed by the same LC method.

Styrene is normally determined by gas chromatography (GC). Headspace sampling techniques, with detectable concentrations in the low ppb range (23), have been used to improve sensitivity. Therefore, to quantitate the amounts of migrating styrene, a headspace GC method compatible with the solutions being studied was developed. The method and results of the migration studies are presented.

Experimental

Reagents

(a) *Ethanol.*—Absolute, USP reagent quality (U.S. Industrial Chemicals Co., New York, NY 10016, or equivalent). Analyze by headspace sampling technique to assure absence of interferences at retention time of styrene.

(b) *Styrene.*—99%, stabilized with 10–15 ppm *p-tert*-butylcatechol (Aldrich Chemical Co., Inc., Milwaukee, WI 53233, No. S497-2). Store in freezer in amber-colored bottle. Confirm purity by GC, using flame ionization detector. In spite of presence of inhibitor, some polymerization may take place. Remove polymer by filtering through Whatman No. 40 paper.

(c) *Water.*—Deionized, distilled, obtained from Milli-Q Water Purification System (Millipore Corp., Bedford, MA 01730), or equivalent. Test water under conditions of analysis to show absence of substances with approximate retention time of styrene.

(d) *Coffee.*—Add ca 1 teaspoon instant coffee to each 200 mL boiling water. Let coffee granules thoroughly dissolve before using mixture for migration studies.

(e) *Tea.*—Add 1 tea bag to each 200 mL boiling water. Let tea brew to medium strength, then remove tea bags.

Apparatus

(a) *Screw-cap glass bottles.*—1 oz narrow-mouth (35 ± 0.5 mL) (Ace Scientific Supply Co., Linden, NJ 07036, No. 10-4257), with screw caps and Teflon-faced septa (Alltech Associates, Inc., Arlington Heights, IL 60004, No. 95953 and No. 9522), or equivalent.

(b) *Syringes.*—10 and 50 μL for liquid delivery (Hamilton Co., Reno, NV 89510, No. 701-N, and No. 705-N, or equivalent). Two mL Series A-2 with Mininert valve for sampling gases (Precision Sampling Corp., Baton Rouge, LA 70895, No. 050034), or equivalent. Ten ml liquid B-D Multifit with Luer-Lok tip (Becton-Dickinson, Rutherford, NJ 07070, No. 2312), or equivalent, equipped with 20-gauge needle (No. 1075).

(c) *Headspace sample vials.*—Glass, 23 mL, with fitted butyl rubber septa and aluminum seals (Perkin-Elmer Corp., Norwalk, CT 06856, No. 105-0118), or equivalent. Fermpress H 207 and HO 207 (Perkin-Elmer No. 105-0106 and No. 105-0107) were used to cap and uncap vials, respectively.

(d) *Forced air oven.*—Blue M Stabil-Therm (Blue M Electric Co., Blue Island, IL 60406), or equivalent.

(e) *Gas chromatograph.*—Hewlett-Packard Model 7620A equipped with flame ionization detector, Model 7128A strip chart recorder (Hewlett-Packard, Avondale, PA 19311), and Varian CDS 111 chromatography data system (Varian Associates, Inc., Palo Alto, CA 94303), or equivalent. Operating conditions: injection port 210°C, column 140°C, detector 230°C; helium carrier flow 50 mL/min, hydrogen 40 mL/min, air 450 mL/min; chart speed 0.25 in./min.

(f) *GC column.*—Coiled 6 ft × 1/8 in. od stainless steel, packed with 6% Carbowax 20M coated on 80–100 mesh Chromosorb 101. Condition column at 200°C overnight or until an acceptable noise level is obtained at lowest attenuation setting of gas chromatograph. To allow for venting, insert a 4-port valve suitable for operation at 140°C at point 2 ft from column end nearest injection. (Only 3 ports were used; the fourth was capped.)

Preparation of Standards

Stock standard solution.—Accurately weigh 1 oz narrow-mouth bottle, screw cap, and septum. Pipet ca 25 mL ethanol into bottle, cap bottle, and reweigh. Using 50 μL liquid syringe, add 45–50 μL styrene to the ethanol by quickly uncapping bottle and injecting the monomer. (This procedure is used to avoid puncturing septum, which would shorten lifetime of standard.) Immediately recap bottle, and thoroughly mix solution by shaking. Reweigh bottle, and calculate styrene concentration (ca 2000 ppm). Store stock solution in freezer to assure useful lifetime of at least 1 month.

Working standard solutions.—Serially dilute

stock standard solution with ethanol to prepare working standard solutions of ca 200 and 20 ppm styrene. Pipet 1 part stock standard solution into 1 oz narrow-mouth bottle containing 9 parts ethanol. Quickly cap bottle and thoroughly mix solution by shaking. Repeat the dilution with an aliquot of this solution. Store these working standard solutions in freezer, to ensure stability of at least 1 month.

Prepare headspace standard solutions in range 10–500 ppb styrene from styrene working standard solutions. Pipet exactly 10 mL food simulant or beverage to be studied into a 23 mL sample vial. (Measure solvent volume for each headspace standard solution under same conditions as respective test aliquot taken at time of sampling. In other words, for extractability test simulating hot-filling or pasteurization above 150°F (65.6°C) (22), temperature of standard solvent should be 100°F (37.8°C), final temperature of migration samples.) Stopper and cap vials. Use a 10 or 50 μL liquid syringe to add predetermined volume of either styrene working standard solution to measured solvent in each vial by injection through septum. Thoroughly mix these solutions by shaking. Use these standard solutions, which should be freshly prepared daily, for no more than two 1 mL injections of headspace.

Headspace Sampling Technique

Place the sealed vials containing 10 mL of sample or standard in a heated forced air oven (at 60 or 90°C, depending on sample) and let equilibrate. After 1 h, sample the headspace, using a gas syringe heated to the same temperature as the vials. Insert the syringe needle through the vial septum. Pump the plunger several times to wet the walls of the syringe with the sample vapors. Draw exactly 1 mL of headspace with the syringe and let the vapors equilibrate between vial and syringe for 1 min in the oven. Close the syringe valve, withdraw the needle from the vial, and quickly transfer the sample to the gas chromatograph. After inserting the syringe needle into the injector, open the valve and inject the headspace.

Before starting daily sample analyses, inject two or three 1 μL liquid aliquots of the stock standard solution to assure suitable instrument response.

Calculation

Quantitate the amount of styrene monomer migrating from a polystyrene container by comparing the sample response to that of standards in a similar solvent. Obtain the peak heights and normalize the measurements to an attenuation of 1×10^{-12}. Construct a calibration curve for each day of data acquisition and use it to determine the amount of styrene leached. From the known volume of liquid added to the container, calculate the total micrograms of styrene monomer that migrated from the container into the solvent. To report the quantity of styrene leached per unit area, divide this value by the container area in contact with the test solvent.

Calculate the inside area of the polystyrene test cup by using the equation for the area of the curved surface of the frustum of a right cone plus the area of a circle:

$$A = \pi (r_1 + r_2) \sqrt{h^2 + (r_1 - r_2)^2} + \tfrac{1}{4} \pi d_2^2$$

where A is the area of the container in contact with the test solvent; r_1 is the inside radius of the cup 1/4 in. from the top; r_2 and d_2 are the inside radius and diameter, respectively, of the base of the container; and h is the inside height of the cup minus 1/4 in.

Migration Studies

Simulation of filling and storage at room temperature.—An extractability test was adapted from the CFR (22) for use in this study. The migration of styrene under conditions simulating filling and storage at room temperature was determined for beverages containing alcohol. An aqueous solution of 8% ethanol (v/v) was the food simulant used for this purpose. Polystyrene test cups containing magnetic stirring bars were filled to within 1/4 in. of the top with measured volumes of 8% ethanol preheated to 120°F (48.9°C). Each sample was covered with Saran Wrap, which was held in place by a rubber band. The containers were maintained for 24 h in a forced air oven at 120°F (48.9°C). After heating, the solutions were stirred, and two 10 mL samples from each covered container were removed with a 10 mL liquid syringe. The aliquots were transferred to glass vials, which were then stoppered and capped. The liquid in each vial was allowed to cool to room temperature, and its styrene content was determined by GC analysis using the headspace sampling technique. The sample vials were equilibrated at 60°C in the heated forced air oven. Immediately after injection of 1 mL of headspace, the GC column was vented for 2 min.

Simulation of hot-filling or pasteurization above

150°F (65.6°C).—By modifying an extractability test from the CFR (22), we developed a method to determine the migration of styrene under conditions simulating hot-filling or pasteurization above 150°F (65.6°C). As specified, water was the solvent used to simulate acidic aqueous food products, acid or non-acid aqueous foods containing free oil or fat, and dairy items and their modifications. To more closely approximate actual usage, coffee and tea were also tested. Polystyrene cups containing magnetic stirring bars were filled to within 1/4 in. of the top with measured volumes of boiling liquid. The samples were allowed to stand at room temperature to cool to 100°F (37.8°C) (ca 1 1/4 h). Each solution was then stirred, and the volume of liquid remaining was measured and recorded.

Two 10 mL aliquots from each container were analyzed for styrene by the headspace GC method. After the liquid had cooled to room temperature, headspace analyses were carried out. Equilibration temperatures for the water and for the coffee and tea migration studies were 90 and 60°C, respectively. One mL portions of headspace were injected into the gas chromatograph, but the column was not vented.

Determination of residual styrene monomer levels.—The concentrations of residual styrene in the polymer test containers were determined by a reverse phase LC method (20).

Results and Discussion

Method Development

Because this study was undertaken to determine the amounts of styrene migrating from food-contact polystyrene packaging materials into food-simulating solvents and beverages, we needed to develop a sensitive and reliable method for its analysis. Previous studies indicated that residual styrene concentrations of 500 ppm produce migration levels in the 5–50 ppb range (24). With conventional GC techniques, the detection limit for the monomer is about 0.5 ppm. Headspace sampling can extend this sensitivity to the low ppb range and was, therefore, the technique chosen.

During the development of the headspace GC method for the determination of styrene, we found that volatile solvents, in particular ethanol, tailed when chromatographed, and that this tailing caused a sloping background for the styrene peak. To alleviate this problem, we tested several different column packings. With Chromosorb 101, 102, and 104, the styrene peak closely followed the tailing ethanol peak.

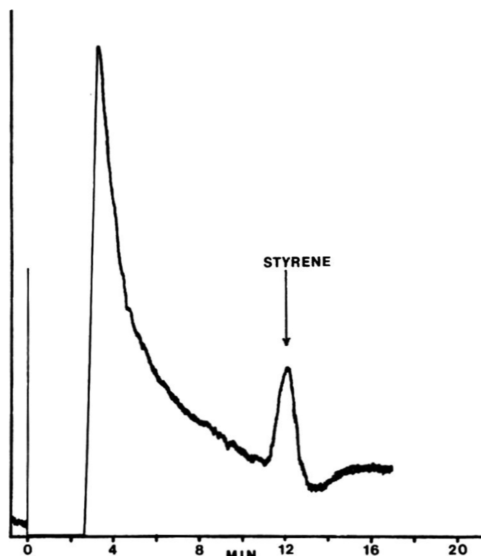

Figure 1. Gas chromatogram of a 1 mL headspace injection from a 10.3 ppb styrene standard solution in 8% ethanol. Peak height is 11.5% full scale deflection.

Lowering the temperature and increasing the carrier flow rate did not sufficiently improve the chromatography. Coating Chromosorb 101 with 6% Carbowax 20M lowered the retention time of styrene and somewhat enhanced the sensitivity, but also increased the background. To completely eliminate the problem, we attempted to reverse the order of elution. We tried several different stationary phases, including 10% Quadrol on Chromosorb W-AW, without success.

As an alternative solution to the problem, the GC column was vented for several minutes immediately after sample injection by opening a valve inserted into the 6 ft column at a point 2 ft from the column end nearest injection. The stationary phase, 6% Carbowax 20M on Chromosorb 101, was selected because it gave the best results in previous tests. Venting the solvent front reduced the sloping background sufficiently to yield reliable results for the quantitation of styrene. A vent time of 2 min ensured that the ethanol peak tailing was reduced and that no styrene was lost. A gas chromatogram obtained with the established conditions is shown in Figure 1. The peak represents styrene in 8% ethanol at the detection limit. With water, tea, or coffee as the solvent, no sloping background was observed so it was unnecessary to vent the column.

To further reduce the possibility of high background, all samples in 8% ethanol were

Table 1. Migration of styrene into 8% ethanol under conditions simulating filling and storage at room temperature [a]

Container	Internal surface area, cm^2	Volume of 8% ethanol, mL	Styrene leached, ppb	Total amt leached, μg	Amt leached per unit area, $\mu g/cm^2$
Foam cup					
1	168	226	28	6.3	0.038
			29	6.6	0.039
2	168	226	24	5.4	0.032
			26	5.9	0.035
Impact cup					
1	157	196	53	10	0.064
			50	10	0.064
2	157	197	53	10	0.064
			53	10	0.064
Crystal cup					
1	196	269	140	37.7	0.192
			167	44.9	0.229
2	196	272	160	43.5	0.222
			152	41.3	0.211
3	196	275	147	40.4	0.206
			141	38.8	0.198
4	196	274	150	41.1	0.210
			151	41.4	0.211

[a] Two 10 mL aliquots from each container were analyzed.

equilibrated at a low temperature (60°C) before headspace sampling. This temperature was also used in the nonalcoholic beverage studies to limit potential interference from other volatile components; the water samples were equilibrated at a higher temperature (90°C) because interfering volatile components were absent.

The time needed to equilibrate the headspace samples in the oven was established by heating styrene working standard solutions at the 20.6 ppb level for periods ranging from 15 to 90 min. Exactly 1 mL of headspace from each vial was injected into the gas chromatograph, and the responses were measured. No changes in peak height were noted for the 60–90 min periods; thus, 1 h was chosen as the equilibration time for the headspace vials.

Several types of septa were used to cap the sample vials. The Teflon-backed septa were inadequate for styrene analysis. After the septa were pierced by the syringe, gradual losses of volatile components from the vials were noted. Fitted butyl rubber septa were adequate under the analytical conditions used; these septa did not introduce interferences and hence were used for all work.

Under the GC analytical conditions chosen, the retention times of styrene with the column vented and unvented were approximately 13 and 14 min, respectively. Detector response was quantitated initially from both height and area measurement of the chromatographic peaks. Peak areas, which were measured by an automatic integrator, gave inconsistent results. Because greater precision was attained with peak heights, all further calculations were made with

Table 2. Migration of styrene into water under conditions simulating hot-filling or pasteurization above 150°F [a] (65.6°C)

Container	Internal surface area, cm^2	Volume of water, mL	Styrene leached, ppb	Total amt leached, μg	Amt leached per unit area, $\mu g/cm^2$
Foam cup					
1	168	206	6.8	1.4	0.0083
			6.1	1.3	0.0077
2	168	208	6.6	1.4	0.0083
			5.8	1.2	0.0071
3	168	202	5.8	1.2	0.0071
			6.6	1.3	0.0077
Average			6.3	1.3	0.0077

[a] Two 10 mL aliquots from each container were analyzed.

Table 3. Migration of styrene into tea under conditions simulating hot-filling or pasteurization above 150°F [a] (65.6°C)

Container	Internal surface area, cm²	Volume of tea, mL	Styrene leached, ppb	Total amt leached, µg	Amt leached per unit area, µg/cm²
Foam cup					
1	168	210	5.8	1.2	0.0071
			5.5	1.2	0.0071
2	168	211	6.4	1.4	0.0083
			6.5	1.4	0.0083
3	168	210	6.4	1.3	0.0077
			6.6	1.4	0.0083
Average			6.2	1.3	0.0078

[a] Two 10 mL aliquots from each container were analyzed.

this measurement.

The linear range of the method for each solvent was determined from analyses of styrene standard solutions. The headspace calibration curves for styrene in the food simulants 8% ethanol and water were linear from at least 500 ppb down to the detection limit. Standard solutions with styrene concentrations greater than 100 ppb were not analyzed with coffee or tea as the solvent, but the standard curves were linear over the range tested. With solutions of 8% ethanol, a styrene level of about 10 ppb gave a peak height of 10% full scale deflection under the conditions used. The same response level was obtained for 5 ppb styrene in coffee and tea. The sensitivity was slightly higher with water as the solvent with a detection limit of about 3 ppb obtained.

Because gas-tight conditions were not used in the migration studies, it was difficult to design meaningful recovery tests; thus, none were performed. Each sample was analyzed in duplicate, and at least 2 polystyrene cups were examined for each set of migration conditions. The close agreement of the data within each set of usage conditions, as shown in Tables 1–4, demonstrates the reproducibility of the method and supports the reliability of the measurements obtained in these studies.

Migration Studies

Previous styrene experiments have shown that the food simulants 3% acetic acid and water yield very similar results (24). Heptane and 50% ethanol caused polystyrene sheet to degrade and distort (25). Therefore, these studies were limited to the food simulants 8% ethanol and water. (Corn oil is also a useful food simulant, but was not included in this work.)

Because these migration studies were intended to simulate actual usage conditions, test containers were left opened to the atmosphere or were covered with Saran Wrap, which was held in place by a rubber band. Thus, there were no gas-tight seals, and the migrating styrene could escape into the air as it would under actual usage conditions.

On the basis of our previous LC determinations of residual styrene levels (20), 3 types of cups, foam, impact, and crystal polystyrene, were chosen for migration studies. To obtain recent values for residual monomer concentrations in the polystyrene test containers, we repeated the

Table 4. Migration of styrene into coffee under conditions simulating hot-filling or pasteurization above 150°F [a] (65.6°C)

Container	Internal surface area, cm²	Volume of coffee, mL	Styrene leached, ppb	Total amt leached, µg	Amt leached per unit area, µg/cm²
Foam cup					
1 [b]	168	210	—	—	—
			6.8	1.4	0.0083
2	168	206	6.8	1.4	0.0083
			6.4	1.3	0.0077
3	168	211	5.9	1.2	0.0071
			6.2	1.3	0.0077
Average			6.4	1.3	0.0078

[a] Two 10 mL aliquots from each container were analyzed.
[b] The data for 1 aliquot from this container were eliminated because of an unreasonably high peak height value.

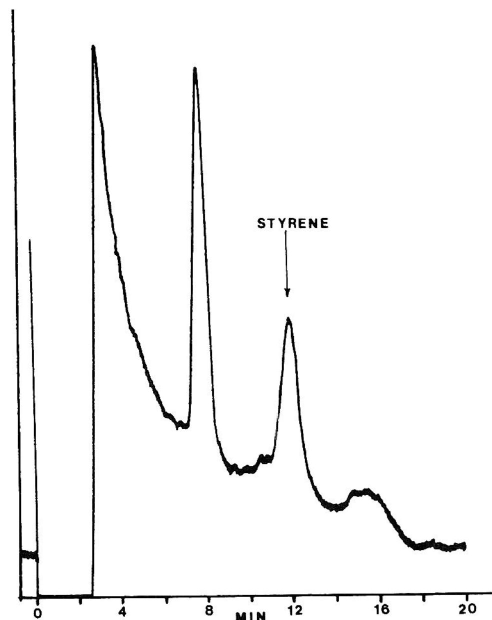

Figure 2. Gas chromatogram of a 1 mL headspace injection of an aliquot from migration study simulating filling and storage at room temperature with the food simulant 8% ethanol. The polystyrene test container was a foam hot drink cup. The peak corresponds to 26 ppb styrene and its height is 17% full scale deflection.

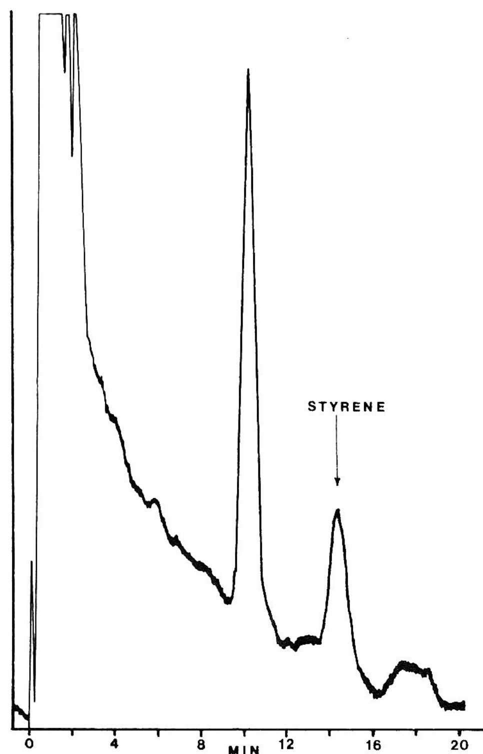

Figure 3. Gas chromatogram of a 1 mL headspace injection of an aliquot from migration study simulating hot-filling or pasteurization above 150°F (65.6°C) with the food simulant water. The polystyrene test container was a foam hot drink cup. The peak corresponds to 5.8 ppb styrene and its height is approximately 16% full scale deflection.

LC determinations before beginning the migration studies. We analyzed a 1 g sample from each of 6 units for the foam and impact polystyrene cups and three 1 g samples from each of 2 crystal polystyrene cups. The average residual levels were 70.8 ppm in the foam containers, 771 ppm in the impact polystyrene cups, and 2261 ppm in the crystal polystyrene glasses with average deviations of 2.9, 23, and 104 ppm, respectively.

Two units each of the 3 types of polystyrene cups were used in the extractability tests with 8% ethanol, which simulated filling and storage at room temperature for alcoholic beverages. Two additional containers at the 2261 ppm residual level were evaluated to determine the consistency of results from separate determinations at this concentration. Two 10 mL aliquots were removed for analysis from each container tested. No apparent interferences were present in the samples from the impact and crystal polystyrene cups. A small peak that appeared on the side of the styrene peak (Figure 2) in the chromatograms of the foam cup solution had no apparent effect on the quantitation of styrene.

The concentration of styrene migrating from the polystyrene containers and remaining in the 8% ethanol was determined from a calibration curve. Alternatively, the height of the styrene peak from a single standard was measured and the nanograms of styrene represented by 1 mm were determined; the monomer concentrations in the sample solutions were then calculated by using this factor. The results from this latter procedure, which were very similar to those obtained from the calibration curve, were, however, somewhat less precise. Therefore, the calibration procedure was used for all remaining calculations.

The results for the migration studies simulating filling and storage at room temperature are presented in Table 1 with values for the following parameters reported: the concentration of styrene extracted into 8% ethanol, the total micrograms migrating, and the amount leached per unit area of cup in contact with the solvent. The average values for micrograms of styrene

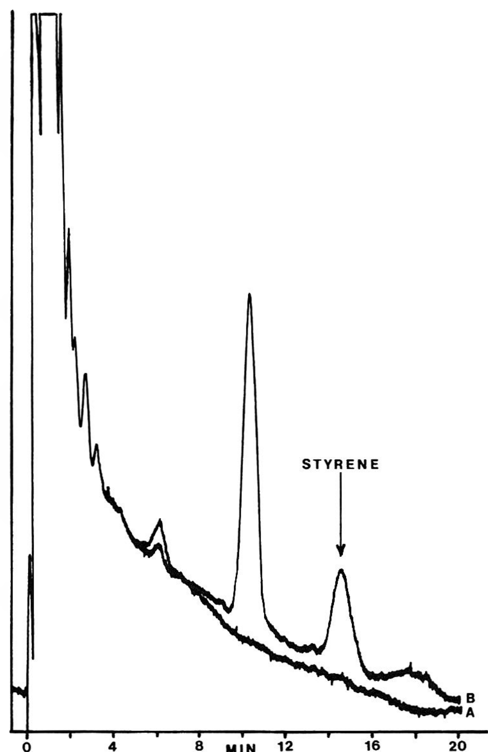

Figure 4. Gas chromatograms of 1 mL headspace injections of a tea blank (A) and an aliquot from migration study simulating hot-filling or pasteurization above 150°F (65.6°C) using tea (B). The polystyrene test container was a foam hot drink cup. In B, the peak corresponds to 5.5 ppb styrene and its height is 10% full scale deflection.

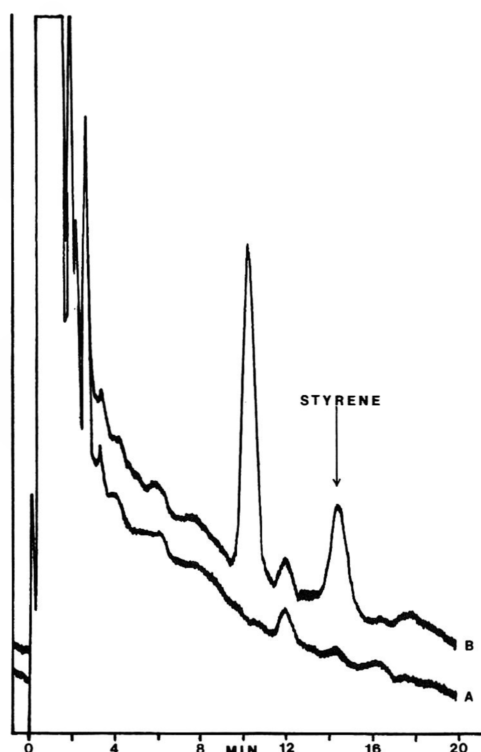

Figure 5. Gas chromatograms of 1 mL headspace injections of a coffee blank (A) and an aliquot from migration study simulating hot-filling or pasteurization above 150°F (65.6°C) using coffee (B). The polystyrene test container was a foam hot drink cup. In B, the peak corresponds to 6.2 ppb styrene and its height is approximately 12% full scale deflection.

migrating per square centimeter of container are 0.036 for the foam cups, 0.064 for the impact polystyrene containers, and 0.210 for the crystal polystyrene glasses. An average deviation of 0.004 was calculated for the crystal polystyrene data. For the nonfoam samples, the amount of styrene leached by 8% ethanol appeared to be proportional to residual monomer levels. Approximately 3 times as much styrene migrated from the crystal polystyrene containers with residual levels of 2261 ppm as from the impact polystyrene cups with residual levels of 771 ppm. In proportion to the residual concentration, the amount of monomer extracted from the foam cups with residual levels of 70.8 ppm styrene was about 6 times that from either of the other sample types. The porosity of the foam polymer, which would increase the actual surface area in contact with the solvent, might account for the apparent higher rate of migration per square centimeter. Another explanation that would account for the higher calculated migration rate is that an adsorbed film of styrene present on the container surface could have been dissolved by the aqueous alcohol to give a higher monomer concentration in the test solution. The presence of such a surface film has been previously suggested by Withey and Collins (26).

In the migration studies with water, tea, and coffee that simulated hot-filling or pasteurization above 150°F (65.6°C), 3 foam cups were tested for each fluid with two 10 mL aliquots analyzed from each unit. In the analyses of water and tea, no apparent interferences were found in either the blank solvent or the migration solution as shown in Figures 3 and 4. The coffee blank chromatogram in Figure 5 shows the presence of a small peak with the same retention time as styrene. This peak, however, does not significantly affect the quantitation of styrene. Calibration curves were used as before to determine the styrene migration levels in these tests. Ta-

bles 2, 3, and 4 show the data from the migration studies with water, tea, and coffee, respectively.

From the average amounts of styrene leached per unit area, it appears that hot coffee and tea extract styrene to the same extent as does hot water. No direct correlations could be drawn between the results obtained with 8% ethanol and those with water because of the different conditions used to carry out the migration studies. It appears, however, that 8% ethanol extracts styrene to a greater extent than does water.

Conclusions

Studies have been conducted under actual usage conditions on the migration of styrene from food-contact packaging materials into food-simulating solvents and beverages. The amounts of styrene leached were determined by a headspace GC method. The test samples included foam, impact, and crystal polystyrene cups with residual monomer levels ranging from about 70 to 2260 ppm. Measurements were made for styrene migration into 8% ethanol, water, coffee, and tea under several different test conditions; in all cases, styrene migrated. The amounts of styrene leached per unit area ranged from 0.0077, 0.0078, and 0.0078 $\mu g/cm^2$ for foam cups with water, tea, and coffee, respectively, as the solvent to 0.036, 0.064, and 0.210 $\mu g/cm^2$ for foam, impact, and crystal polystyrene cups, respectively, with 8% ethanol as the solvent.

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Temperature-Programmed Gas Chromatographic Determination of Polychlorinated and Polybrominated Biphenyls in Serum

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An analytical method was developed to quantitate polychlorinated and polybrominated biphenyls (PCBs and PBBs, respectively) in human serum. The method includes denaturation of the proteins in serum, extraction, adsorption chromatography, and gas chromatography with electron capture detection. The coefficients of variation for determining the in vivo bound PCBs and PBBs ranged from 11.7 to 29.8% and 7.1 to 14.0%, respectively. The method is capable of measuring 10 ng PCBs and PBBs/mL in 4 mL serum.

As recognition of the extent of environmental contamination from chemicals increases, so will the need to analyze both environmental and human specimens for these contaminants. Because these compounds occur as mixtures, methods for analyzing the entire group are needed. The method reported here analyzes human serum for a series of compounds, polychlorinated biphenyls (PCBs), that have been released into the environment over a period of decades and for a smaller series of compounds, polybrominated biphenyls (PBBs), that have presumably contaminated a much more limited area. The Michigan Department of Public Health and the Center for Disease Control are using this method to monitor PCB and PBB levels in Michigan residents.

Commercial PCB mixtures vary in percentage by weight of chlorination, depending on the properties needed for a particular use; however, higher forms of mammalian life that have been exposed to all these mixtures selectively accumulate the more highly chlorinated components (1). In this study, PCBs were quantitated as Aroclor 1254, of which 54% of its weight is chlorine.

The decision to use Aroclor 1254 for quantitation was made on the following basis. Milk and fish products are reportedly the major sources of PCBs in humans (2), and market basket surveys conducted by the Food and Drug Administration showed that the gas chromatographic (GC) patterns of PCBs found in dairy and fish products most closely resembled Aroclors 1254 and 1260 (3). In a study conducted in the State of Michigan concerning PCB levels of residents who consumed fish from Lake Michigan, the PCB patterns in adipose tissue resembled Aroclors 1254 and 1260 (4). A source of the PCB contamination of dairy cattle was found to be silage that had been stored in PCB-coated silos; this PCB coating contained Aroclor 1254 (5). Another reason Aroclor 1254 was used in this study was that a preliminary investigation of a select number of serum samples from Michigan residents gave a GC pattern essentially similar to that found in goats that had been dosed with Aroclor 1254.

We were interested in developing a method that would analyze serum extracts for PCBs and PBBs so that the PBB results would reproduce those obtained from the same human serum that had previously been analyzed by isothermal GC with electron capture detection (ECD) (6). Preliminary attempts to separate the analytes by using adsorption chromatographic techniques and the more readily available adsorbents, Florisil, silica gel, silicic acid, and alumina, were unsuccessful.

We then considered approaches that would take advantage of differences in the ionic properties and molecular size of chlorine- and bromine-substituted biphenyls (7). Ion exchange resins utilize the slight difference in ionic character, but were not used because of the unpredictable character of these resins (8). Exclusion chromatography (9) utilizes the difference in molecular size and was evaluated by using 100 Å μ-Styragel columns in a high performance liquid chromatographic system; however, this system did not completely separate PCBs from PBBs.

Following these preliminary studies, we tentatively decided to follow an analytical approach that would provide simultaneous determination of both analytes. The objective then became the development of a method that would extract PCBs and PBBs from serum, provide an extract compatible with GC/ECD analysis and subsequent quantitation, and reproduce the previous

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PBB results. Previous data (4, 6) had established the efficiency of a hexane-ethyl ether extraction of methanol-denatured serum for PCBs and PBBs. Evaluation of the use of silica gel in various forms and stages of activation and deactivation (10-13) produced varying degrees of success in separating the commonly occurring chlorinated hydrocarbon insecticides from the halogenated biphenyls. On the basis of the evaluation, it appeared that procedures using deactivated silica gel provided a separation of most chlorinated hydrocarbons from halogenated biphenyls, good transferability, and processed a high volume of samples.

Evaluation of GC column packings to determine suitability for simultaneous analysis of PCBs and PBBs was done in the order of ascending value for McReynolds' constants of each liquid phase. The evaluation criteria consisted of: (*1*) peak-to-valley resolution of analytes; (*2*) total retention time; and (*3*) ability to withstand rapid programming and high temperatures with little column bleed. We noted that those liquid phases with the lowest McReynolds' constants, the methyl silicones, gave the best overall performance on the basis of our criteria.

A major problem in determining PCBs is the quantitation step, which is made increasingly difficult in biological samples by metabolism and/or elimination of some of the PCBs, so that the extracted product does not replicate any of the commercial PCB mixtures. Other studies (14-16) have shown that when gas chromatography is used with an electron capture detector, the method of Webb and McCall (17) gives improved interlaboratory precision and/or accuracy in quantitating PCBs as a particular Aroclor over methods that measure total peak height (or area) of all peaks or selected peaks.

On the other hand, PBB mixtures show fewer peaks than do PCBs when determined by packed column GC/ECD. Moreover, since the commercial product (Firemaster FF-1) consists largely of 2,2',4,4',5,5'-hexabromobiphenyl (18), quantitation is based on comparing the size of the hexabromobiphenyl peak in the sample to the size of this peak in a known weight of the commercial material (6).

For the quantitation of PCBs, we adapted the method to a microprocessor-controlled data system. The quantitation of PBBs is based on the largest peak in the Firemaster standard and is also accomplished by the microprocessor-controlled data system. This system has been described elsewhere (19).

METHOD

Apparatus

(a) *GLC/data system*.—Varian 3700 gas chromatograph (Walnut Creek, CA) equipped with Hewlett-Packard Model 7671A automatic injector (Avondale, PA), constant current ^{63}Ni electron capture detector, and Varian Model A-25 strip chart recorder. Chromatograph was controlled and data were calculated by Varian CDS 111(c) microprocessor.[2] Operating conditions: temperatures—column held at 190°C and programmed after PCBs elute (ca 21 min) to 230°C at 10°/min; detector 330°C, injection port 250°C; nitrogen flow 20 mL/min.

(b) *GLC column*.—Glass, 6 ft × 1/8 in. id, packed with performance-tested 3% SE-30 on 80-100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA).

(c) *Chromatographic column*.—Glass, 18 cm × 9 mm, packed with 3.0 g 70-150 mesh silica gel (Woelm) containing 3% water and sandwiched between 1 cm layers of anhydrous Na_2SO_4.

Reagents

(a) *Solvents*.—*n*-Hexane, methanol, and ethyl ether (Distilled-in-Glass, Burdick and Jackson, Muskegon, MI).

(b) *Sodium sulfate*.—Anhydrous, reagent grade, washed with hexane and continually oven-dried at 130°C. Remove from oven and let cool in desiccator before use.

(c) *Silica gel*.—Weigh silica gel into beaker, cover with aluminum foil (punch several holes in foil) and let stand in 130°C oven for at least 24 h. Let silica gel cool in vacuum desiccator. Weigh dried gel into flask with Teflon-lined screw cap. Add water down sides of flask in amount to constitute 3% of total weight. Extract water beforehand 3 times with hexane. Shake wetted gel until there is no evidence of clumping. Rotate 3 h on mechanical rotator and let stand tightly capped overnight before use. Silica gel prepared in this manner maintains its elution characteristics for at least 7 days.

(d) *Standards*.—PCB (AR 1254, EPA STD 5700E) was provided by Environmental Protection Agency. PBB (Firemaster FF-1, Lot 7042) was provided by Farm Bureau Services, Inc. (Lansing, MI) via the Food and Drug Administration. Standard solutions, from which cali-

Use of a trade name is for identification only and does not constitute an endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

[2] A complete description of programming the Varian CDS 111(c) for this determination can be obtained from the authors.

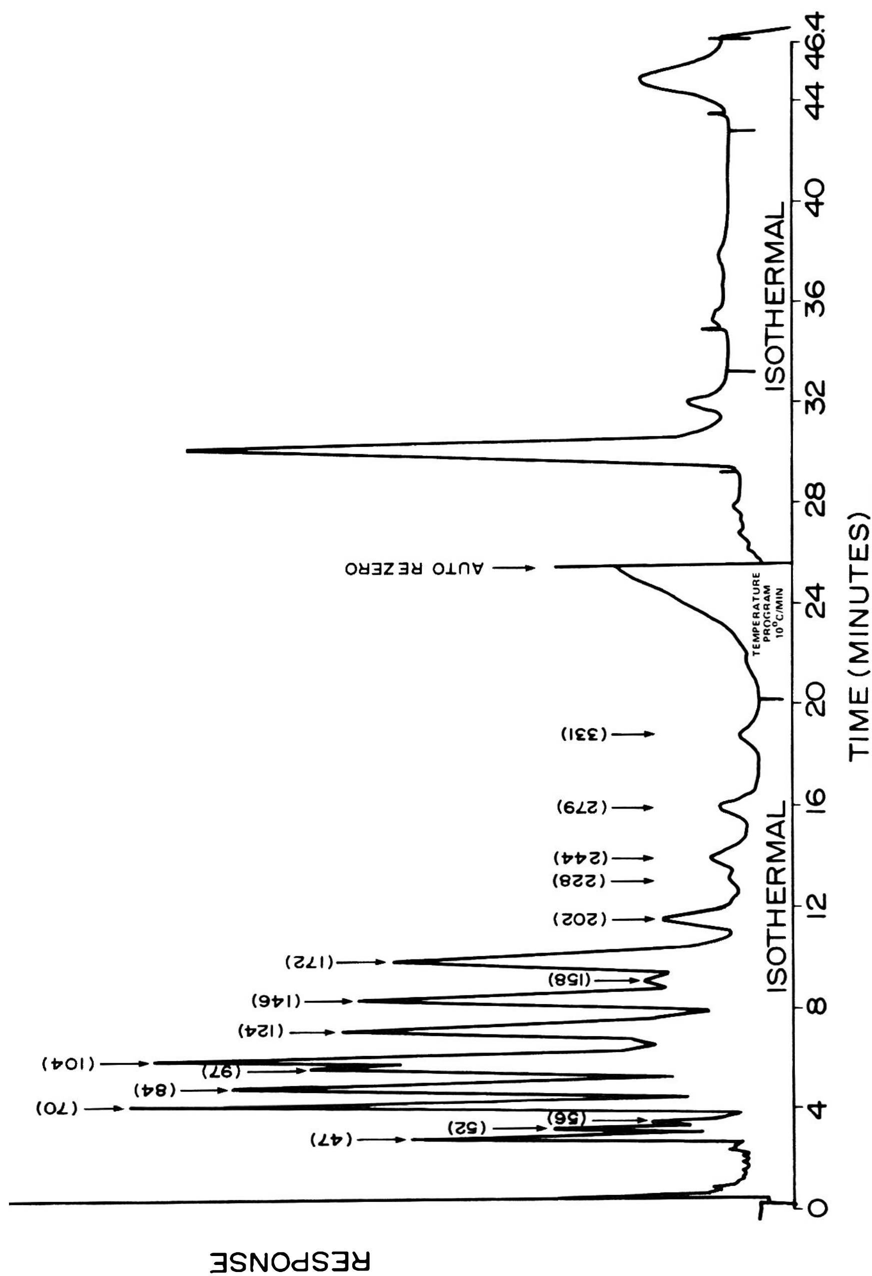

Figure 1. GC chromatogram of 3 μL of a mixed PCB/PBB standard containing 143.5 pg PCB/μL and 23.8 pg PBB/μL. Conditions as described in text.

Table 1. Estimates of precision on analyses [a] of in vivo pools containing PCBs and PBBs

		PCB			PBB		
Pool	N	Target,[b] ppb	Obtained (mean), ppb	CV, %	Target,[b] ppb	Obtained (mean), ppb	CV, %
A	49	10	10.5	13.1	10	8.6	8.4
B	48	42	41.2	15.2	164	138.6	8.7
C	46	10	12.7	23.7	41	34.8	14.0
D	47	100	95.3	11.7	10	8.9	13.8
E	44	10	12.5	29.8	484	426.9	7.1

[a] See text for pool description. Data analyzed by one-way analysis of variance.
[b] Target value is defined as the projected concentration resulting from the dilution of serum taken from cattle exposed to Aroclor 1254 and Firemaster FF-1 with PCB/PBB-free bovine serum.

bration factors are obtained, were prepared in hexane. These 4 mixed standards ranged from 430.5 pg AR 1254/μL and 95.1 pg PBB/μL to 41.0 pg AR 1254/μL and 9.5 pg PBB/μL.

Extraction

Accurately pipet 4 mL serum into 16 × 125 mm culture tube, add 2 mL methanol, and swirl briefly by vortex. Add 5 mL hexane–ethyl ether

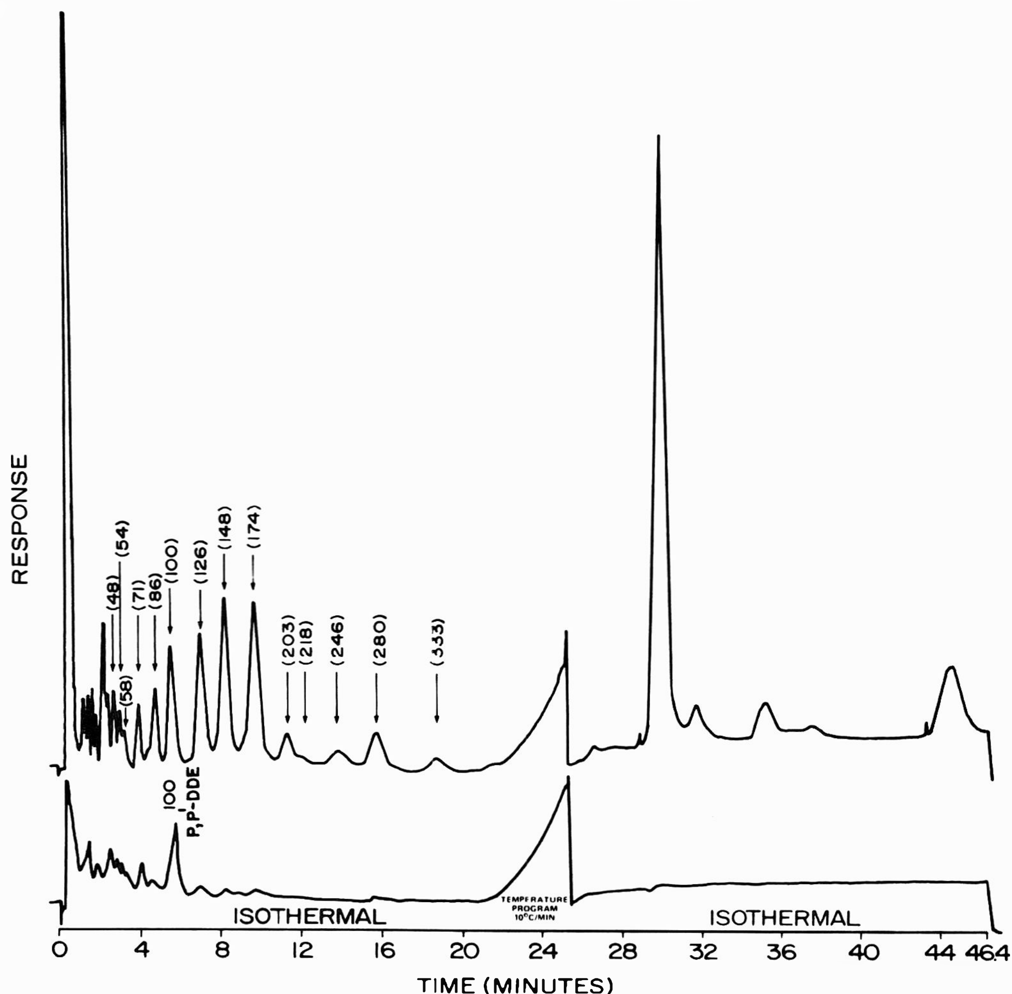

Figure 2. GC chromatograms of in vivo Pool A (upper trace) and control serum (lower trace). As shown in Table 1, the concentrations of PCBs and PBBs were approximately 10 ppb in this serum pool. Injection volume was equivalent to 12 mg of sample. Conditions as described in text.

(1 + 1), agitate 15 min at 50–55 rpm on rotary mixer, centrifuge 6 min at 1800 rpm, and transfer supernatant to 20 × 150 mm culture tube. Repeat this extraction step twice and combine extracts. Reduce solvent volume, at room temperature, to ca 0.5 mL under gentle stream of nitrogen.

Adsorption Chromatography

Prewash column containing silica gel with 20 mL hexane. Transfer concentrated extract to top of column. Rinse sample tube with three 0.5 mL portions of hexane and transfer each wash to head of column. Elute column with 5 mL hexane; discard first 7 mL; collect 15 mL in conical tube which has been calibrated to 1 mL. Concentrate eluate to 1 mL by blowing gentle stream of nitrogen into eluate which is heated to 40°C in a water bath. Analyze by GC/ECD.

Gas Chromatography

Inject 3 μL of PCB and PBB mixed standards. Having calculated various absolute calibration factors by external standard method (19), inject 3 μL of an appropriate aliquot of extract. Derive PCB and PBB contents of unknowns by comparing their peak areas to that of the mixed standards.

Results and Discussion

Our chromatogram of Aroclor 1254 (Figure 1) compares favorably with that of Webb and McCall (17), to which they assigned their factors. The main difference is that our chromatogram shows 3 additional peaks at relative retention times of 244, 279, and 331. Also, our chromatogram includes the response for PBB. However, the peaks 244, 279, and 331 are not characteristic of PBBs, because our GC recordings of PCB standards without added PBB give these 3 peaks. We believe the differences may be attributed to any one or combination of the following: (*1*) differences in the lots of AR 1254 employed in the 2 studies; (*2*) differences in resolution of the GC columns used; (*3*) differences in the response characteristics of the different electron capture detectors to certain PCB isomers.

Certain characteristics of the GC tracing led to difficulty in obtaining reproducible calibration factors (or response factors) for certain peaks, i.e., the CDC 111(c) microprocessor did not consistently detect baseline at the same point within concentrations and especially among concentrations of the Aroclor 1254 standards. This resulted in considerable variation by area counts for the same peak. This problem was especially

Table 2. Statistics[a] associated with calibration factors at 4 concentration levels

	Within concentration[b]												Among concentrations[b]		
	Std A			Std B			Std C			Std D			Stds A,B,C,D		
Webb-McCall ID No.	$\bar{X}$	CV (%)	*N*	$\bar{X}$	CV (%)	*N*	$\bar{X}$	CV (%)	*N*	$\bar{X}$	CV (%)	*N*	$\bar{X}$	CV (%)	*N*
47, 54, 58 group	4.812	6.1	14	4.701	6.4	13	4.506	7.0	15	4.319	8.2	13	4.182	12.1	55
70	3.988	5.4	14	3.882	5.5	13	3.736	6.2	15	3.684	8.9	13	3.822	7.2	55
84	3.752	5.5	14	3.632	6.3	13	3.483	6.8	15	3.435	9.9	13	3.576	7.6	55
98	2.378	6.0	14	2.334	5.4	13	2.246	6.2	15	2.069	17.4	13	2.258	10.4	55
104	3.055	4.8	14	2.946	6.2	13	2.838	7.1	15	2.759	11.0	12[c]	2.903	8.1	54
125 group	2.462	6.3	14	2.418	6.8	13	2.366	6.2	15	2.550	10.2	13	2.446	7.8	55
146, 160 group	1.836	5.8	14	1.802	7.0	13	1.763	6.8	15	1.888	9.8	13	1.816	8.2	55
174 group	1.318	6.3	12[c]	1.258	11.7	13	1.404	16.9	13[c]	1.490	21.0	8[c]	1.355	15.6	46
203, 232 group	0.839	9.8	14	0.874	8.8	13	0.880	8.7	15	1.045	17.2	12[c]	0.904	14.5	54
PBB (major peak)	2.523	4.2	14	2.442	5.2	13	2.255	8.2	15	2.144	13.8	13	2.341	10.2	55

[a] Among run statistics include: $\bar{X}$ = mean of calibration factors; CV (%) = coefficient of variation; *N* = number of determinations.

[b] Concentration (pg/μL hexane) of standards A, B, C, and D were: (A) PCB = 430.5, PBB = 95.1; (B) PCB = 287, PBB = 47.6; (C) PCB = 143.5, PBB = 23.8; and (D) PCB = 41, PBB = 9.5.

[c] Integration malfunction resulted in a smaller *N*.

Table 3. Potential environmental interferences calculated as PCBs and/or PBBs

Compound	Level of in vitro spike (ppb)	Total PCB level (ppb)[a]	PBB level (ppb)[a]	PCB interference factor	PBB interference factor
Halowax 1001	48.8	11.06	0.07	0.2266	0.0014
Decachlorobiphenyl	50.0	1.77	11.24	0.0354	0.2248
Halowax 1051	51.2	6.95	N.P.D.	0.1357	0
AR 5432	47.8	1.86	N.P.D.	0.0390	0
AR 5442	51.0	1.16	0.015	0.0242	0.00029
AR 5460	60.0	1.34	0.035	0.0223	0.0006
Butylphthalyl butylglycolate	150 000	1.10	0.09	7.3×10^{-6}	6×10^{-7}
Di-2-ethyl hexyl phthalate	138 800	1.54	0.02	11×10^{-6}	1×10^{-7}
Di-2-ethyl hexyl adipate	140 000	1.16	0.035	8.2×10^{-6}	2×10^{-7}
Mixed chlorinated hydrocarbon standard[b]	10 (each component)	35.06[c]	0[d]	0.3506[d]; 3.506[e]	0
trans-Nonachlor	6.0	0[f]	0	0	0
Oxychlordane	2.5	0	0	0	0
Toxaphene	100	3.40	0	0.034	0
Mirex	12.2	4.2	0	0.34	0

[a] Average of duplicates.
[b] Hexachlorobenzene, γ-hexachlorocyclohexane, β-hexachlorocyclohexane, heptachlor epoxide, *o,p'*-DDE, *p,p'*-DDE, dieldrin, *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDT.
[c] Single value, *p,p'*-DDE accounted for 94.8% of the PCB concentration.
[d] Considering concentration of all 10 components.
[e] Considering concentration of only *p,p'*-DDE.
[f] Elutes from adsorption column with PCBs but GC retention time precludes calculation as a PCB.

evident for PCB peaks 56, 124, 146, 158, and 172. However, this problem was overcome through grouping. Grouping is a process whereby areas of several peaks or a broad peak are combined and treated as a single peak; the retention time assigned is that of the midpoint of that group. Peaks 244, 279, and 331, for which there were no assigned Webb and McCall factors, were combined with peaks 202 and 228. Since Webb and McCall's mean weight percent factor for Aroclor 1254 accounted for 100% of the PCBs, this group was assigned a mean weight percent value of 2.8, which is the sum of values (1.8 + 1.0) originally assigned by Webb and McCall for peaks 203 and 232, respectively.

To better estimate the precision of this method, pooled material containing in vivo bound PCBs (as AR 1254) and PBBs was repeatedly evaluated before and during the analysis of unknowns. In the data shown in Table 1, each of the pools was analyzed in duplicate on 11 different days before the analyses of unknown samples, and 3 pools were included with each run of unknowns; this resulted in a minimum of 44 analyses for each pool. In general, the coefficients of variation (CV) were lower for the PBBs. Figure 2 is a typical gas chromatogram of Pool A (see Table 1) and the bovine control.

The variation in any procedure is the sum of all variations from various aspects of the method. We have evaluated the contribution to the total variation made by the automated instrumentation through the statistical examination of calibration factors for standards at 4 concentration levels. These calibration factors were generated over a series of analytical runs and are displayed in Table 2. The CV determined from the among-run "within-concentration" statistics increased for all analytes as the concentration of the standard decreased. The among-run "among-concentration" statistics indicate a CV of ≥10% for grouped peaks 47–58, 174, and 203–232; for the PCB peak 98; and for the major peak in PBB. Nonetheless, this agreement among the calibration factors demonstrates the linearity of the system over the concentration range studied.

We also evaluated the contribution of the adsorption chromatographic step to the total variation in the PCB and PBB data. This was done by monitoring the recoveries from the elution step of an extract of serum that originally had been PCB/PBB-free, but after extraction was fortified with PCBs and PBBs at 26 and 119 ppb, respectively. The PCB and PBB recoveries were measured daily for 7 days following silica gel deactivation; the analyses of both PCBs and PBBs had CVs of 5.9%.

To determine if the method gave false-positive results for PCBs and PBBs, we analyzed sera from 25 employees of the Center for Disease Control. The PBB values ranged from undetectable to <1 ppb, and the PCB values ranged from 2 to 18 ppb. These data confirm the ubiquitous character of PCBs and indicate that exposure to PBBs is much more limited geographically.

Table 3 shows the results of an experiment designed to evaluate the quantitative effect on the PCB and PBB values by potential interferants by means of an "interference factor," i.e., the ratio of the apparent PCB or PBB concentration measured to the concentration of the interfering substance. For example, a value of 1.0 for the PCB interference factor for a contaminant indicates that the method gives the same response for that analyte when quantitated as Ar 1254 as does an equal amount of AR 1254. Decachlorobiphenyl gave the greatest interference for PBBs, while Halowax 1001, Halowax 1051, the mixed chlorinated hydrocarbon standard (particularly *p,p'*-DDE), and mirex significantly interfered with the quantitation of AR 1254.

In our use of this method, we routinely analyze control bovine serum that has been fortified with chlorinated hydrocarbons at levels comparable with the general population (20) so that we can detect any undesirable elution characteristics exhibited by the silica gel. This method is capable of determining 10 ng PCBs and PBBs/mL in 4 mL serum; however, based on the response of in vivo Pool A (Figure 2), lower detection limits of 5 and 1 ng/mL are achievable for PCBs and PBBs, respectively.

Acknowledgment

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ALCOHOLIC BEVERAGES

Analysis and Evaluation of Barley Used in the Malt Beverage Industry

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The American Society of Brewing Chemists methods for barley moisture, extract, and potential diastatic power are presented. These methods were reviewed by a Barley Analysis Subcommittee during 1975-1980 and published as recommended procedures. The moisture determination has been adopted as an official first action ASBC-AOAC method.

The American Society of Brewing Chemists (ASBC) has had methods for the analysis and evaluation of barley in its *Methods of Analysis* since the fifth edition, published in 1949, but these procedures have not previously been presented to AOAC. In this report, methods are presented for moisture in barley, extract from malting barley, and potential diastatic power of barley.

The majority of malting grade barley grown in the United States is used by the malt beverage industry in the form of malted barley. According to figures released by the U.S. Treasury, Bureau of Alcohol, Tobacco and Firearms, 4 898 767 348 lb of barley malt and malt products were used in 1979 by the industry. A lesser amount, 23 049 020 lb, of unmalted barley was also used by brewers. These figures are cited to emphasize that malting barley is an important agricultural commodity in the United States.

The malting industry and the malt beverage industry are interested in the analytical as well as the varietal and physical properties of barley. Only malting barley varieties accepted by joint committees of plant breeders, brewers, and maltsters are grown by farmers and purchased by maltsters for malting purposes. New varieties of barley are developed by plant breeders and evaluated by agricultural stations and brewing industry-sponsored groups each year. The analytical properties of the barleys play an important role in determining their acceptance by the industry.

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This report of the Associate Referee was presented at the 94th Annual Meeting of the AOAC, Oct 20-23, 1980, at Washington, DC.
The recommendation of the Associate Referee for adoption of the method for moisture in barley was approved by the General Referee and Commiteee D and was adopted by the Association. See *J. Assoc. Off. Anal. Chem.* **64**, 429 (1981).

Moisture, or loss on drying, is an obvious factor in the purchase and use of barley. Not only does it affect the storage properties of the grain and the solids content of the barley, it also affects the malting properties of the barley.

To the maltster and brewer, extract in cereal grains means the total amount of soluble substance derived from the grain by enzymatic action and by simple water solution. Raw barley contains some active enzyme systems but many more are formed or released during the germination part of the malting process. Barley extract as defined in this report is a predictive test to give information on the relative amount of extract contained in the barley that will be available after the completion of the malting process. Because of differences in commercial malting methods, the barley extract values do not necessarily correlate closely with extract yields determined on malt made from the barley. However, the values are useful for comparing barleys of different varieties or different lots of the same variety. Barley extract is evaluated by digesting ground barley with a standardized mixture of enzymes for stated time periods at specified temperatures. The extract released is then separated by filtration, and the specific gravity of the clear filtrate is determined.

Potential diastatic power of barley is another important determination of quality. A substantial portion of the starch-saccharifying enzyme of barley (β-amylase) is in bound form. This can be released by digesting with a suspension of proteolytic enzyme in a way similar to the release in malting by the natural proteolytic enzymes evolving during germination. Diastatic power of malt results from the combined actions on starch of β-amylase, and the dextrinizing enzyme α-amylase produced during germination of barley. However, a sufficiently high percentage of malt diastatic power is attributed to β-amylase to make an evaluation of this enzyme in barley of predictive value.

Moisture Procedure

Barley moisture or loss on drying is a grading factor and its determination is prescribed in the

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Table 1. Statistical summary of barley moisture content by ASBC malt oven method and USDA barley oven method (units of % moisture) (from ref. 1)

Pair	Method	No. labs. (n)	Moisture level	Grand mean[a]	Within-lab. error[b]	Between-lab. error[c]	Combined error[d]	CV[e]	Calc. F[f]	Critical F at $P = 0.01$[g]
1,2	ASBC	16	medium	12.06	0.10	0.37	0.38	3.13	29.132	3.522
	USDA	16	medium	12.40	0.10	0.29	0.30	2.46	16.183	3.522
3,4	ASBC	16	low	10.19	0.04	0.22	0.22	2.17	73.762	3.522
	USDA	16	low	10.52	0.04	0.15	0.15	1.47	24.000	3.522
5,6	ASBC	13	high	14.10	0.14	1.52	1.53	10.85	224.802	4.155
	USDA	13	high	14.50	0.15	1.56	1.56	10.79	219.815	4.155

[a] Grand mean = GM = $(\bar{X} + \bar{Y})/2$.
[b] Within-lab. error = $s_r = \sqrt{S_r^2}$.
[c] Between-lab. error = $s_b = (\sqrt{S_t^2 - S_r^2})/2$.
[d] Combined error = $s_c = \sqrt{S_b^2 + S_r^2}$.
[e] CV = coefficient of variation of $s_c = 100s_c/\mathrm{GM}$.
[f] Calculated $F = S_t^2/S_r^2$.
[g] Critical F from tables with $n - 1$ and $n - 1$ degrees of freedom.

United States by the U.S. Department of Agriculture and in Canada by the Canadian Grain Commission. The Standard Reference Method (SRM) prescribed in the United States is the same as AOAC method **14.004** and the ASBC air-oven method (Standard Reference Method). The procedure specifies 55 × 15 mm metal dishes and a temperature of 130°C for 1 h.

According to ASBC moisture procedures, other methods may be used if they give equivalent results to the SRM method.

A collaborative study by 17 collaborators on 2 methods for barley moisture was reported in the ASBC Journal (1). The study used 3 sample pairs with medium, low, and high moisture contents. The USDA (ASBC barley) method produced higher moisture contents than the ASBC malt method. The statistical summary of this collaborative study is given in Table 1.

Extract Procedure

Collaborative studies of extract methods were made in 1945, 1946, and 1947 by an ASBC Subcommittee on Methods of Barley Analysis. It is unfortunate that at that time, 35 years ago, no real thought was given to statistically designed experiments and very little statistical analysis of data was made.

The 1945 study was done on 3 barley samples by 7 collaborators. Three published methods for extract were tested to see if one was suitable for further investigation. None of the 3 procedures was the same as the one presented in this report; therefore, the data obtained are irrelevant.

For the 1946 study, the committee modified one of the 1945 procedures and subjected it to collaborative study on 8 barley samples by 8 collaborators. Each collaborator was requested to perform duplicate determinations. The extract method used was the same as the one submitted in this report. Mean extract values for the 8 samples ranged from 75.3 to 80.5%, and the range of values among the 8 collaborators was 1.2–3.5.

The 1947 collaborative study used the same procedure used in 1946. Eight barley samples were tested in duplicate by 9 collaborators. Mean extract values for the 8 samples ranged from 75.1 to 80.9%, and the range of values between the 9 collaborators was 1.7–4.4. The range of each collaborator replication was 0.4–1.1% with a grand mean of 0.33%.

As a result of the 1946 and 1947 studies, the Subcommittee recommended that the method used be accepted by ASBC. The ASBC Technical and Executive Committees concurred and the method was printed in the 1949 edition of ASBC *Methods of Analysis*.

Potential Diastatic Power Procedure

Collaborative studies of this procedure were conducted by ASBC Barley Analysis Subcommittees during 1945 and 1948.

In 1945, 5 barley samples were tested by 9 collaborators. The potential diastatic power (DP) of the barleys ranged from 81 to 195. The values among collaborators ranged from 17 to 25 with the higher DP samples having greater ranges.

The 1948 series was done by 10 collaborators on 4 samples of barley. The main purpose of this study was to determine the effect of different papains on the DP results. Three papains were used by each collaborator, 2 furnished by the chairman and one of the collaborator's choice.

The grand average of all DP results was 116.8 and the averages of the 3 papains were 115.5, 116.0, and 119.0, indicating little variation caused by papain. The ranges of values among collaborators were 13.7, 16.2, and 15.4.

As a result of these collaborative studies, the Subcommittee recommended and the ASBC Technical and Executive Committees agreed that the procedure be accepted. The method was printed in the 1949 edition of ASBC *Methods of Analysis*.

In May 1975, a new Barley Analysis Subcommittee was formed by ASBC and charged, among other items, with determining which barley analysis items were being used by the industry and whether the older procedures were acceptable as printed or needed revision. A questionnaire sent to the major malting companies disclosed that 7 of 10 who replied used the ASBC moisture method, 6 of 7 used the extract procedure, and 7 of 7 used the potential DP method. These returns indicated to the committee that the procedures as presented are valid after more than 30 years of use.

Recommendation

It is recommended that the following methods for moisture, extract, and potential diastatic power of malting barley be adopted offical first action.

Barley Potential Diastatic Power

Reagents

Revise method **10.117-10.120** as follows:

Add the following reagent:

(i) *Papain suspension.*—1%. Use only papain of high proteolytic activity that has been tested for absence of amylolytic activity. Weigh papain into beaker and work into smooth paste with glass rod and water, carefully added 2-3 drops at a time. When papain is thoroughly wetted and in suspension or solution, transfer to suitable volumetric flask and dilute to volume with water. Let stand 2 h before use.

Determination

(*1*) *Malt.*

(*2*) *Barley.*—Prepare sample as in **14.062.** Weigh 5 ± 0.005 g well mixed ground barley into dry 250 mL flask. Pipet 100 mL well mixed papain suspension onto barley in flask, agitating carefully to avoid sticking barley particles to wall of flask. Stopper flask and digest 20-22 h at 20°C. Filter well mixed infusion through 15 cm fluted paper (S&S No. 588, or equivalent), pouring back first 25 mL filtrate.

Immediately dilute 20 mL infusion (*1*) or (*2*) above . . .

Malting Barley — Moisture (Loss on Drying) (1)

Official First Action — ASBC-AOAC Method

10.B01 ***Determination***

Proceed as in **14.004.** Use gravity convection or forced-draft oven, regulated to maintain temp. 130 ± 1°. Thermometer bulb is at level of shelf where dishes are placed. Report moisture loss on drying to 0.1%.

Extract from Malting Barley (2, 3)

Principle

Ground barley is digested with standardized mixture of enzymes for stated period at specified temperature. Released extract is filtered, and sp. gr. is determined.

Reagents

(**a**) *Malt diastase.*—Analytical grade (Sturge Enzymes, 750 Third Ave, New York, NY 10017).

(**b**) *α-Amylase.*—Analytical grade (Sturge Enzymes).

Apparatus

See **10.106(c)**, **(d)**, **(f)**, **(g)**, **(i)**, **(k)**.

Preparation of Sample

See **14.062.**

Determination

Weigh 50 ± 0.05 g ground barley into mash beaker. Add 2.5 ± 0.0005 g malt diastase and 0.5 ± 0.0005 g α-amylase. Mix well with stirring rod. Add 200 mL water in 3 portions. Mix meal and first portion of water to form uniform slurry. Use second portion to wash sides of beaker and use third portion to rinse stirring rod. Place mash beaker in 20°C water bath and let stand overnight (15-16 h).

Place mash beaker in mashing apparatus maintained at 70°C. Start stirrer in mash beaker and stir continuously 60 min at 70°C. Continue as in **10.108(c)**, *Cooling and filtration*, and **10.108(d)**, *Specific gravity*. Determine Plato value from **52.009.**

Determine extract value of enzyme prepared by carrying through blank of enzymes and water

only. Determine sp. gr. and Plato value as with barley mash.

Calculations

(a) Calculate extract correction for enzyme blank:

$$E = [Pe\ (900 - 2D)]/(100 - Pe)$$

where E = extract correction; Pe = Plato value of enzyme blank filtrate; D = g enzyme preparation used = 3.0 g.

(b) Calculate extract in barley, as-is basis:

$$BE\text{, as-is, \%} = \left|\frac{P(800 + M - 2D)}{100 - P}\right| - E$$

where P = Plato value of barley mash filtrate; M = barley moisture, %.

(c) Calculate extract in barley, dry basis:

$$BE\text{, dry basis, \%} = [BE(\text{as-is, \%}) \times 100]/(100 - M)$$

Report barley extract to 0.1%.

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Liquid Scintillation Counting of ^{14}C for Differentiation of Synthetic Ethanol from Ethanol of Fermentation

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Samples containing ethanol are fractionated on a column so that the resultant ethanol content is >93%. Determination of ^{14}C by liquid scintillation counting on the ethanol fraction differentiates ethanol produced by fermentation from synthetic ethanol produced from fossil fuel sources. Twenty-seven samples were fractionated and analyzed for the ^{14}C isotope. Six samples were synthetic ethanol derived from ethylene gas (direct and indirect process), and yielded a mean value for ^{14}C isotope of 0.167 dpm/g carbon with a standard deviation (SD) of 0.066 dpm/g carbon (disintegrations per minute per gram of carbon). The remaining samples were ethanol derived from the fermentation of natural materials, such as corn, pear, sugar cane, grape, cherry, and blackberry, and yielded a mean value for ^{14}C isotope of 16.11 dpm/g carbon with an SD of 1.27. The ^{14}C values for specific mixtures of a synthetic and a natural ethanol compare favorably with the analytical values obtained by this procedure.

Ethanol can be derived by fermentation of any material in which carbohydrates are present in the form of simple sugars or polysaccharides. Simple sugars (sugar cane, sugar beets, molasses, and fruit) may be converted directly by fermentation. Polysaccharides (starches, cellulose) from grains or root crops must first be hydrolyzed to simple fermentable sugars by the action of enzymes from malt or molds.

There are 2 main processes for synthesizing ethanol from ethylene. The earliest developed, the indirect hydration process, requires 2 steps: absorption of ethylene in concentrated sulfuric acid to form mono- and diethyl sulfates, and then hydrolysis of the ethyl sulfates to ethanol. The second, the direct hydration process, is designed to eliminate the use of sulfuric acid. This process involves the catalytic addition of water to ethylene under high pressure and temperature. Phosphoric acid, impregnated on an inert support such as Celite diatomite, is commonly used as a catalyst.

The measurement of the radioactive carbon isotope ^{14}C provides a direct means of differentiating products derived from fossil fuels and those derived from present-day natural products. The source of ^{14}C present in organic plant material is the uptake of atmospheric $^{14}CO_2$, a process that has been relatively constant for many thousands of years.

All ^{14}C originally present in fossil fuel, which once consisted of living plant material, has long since decayed. By contrast, the level of ^{14}C in modern organic plant material reflects an equilibrium with atmospheric $^{14}CO_2$ of 15–18 dpm/g carbon. Variations in these values are attributable to latitude, elevation, fossil fuel combustion, nuclear bomb testing, and preferential fractionation by plant material of carbon isotopes during photosynthesis.

Because synthetic ethanol is produced from dead carbon (petroleum products void of ^{14}C), radiocarbon liquid scintillation counting is an ideal method for differentiating natural and synthetic compounds (1, 2). The biosynthesized compound has a relatively constant level of ^{14}C because of the uniform distribution and uptake of this carbon isotope by plants in the form of $^{14}CO_2$. Quantitation of ^{14}C present in an ethanol sample, compared with the value established for a 100% natural ethanol standard, enables determination of the presence of natural and/or synthetic petroleum-derived ethanol.

The ^{14}C technique has been applied to caffeine to ascertain whether it originates from a natural product or from petroleum synthesis (3–5). The ^{14}C procedure has also been used to determine the age of wine (6, 7).

Experimental

Ethanol was isolated from fermentation products of various natural sources, i.e., corn, rye, grape, whey, and cherry, and also from synthetic ethanol produced from ethylene gas by direct or indirect process.

Samples were fractionated on a distillation column, ca 3 ft × 1 in. diameter, containing a glass helix (ca 25–50 theoretical plates). With the temperature maintained at 78°C, and a reflux

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Table 1. Liquid scintillation counting of ^{14}C from ethanol derived from fruit

Origin	Sample	Ethanol in distillate, %	Specific activity, dpm/g carbon	SD	Modern C, %
Apricot	1	95.79	16.21	0.14	100.5
Apple	2	95.69	15.69	0.23	97.3
Blackberry	3	92.60	16.46	0.20	102.0
Cherry	4	93.44	15.53	0.25	96.3
Cherry	5	94.06	15.32	0.17	95.0
Grape	6	97.03	14.52	0.12	95.6
Grape	7	95.55	14.92	0.16	92.5
Grape	8	95.70	15.44	0.14	95.7
Pear	9	95.68	15.47	0.17	95.9
Mean			15.51		
SD			0.59		
CV, %			3.80		

ratio of 5 to 1, the ethanol concentration in the distillate was ca 93%. After each sample was collected, the column was rinsed with distilled water and dried under vacuum. Approximately 10–20 mL ethanol was collected for each analysis.

^{14}C Analysis

^{14}C content was determined by using a low level liquid scintillation counting technique. The isolated ethanol was ignited, and the combustion products (CO_2 and H_2O vapor) were separated. CO_2 was converted to acetylene via lithium carbide. Acetylene gas was then catalytically trimerized to benzene. ^{14}C was determined on the benzene sample dissolved in liquid scintillation cocktail (8). A special Teflon lead-shielded vial was used to reduce background of the counter (9). The discrimination windows of the liquid scintillation counter (Picker 220) were set between the E_{max} energy of ^{3}H and ^{14}C to eliminate the introduction of any ^{3}H activity into the ^{14}C determination. Counting efficiency for the ^{14}C energy window setting was 68%, with background averaging 3.12 counts/min. A liquid scintillation standard was prepared from ^{14}C-labeled toluene (New England Nuclear).

Results and Discussion

The determination of ^{14}C in ethanol derived from fruits gave a mean value of 15.51 dpm/g carbon with a standard deviation (SD) and coefficient of variation (CV) of 0.59 and 3.80%, respectively (Table 1). The 100% modern figure is based on a mean value for natural ethanol of 16.1 dpm/g carbon. Percent modern calculated for ethanol derived from fruit ranged from 92.5 to 102.0.

Table 2. Liquid scintillation counting of ^{14}C from ethanol of nonfruit origin

Origin	Sample	Ethanol in distillate, %	Specific activity, dpm/g carbon	SD	Modern C, %
Sugar cane	10	95.15	16.16	0.11	100.2
Sugar cane	11	90.00	15.43	0.19	95.7
Rum (first detn)	12	94.20	20.13	0.18	124.8
Rum (second detn)	13	94.20	20.33	0.15	
Corn	14	94.01	16.34	0.16	101.3
Corn	15	99.09	15.74	0.23	97.6
Corn	16	94.02	17.25	0.22	106.9
Sake	17	96.44	15.65	0.18	97.0
Sake	18	96.37	15.87	0.14	98.4
Rye	19	94.01	15.98	0.16	116.9
Whey	20	96.58	15.37	0.14	95.3
Whey	21	96.50	15.34	0.09	95.1
Mean			16.63		
SD			1.76		
CV, %			10.58		

Liquid scintillation counting of natural ethanol of nonfruit origin gave a mean value of 16.63 dpm/g carbon (Table 2). All values reported in dpm/g carbon were very close except on one sample which was analyzed twice and gave values of 20.13 and 20.33 (Nos. 12 and 13). There was no explanation for this high value. The range in percent modern for natural ethanol (nonfruit origin) was from 95.1 to 124.8.

Synthetic ethanol (direct or indirect process) gave a mean value of 0.167 dpm/g carbon with SD and CV values of 0.066 and 39.52%, respectively (Table 3). The CV value was high because the dpm/g carbon values were very small with large range. The low specific activity values probably reflect the statistical uncertainty in variations in long background count times rather than the actual presence of low concentrations of ^{14}C. The calculated percent modern for synthetic ethanol (indirect or direct process) ranged from 0.6 to 1.7.

Mixtures of natural and synthetic ethanol of known ^{14}C content were blended in various proportions, and analytical values were compared with the theoretical value of the mixtures. Table 4 compares the analytical values obtained for specific mixtures of synthetic and natural ethanol, 0.11 and 15.69 dpm/g carbon, respectively, with the theoretical value.

In general, good agreement was obtained, demonstrating that the technique is suitable for identifying added synthetic alcohol in the presence of ethanol produced by fermentation, as for example, in a wine fortified by the addition of synthetically produced alcohol.

Table 3. Liquid scintillation counting of ^{14}C in synthetic ethanol (petroleum base)

Ethanol process hydration	Sample	Ethanol in distillate, %	Specific activity, dpm/g carbon	SD	Modern C, %
Direct	22	99.09	0.16	0.04	1.0
Direct	23	94.84	0.27	0.09	1.7
Indirect	24	95.09	0.16	0.06	1.0
Indirect	25	99.09	0.11	0.04	0.7
Direct	26	99.09	0.09	0.04	0.6
Indirect	27	99.09	0.21	0.06	1.3
Mean			0.167		
SD			0.066		
CV, %			39.52		

Table 4. Liquid scintillation counting of ^{14}C in mixtures of synthetic and natural ethanol

Composition	^{14}C Specific activity, dpm/g carbon		Modern C, %	
	Anal.	Theor.	Anal.	Theor.
100 % natural	15.69	—	97.2	—
Natural +				
synthetic (90 + 10)	13.85	14.13	85.8	87.6
(80 + 20)	12.34	12.57	76.5	77.9
(70 + 30)	10.51	11.01	65.2	68.3
(50 + 50)	7.96	7.90	49.3	49.0
(30 + 70)	4.82	4.78	29.9	29.6
100 % synthetic	0.11	—	0.6	—

No carbon isotope fractionation is present when the carbonaceous samples are converted to benzene. This was studied in detail early in the conception of the benzene method (8).

The slight differences noted in the modern carbon as reported by McWeeny and Bates (10) and this paper are most likely explained in the variations in natural radioactivity of the samples tested. However, counting ethanol samples in the benzene form should be superior because the exact carbon content is known. There is no quenching effect in the liquid scintillation cocktail (benzene is sample and solvent) and, therefore, counting is more efficient. Also, because liquid scintillation counters are volume-sensitive to background, it is probable that counting a 5 mL sample vs a 20 mL sample resulted in a 3-4X background reduction, thereby reducing error by shortening count time, increasing signal-to-noise ratio, and improving counting statistics for equal count time.

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Colorimetric Method for Determination of Ethanol in Presence of Methanol

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A method is described for the determination of ethanol in the presence of methanol without pre-separation. The sample is oxidized to give acetaldehyde and formaldehyde. 2-Thiobarbituric acid reagent is added and selectively forms a pink adduct with acetaldehyde, but not with formaldehyde. The method is sensitive, obeys Beer's law in the concentration range 18-80 μg/mL, and has an advantage over other methods because it does not detect any "apparent normal value" of alcohol in blood.

The official AOAC method (1) for the quantitative determination of ethanol in distilled spirits is based on distillation and measurement of specific gravity. This method cannot be used if other alcohols such as methanol are present. Determination of alcohol by measuring its volume in pure form (2) is a lengthy procedure that requires considerable technique and is not suitable for routine analysis. Determination of alcohol in the distillate by measuring physical properties such as specific gravity (1) and refractive index (3-5) is not well suited to very small quantities, or to mixtures containing other volatile ingredients. There are a number of methods based on oxidation with potassium permanganate (6), iodine pentoxide (7), or potassium dichromate (8-17). Other methods include bromometric measurement of ethylene formed by pyrolytic dehydration of alcohol vapors (18), conversion of alcohol to ethyl nitrite and measurement of nitrite formed (19), spectrophotometric determination of reduced trivalent chromium (5), or determination by enzymic (20), amperometric (21), interferometric (22), and spectrophotometric (23) methods. These methods are not specific because compounds such as methyl alcohol ether, chloroform, or formaldehyde interfere. We have found that 2-thiobarbituric acid does not react with methanol, formaldehyde, chloroform, or acetone but gives a colored adduct only with acetaldehyde (24). A method has been developed to convert ethanol to acetaldehyde for treatment with 2-thiobarbituric acid in an acidic medium. This enables determination of ethanol in the presence of methanol, formaldehyde, etc., without pre-separation from the system. The reaction is shown in Figure 1.

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Experimental

Reagents

(a) *Saturated aqueous mercuric chloride solution.*—7.5 g $HgCl_2$/100 mL.

(b) *Aqueous sodium hydroxide solution.*—20%.

(c) *Aqueous potassium dichromate solution.*—2N.

(d) *Ethanol.*—98%.

(e) *Sulfuric acid.*—2N.

(f) *2-Thiobarbituric acid reagent.*—Dissolve 2.88 g 2-thiobarbituric acid in ca 70 mL glacial acetic acid, and add 30 mL concentrated sulfuric acid. Heat, if necessary, until solution becomes clear. Cool to room temperature, transfer to 1 L volumetric flask, and dilute to volume with water.

Preparation of Sample

Visceral/stomach contents.—Macerate or homogenize 50 g accurately weighed sample. Add 10 mL saturated mercuric chloride solution and 10 mL 20% NaOH. Heat to boiling in distillation flask, and collect 10 mL distillate.

Blood or urine.—To 1-2 mL blood or urine in 50 mL distillation flask, add 10 mL saturated mercuric chloride solution and 10 mL 20% NaOH. Distill, and collect 10 mL distillate.

Blended preparations and drinks.—Dilute sample appropriately to bring alcohol content to ca 1%. Add 10 mL saturated mercuric chloride solution and 10 mL 20% NaOH. Distill, and collect 10 mL distillate.

Conversion of Alcohol to Aldehyde

To 10 mL distillate, in 50 mL distillation flask, add 10 mL 2N potassium dichromate solution and 10 mL 2N sulfuric acid. Distill, and collect 2 mL distillate.

Determination

Transfer 1 mL distillate from conversion step into 10 mL centrifuge tube. Add 1 mL 2-thiobarbituric acid reagent and place tube in boiling

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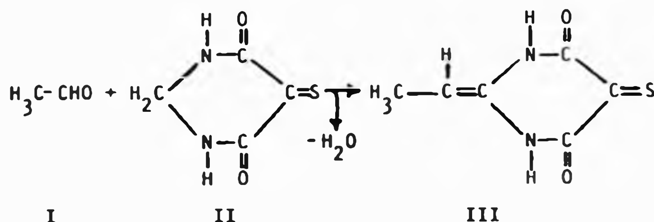

Figure 1. Reaction of acetaldehyde (I) with 2-thiobarbituric acid (II) to give colored reaction product (III).

water bath 30 min. Cool to room temperature, and dilute to 3 mL with distilled water. Centrifuge, and read absorption of supernatant liquid at 504 nm. Treat 1 mL distilled water similarly to serve as reagent blank.

Prepare standard curve by carrying out procedure on series of standards from 50 to 250 μg.

For biological specimens, perform another analysis using 0.2 mL distillate to confirm level determined. For example, a blood specimen containing 0.3 g % ethanol will not give a correct reading with 1 mL distillate, but 0.2 mL of distillate will give a reading within the range 18–80 μg/mL which follows Beer's law.

Results and Discussion

It is necessary to remove sulfides, aldehydes, and similar decomposition products such as lactic acid from putrified blood or tissue specimens before analysis. In the first distillation, all volatile acids (lactic acid, acetic acid, etc.) and other impurities such as aldehydes, sulfides, and chloral hydrate, will be eliminated (25), while the second distillate will contain converted acetaldehyde and formaldehyde (4). Distillates of

Table 1. Determination of ethanol in blended preparations

Sample	Blend	Declared concn, % w/v (g/100 mL)	Detd by AOAC method (ref. 1)	% of declared	Concn detd by proposed method, % w/v (g/100 mL)	% of declared
1	rum	33.30	33.20	99.70	33.00	99.10
2	brandy	33.30	33.20	99.70	33.00	99.10
3	whisky	33.30	33.10	99.40	33.05	99.25
4	gin	33.30	33.20	99.70	33.05	99.25
5	vodka	31.04	30.90	99.55	30.80	99.23
6	wine	14.63	14.70	100.48	14.30	97.74
7	feni	33.30	33.20	99.70	33.05	99.25
8	industrial methylated spirit (contained 5% wood naphtha)	95.00	99.80	105.05	92.50	97.37

Table 2. Recovery of ethanol in presence of methanol added to blended preparations

Sample[a]	Declared ethanol concn, g/100 mL	Added, mg/100 mL		Total ethanol concn, g/100 mL	Ethanol detd by proposed method, g/100 mL	Rec., %
		Ethanol	Methanol			
1	33.30	100.00	100.00	33.40	33.13	99.19
2	33.30	100.00	100.00	33.40	33.13	99.19
3	33.30	200.00	200.00	33.50	33.75	100.75
4	33.30	200.00	200.00	33.50	33.13	98.90
5	31.04	200.00	200.00	31.24	31.00	99.23
6	14.63	300.00	300.00	14.93	14.50	97.12
7	33.30	200.00	200.00	33.50	33.75	100.75

[a] See Table 1 for sample description.

Table 3. Recovery of ethanol in presence of methanol from biological specimens

Sample	Ethanol added	Methanol added	Ethanol by proposed method	Rec., %
Viscera (stomach/intestine)	200 mg/100 g	200 mg/100 g	196.6 mg/100 g	98.3
Viscera (liver/spleen/kidney)	150 mg/100 g	150 mg/100 g	148.8 mg/100 g	99.2
Blood	100 mg/100 g	100 mg/100 g	100.6 mg/100 g	100.6
Urine	125 mg/100 mL	125 mg/100 mL	122.5 mg/100 mL	98.0
Stomach contents	150 mg/100 g	150 mg/100 g	146.0 mg/100 g	97.3

body tissues and fluids contain trace materials that reduce dichromate. Many workers concluded that this volatile reducing substance is "normal alcohol" which ranges from 0.5 to 10 mg % (26). However, later studies with the alcoholic dehydrogenase method (27, 28) have shown that fresh blood and tissues contain not more than 0.2 mg % alcohol. The 2-thiobarbituric acid method does not detect any "normal alcohol." The results of determining ethanol in different samples are shown in Tables 1–3.

Dagani and Archer (29) reported that removal of the larger amounts of formaldehyde may require somewhat longer reaction times, higher temperatures, or higher concentrations of 2,4-pentanedione. The unconsumed formaldehyde will give a blue color (λ max. 600 nm) with *p*-phenylphenol, thereby seriously interfering in the acetaldehyde determination (λ max. 560 nm). This makes reaction conditions more critical.

Barker (30) noted that copper–lime treatment of blood or red cells liberates acetaldehyde. The amount of such "bound acetaldehyde" liberated from human blood by this treatment depends on the dilution of the blood, concentration of reagents, and especially the time and temperature of the copper–lime treatment. Values as high as 90 mg % were obtained by copper–lime treatment in a water bath. Such a value may be too high and would be misleading if used for ethanol determination in traffic offenses due to drunken driving. An excellent review (31) of gas-liquid chromatographic (GLC) methods shows that ethanol can be determined quantitatively in the presence of methanol by GLC. The colorimetric method described here will be useful in laboratories not equipped for GLC analyses.

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SYMPOSIUM

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ANALYTICAL METHODOLOGY FOR LEAD IN FOODS

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FLAVORS AND NONALCOHOLIC BEVERAGES

Stable Isotope Ratio Determination of the Origin of Vanillin in Vanilla Extracts and Its Relationship to Vanillin/Potassium Ratios

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A method is described for isolating vanillin from vanilla extract, followed by stable isotope ratio analysis to determine the amount of natural vanillin contained in adulterated vanilla extracts. After the potassium content is determined, the percent Madagascar and/or Java vanilla beans incorporated into the extract may then be approximated from the vanillin/potassium ratio.

Vanilla extract, as defined by Food and Drug Administration standards, is a solution of the sapid and odorous principles extracted from one or more units of vanilla constituent and containing not less than 35% ethanol. One unit of vanilla constituent is 13.35 oz vanilla beans containing not more than 25% moisture. When the extract material from a unit is contained in one gallon, it is considered a single-fold vanilla extract. No addition of artificial vanillin is permitted in products designated vanilla extracts. These criteria are followed in procedures in both the *National Formulary* and *Official Methods of Analysis*. Many factors have been used to determine the authenticity of vanilla extracts. For example, lead number, which is directly related to the quantity of organic acid present, has been used extensively to establish purity (**19.025-19.028**) (1). Likewise, the amounts of nitrogen, phosphate, and potassium are directly related to the quantity of vanilla beans used to make the single- or various multifold vanilla extracts (2, 3). In addition, if the bean composition of the vanilla extract is known, the vanillin/potassium ratio is indicative of the presence or absence of added vanillin. None of these methods, however, provides positive confirmation of the natural origin of the vanillin present in vanilla extracts.

To date, a considerable amount of work has been done with radio carbon techniques, and Bricout et al. (4) and Hoffman and Salb (5) used the technique to determine the origin of vanillin. Plants fix CO_2 by one of 3 metabolic pathways (Calvin synthesis, Hatch-Slack pathway, or Crassulacean acid metabolism), each of which results in natural products with different $^{13}C/^{12}C$ ratios; therefore, the $^{13}C/^{12}C$ ratio can be used to determine the CO_2 fixation pathway of the carbon in a given natural product. In the Calvin synthesis, which is common to most plants, CO_2 is fixed by the carboxylation of ribulose-1,5-diphosphate (RUDP) (6) to give 3-phosphoglycerate. The carbon isotope fractionation occurs at this enzymatic step (7).

The Hatch-Slack pathway is used by some plants to fix atmospheric CO_2 by the carboxylation of phosphoenolpyruvate (PEP) (8), an enzymatic reaction which occurs with low carbon isotope fractionation (9). Plants which fix atmospheric CO_2 by this pathway are *Zea mays* (corn), *Saccharum*, and *Cymbopogon citratus* (lemon grass) (10).

The Crassulacean acid metabolic pathway is characteristic of *Vanilla planifolia*. Carbon assimilation occurs at night by carboxylation of PEP, with accumulation of malic acid which is decarboxylated in the presence of light; liberated CO_2 is fixed by RUDP. Carboxylation of PEP at night varies under the influence of environmental factors and can be estimated by carbon isotope composition (11).

Making use of methods described by Martin et al. (2, 3), Bricout et al. (4), and Hoffman and Salb (5), we developed a procedure which indicates the quantity of artificial vanillin added, as well as the origin of vanilla beans (Madagascar and/or Java) used to produce a single- or multifold extract.

METHODS

Ultraviolet Screening Method

Determine vanillin as described in *Official Methods of Analysis*, sec. **19.010** (1).

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Potassium Analysis

Perform potassium analyses by flame emission spectrophotometry, using Instrumentation Laboratory Model 343 digital flame photometer equipped with Model 444 automatic sample dilutor, or equivalent. Dilutor automatically performs 1:200 dilution of aspirated solution and adds internal standard (lithium, 3000 meq.). Assay a series of 4 standards (5, 50, 100, and 140 meq.) and calibrate instrument for direct readout of potassium values in concentration range 0–140 meq. Dilute samples with double-distilled water (potassium-free) if necessary to assay within this concentration range.

Vanillin Isolation Procedure

Mix 200 mL single-fold vanilla extract or equivalent and 50 mL lead acetate solution (8 g lead acetate and 1 mL glacial acetic acid diluted to 100 mL with water) and refrigerate 2 h to allow precipitate formation; remove precipitate, and add additional lead acetate solution until no further precipitate forms. Place in refrigerator an additional 2 h, remove, and centrifuge at 5000 rpm 5 min. Discard precipitate. Dilute 50 mL aliquot of supernate in separatory funnel to 200 mL with water and add 5 mL chloroform. Shake vigorously 5–10 min and let layers separate. Collect chloroform layer in test tube, along with any emulsion. Centrifuge to break emulsion. Remove chloroform phase, and let evaporate at room temperature.

TLC Procedure

Dissolve residue in ca 2 mL methanol. Streak methanol solution across a silica gel 60 TLC plate (E. Merck, 2 mm thick, on 20 × 20 cm glass support) ca 1 in. from bottom (no more than 0.5 mL per plate of 2 mL total volume obtained from extraction of 200 mL single-fold vanilla extract equal to ca 0.1 g vanillin). Let plate dry. Place plate in developing tank containing benzene–methanol (97 + 3). Let plate develop ca 3 h, remove, and let benzene–methanol solution evaporate under hood. View plate under long-wave UV light and compare sample plate to standard plate which has been streaked with 0.5 mL solution of 2000 ppm vanillin. Locate vanillin by its dark blue fluorescence. Scrape vanillin band from sample plate and collect in test tube.

Extract vanillin from silica gel with 2–3 mL methanol. Centrifuge, and decant methanol solution into evaporating dish. Re-extract vanillin from silica gel with 2 more portions of methanol. Collect methanol portions in evaporating dish and let evaporate in refrigerator overnight. Crystals should form. If crystals fail to form, dissolve material in 2–3 mL methanol and repeat TLC procedure.

Stable Isotope Ratio Analysis (SIRA)

Oxidize vanillin (5–10 mg) in closed oxygen atmosphere maintained at 600°C. Collect resulting carbon dioxide and water in liquid nitrogen trap and remove oxygen by vacuum. Heat nitrogen trap in dry ice and recondense carbon dioxide on sample tube.

Measure $^{13}C/^{12}C$ isotopic ratio of carbon dioxide from sample tube with mass spectrometer equipped with dual inlet system and double collector. Report results in δPDB ^{13}C units expressed in parts per thousand compared with reference standard Pee Dee Belemnite (PDB) calculated according to following equation:

$$\delta^{13}C(‰) = \left| \frac{(^{13}C/^{12}C \text{ sample})}{(^{13}C/^{12}C \text{ standard})} - 1 \right| \times 1000$$

Results

The $^{13}C/^{12}C$ analyses were performed by Geochron Laboratories Division, Krueger Enterprises Inc., Cambridge, MA, using a Micromass 602 D spectrometer.

The authors have found $\delta^{13}C$ values for vanillin from lignin to be −26.9. This is similar to the values of −27.7 and −27.0 found by Bricout et al. (4) and Hoffman and Salb (5), respectively. Hoffman and Salb reported mean values for $\delta^{13}C$ of −18.7 and −20.4 for vanillin derived from Java and Madagascar vanilla beans, respectively. Corresponding values found by the authors were −18.7 and −20.7.

The standard single-fold Madagascar vanilla extract contained 1975 ppm vanillin and 1353 ppm potassium and had a vanillin/potassium ratio of 1.46. The standard single-fold Java vanilla extract contained 362 ppm vanillin and 1833 ppm potassium and had a vanillin/potassium ratio of 0.197. The calculated vanillin and potassium content for blends of 20/80, 40/60, 60/40, and 80/20 of the standard Madagascar and Java single-fold vanilla extracts were 684, 1007, 1330, and 1652 ppm vanillin and 1736, 1641, 1547, and 1449 ppm potassium, respectively. These values were used to obtain the vanillin/potassium ratios for the blends of Madagascar and Java vanilla extracts and are plotted in Figure 1 and appear in Table 1.

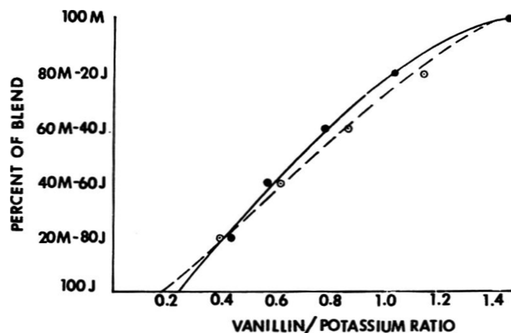

Figure 1. Corrected vanillin/potassium ratio of adulterated vanilla extract standards by $\delta^{13}C$ (—). Vanillin/potassium ratio of standard vanilla extracts based on known natural vanillin content (---).

The theoretical $\delta^{13}C$ values for the various blends (20/80, 40/60, 60/40, and 80/20) of Madagascar and Java were calculated using the $\delta^{13}C$ values obtained for pure Java and Madagascar vanillin, according to the following equation:

$$\text{Theor. } \delta^{13}C = \frac{\text{ppm natural vanillin from Madagascar } (-20.7)}{\text{ppm total natural vanillin}} + \frac{\text{ppm natural vanillin from Java } (-18.7)}{\text{ppm total natural vanillin}}$$

This gave calculated $\delta^{13}C$ values for 20/80, 40/60, 60/40, and 80/20 blends of Madagscar and Java vanilla extracts of −19.85, −20.25, −20.48, and −20.6, for a weighted average of −20.3.

Using the weighted average of −20.3, the following equation was developed to determine the natural vanillin content of adulterated vanilla extracts with $\delta^{13}C$ less than −21.0:

$$\text{Natural vanillin concn in sample} = \text{vanillin concn of sample} \times (\delta^{13}C_1 - \delta^{13}C_2)/\Delta\delta^{13}C$$

where $\delta^{13}C_1$ = value for lignin vanillin (−26.9); $\delta^{13}C_2$ = value for vanillin in sample; $\Delta\delta^{13}C$ = value for lignin vanillin (−26.9) minus weighted average (−20.3) for ($\Delta\delta^{13}C$) = −6.6.

To test the above equation, 1613 ppm vanillin from lignin was added to the standard single-fold Java vanilla extract which contained 362 ppm vanillin, to yield a total concentration of 1975 ppm vanillin. The extract contained a concentration of 1833 ppm potassium with a resulting vanillin/potassium ratio of 1.07. This adulterated Java vanilla extract was then blended with the standard Madagascar extract in the proportions 20/80, 40/60, 60/40, and 80/20. The resulting vanillin/potassium ratios of these blends were 1.363, 1.277, 1.203, and 1.138, respectively. These adulterated blends of Java/Madagascar yielded $\delta^{13}C$ values of −21.9, −22.9, −23.8, and −24.4, respectively.The $\delta^{13}C$ values were then used to calculate the amount of natural vanillin in the adulterated blends, and the corrected vanillin/potassium ratio was obtained using the formula found above. These values are shown in Table 1 and plotted as the corrected vanillin/potassium ratio curve in Figure 1.

Table 2 represents 30 samples of vanilla extracts with $\delta^{13}C$ value determined. Samples 1 through 8 had $\delta^{13}C$ values greater than −21.0, indicating pure vanilla extract, and vanillin/potassium ratios from 1.23 to 1.56, indicating pure vanilla extracts of Madagascar origin.

Samples 9 through 30 represent 22 samples of vanilla extracts with $\delta^{13}C$ values less than −21.0, indicating addition of artificial vanillin. The natural vanillin content was determined by the formula above, and the corrected vanillin/potassium ratio was obtained. These values were then extrapolated from the vanillin/potassium ratio curve in Figure 1. Samples 9-21 had corrected vanillin/potassium ratios indicating concentrations of Madagascar between 4 and 90%

Table 1. Elemental analysis of standard and adulterated vanillin extract blends based on known natural composition and corrected vanillin/potassium ratio based on $\delta^{13}C$

	Known composition			$\delta^{13}C$ analysis				
Vanilla ext blend,%	Potassium, ppm	Natural vanillin, ppm	Natural vanillin/potassium	Total vanillin, ppm	Total vanillin/potassium	$\delta^{13}C$	Natural vanillin by $\delta^{13}C$, ppm	Corrected vanillin/potassium
100J	1833	362	0.197	362	0.197	−18.7	362	0.197
20M–80J	1736	684	0.394	1975	1.138	−24.4	748	0.431
40M–60J	1641	1007	0.613	1975	1.203	−23.8	927	0.565
60M–40J	1547	1330	0.860	1975	1.277	−22.9	1197	0.774
80M–20J	1449	1652	1.140	1975	1.363	−21.9	1496	1.032
100M	1353	1975	1.460	1975	1.460	−20.7	1975	1.460

Table 2. $\delta^{13}C$ analysis of vanilla extracts

Sample	^{13}C	Vanillin, ppm	Potassium, ppm	Vanillin/ potassium	Corrected vanillin, ppm	Corrected vanillin/ potassium	Bean composition
				Pure Vanilla Extracts			
1	−20.0	18285	12129	1.51			100 M
2	−20.5	1820	1361	1.35			100 M
3	−20.9	1878	1435	1.35			100 M
4	−20.5	1756	1417	1.23			100 M
5	−20.9	1935	1353	1.49			100 M
6	−20.6	1969	1264	1.56			100 M
7	−20.6	4817	3580	1.35			100 M
8	−20.9	4526	2952	1.53			100 M
				Adulterated Madagascar and Java Blends			
9	−22.4	2180	1400	1.56	1486	1.06	82M–18J
10	−22.0	2101	1412	1.54	1560	1.10	85M–15J
11	−23.1	17233	10140	1.70	9922	0.98	76M–24J
12	−25.5	7603	1520	4.97	1613	1.06	82M–18J
13	−23.6	18862	14313	1.32	8574	0.60	40M–60J
14[a]	−22.6	7674	5655	1.36	5000	0.88	68M–32J
15[a]	−23.0	3718	2586	1.44	2197	0.85	66M–34J
16	−25.7	8882	6131	1.45	1615	0.26	2M–98J
17	−24.8	2048	1503	1.36	650	0.43	22M–78J
18	−25.7	2415	1509	1.60	439	0.29	10M–90J
19	−25.1	9237	6299	1.47	2519	0.40	20M–80J
20	−25.4	4239	3012	1.36	963	0.32	10M–90J
21	−25.1	19821	6708	2.95	5405	0.81	62M–38J
				Adulterated Java Vanilla Extracts			
22	−26.0	1906	1587	1.20	260	0.16	100 J
23	−26.4	7863	6240	1.26	597	0.10	100 J
24	−24.1	1116	2999	0.37	473	0.16	100 J
25	−26.1	1437	1256	1.14	175	0.14	100 J
26[b]	−27.1	36505	8229	4.44	—	—	100 J
27[b]	−27.3	3543	834	4.10	—	—	100 J
28[b]	−26.6	21929	10413	2.11	997	0.10	100 J
29[b]	−27.2	4087	1743	2.34	—	—	100 J
30[b]	−27.0	4620	2297	2.01	—	—	100 J
				Pure Vanilla Extracts, Ratios >1.60			
31	−20.2	2282	1080	2.10			
32	−20.9	2846	1673	1.70			
33	−20.2	2531	1474	1.72			
34	−20.9	3984	2184	1.82			
35	−20.1	22231	11232	1.90			

[a] Known composition of ⅔ Madagascar to ⅓ Java.
[b] Within experimental error of method.

and Java between 10 and 96%, respectively. Samples 22–30 had corrected vanillin/potassium ratios of 0.16 or less, indicating Java vanilla extracts.

From the results, it is apparent that the addition of artificial vanillin is most often practiced with extracts made with Java vanilla beans, ranging in amounts from 4 to 90%. The majority of the original vanillin/potassium ratios fall within the acceptable range for Madagascar vanilla extracts. However, the corrected vanillin/potassium ratios clearly indicate Java vanilla bean contents between 4 and 96%.

This examination of vanilla extracts also produced 5 samples (31–35) with vanillin/potassium ratios from 1.7 to 2.1. All samples exhibited lower potassium values than would normally be found in single-fold pure vanilla extracts when potassium value was corrected to the single-fold level. Samples 31–35 are known to have undergone intricate extractions, incorporating a procedure that washes the bean after extraction is complete.

High vanillin/potassium ratios have also been

observed in vanilla products produced from oleoresin that has been extracted with isopropanol rather than ethanol. Because vanilla products produced from oleoresin of this type cannot be classed as pure vanilla extracts, they have not been considered in this work.

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DRUGS

Nuclear Magnetic Resonance Spectroscopic Determination of Methoxyflurane

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A simple, rapid nuclear magnetic resonance (NMR) spectroscopic method for quantitatively determining methoxyflurane was developed. The proton NMR spectrum of methoxyflurane in carbon tetrachloride exhibited a well defined triplet at 5.67 ppm and a sharp singlet at 3.68 ppm. Using dimethyl sulfoxide (DMSO) as an internal standard, methoxyflurane was quantitated by comparing the integral for the singlet of methoxyflurane with the integral for the singlet of DMSO at 2.60 ppm. The method was accurate to concentrations as low as 1-2%, and results agreed with those obtained by the NF XIV method.

Methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) is a clear, mobile liquid used as a general inhalation anesthetic in obstetrics and surgery (1, 2).

The official assay method for methoxyflurane (3) involves gas-liquid chromatography (GLC) with thermal conductivity detection The method described here uses nuclear magnetic resonance (NMR) spectroscopy, a technique devoid of many of the instrumental and procedural variables inherent to GLC.

In the proposed method the methoxyflurane sample and an internal standard are dissolved in a suitable solvent, the proton NMR spectra are recorded, and the integrals are obtained for the appropriate resonance signals of the test material and internal standard. The method requires no sample preparation, and the NMR spectra can also be used to identify methoxyflurane in the test sample.

Experimental

Apparatus and Reagents

(a) *NMR spectrometer.*—Model EM-390, 90 MHz (Varian Associates, Palo Alto, CA 94303).

(b) *NMR sample tubes.*—Precision type, 5 mm (Kontes, Vineland, NJ 08360).

(c) *Microtubes.*—Microflex, with quick access pressure valve (Kontes).

(d) *Microliter syringe.*—100 and 200 μL (Hamilton Co., Reno, NV 89510).

(e) *Internal standard.*—Dimethyl sulfoxide (DMSO), ACS-grade (Fisher Scientific Co., Pittsburgh, PA 15219).

(f) *Tetramethylsilane (TMS).*—Wilmad Glass Co., Inc., Buena, NJ 08310.

(g) *Reference standard.*—USP Methoxyflurane Reference Standard.

Procedure

Transfer ca 40–70 mg DMSO, accurately weighed, into tared microtube. Add 0.8–1.0 mL carbon tetrachloride, and close container with pressure valve. Using 100 μL syringe, inject ca 170 μL sample through valve septum. Close valve and weigh microtube. Thoroughly mix contents. Using syringe with long needle, withdraw ca 0.5 mL solution and transfer to precision NMR tube. Cap tube and record proton NMR spectrum at 90 MHz. Integrate at least 5 times the singlet for methoxyflurane at 3.70 ppm and the singlet for DMSO at 2.60 ppm. Obtain average integral values. Amount of methoxyflurane is calculated from:

$$\text{Methoxyflurane, \%} = (A_M/A_D) \times (EW_M/EW_D) \times (M_D/M_S) \times 100$$

where A_M = integral height of methoxyflurane; A_D = integral height of DMSO; EW_M = equivalent weight of methoxyflurane (54.99); EW_D = equivalent weight of DMSO (13.02); M_D = weight of added DMSO, mg; M_S = weight of sample taken, mg.

Results and Discussion

The choice of solvent and internal standard is of primary importance in any quantitative NMR procedure. Since both methoxyflurane and DMSO are freely miscible with carbon tetrachloride, this substance was the solvent of choice. Furthermore, carbon tetrachloride will not interfere with the integration and subsequent quantitation of methoxyflurane because it is free of protons.

DMSO has been previously shown to be a suitable internal standard (4). In the present case

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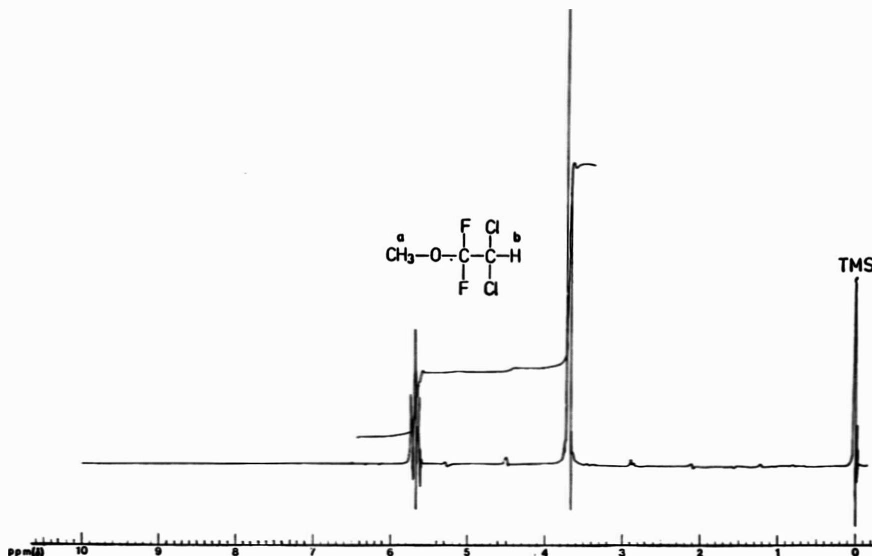

Figure 1. Proton NMR spectrum (90 MHz) of methoxyflurane. Solvent carbon tetrachloride. TMS = tetramethylsilane.

it proved advantageous, not only because of its compatibility with carbon tetrachloride, but also because it generated a strong absorption signal at 2.60 ppm, well separated from the spectral signals of methoxyflurane.

The proton NMR spectrum of methoxyflurane (Figure 1) exhibited a typical triplet centered at ca 5.67–5.70 ppm and a sharp singlet at ca 3.68–3.70 ppm. The triplet is ascribed to the methine group of this compound, whose resonance undergoes splitting by the fluorine atoms attached to the neighboring carbon. The singlet is assigned to the methyl group. The resonance signal for DMSO was observed at 4.60 ppm, as a singlet integrating for 6 methyl protons.

Butylated hydroxytoluene (BHT; 2,6-bis(1,1-dimethyl-ethyl)-4-methylphenol), in 0.01% concentration, is added to methoxyflurane to ensure its stability on standing (5, 6). BHT yielded a proton NMR spectrum (Figure 2) with singlets at 6.87, 4.78, 2.2, and 1.4 ppm relative to TMS. The resonance signal at 1.4 ppm was the most intense, but it did not appear in the spectrum at the concentrations used. Signals occurring in the 2.5–4.0 ppm region did not interfere with the analysis of the sample and internal standard.

Table 1 summarizes the results of a compara-

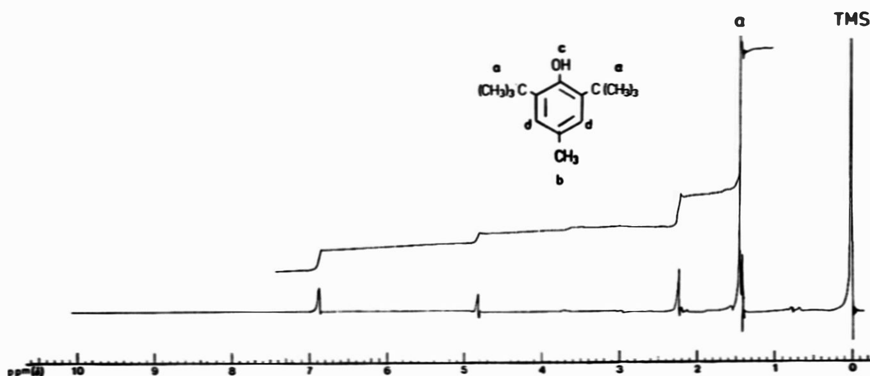

Figure 2. Proton NMR spectrum (90 MHz) of butylated hydroxytoluene. Solvent carbon tetrachloride. TMS = tetramethylsilane.

Table 1. Determination (%) of methoxyflurane in 2 commercial lots by NMR spectroscopic and NF XIV methods

	Lot 1			Lot 2		
Sample No.	Int. std added, mg	NMR	NF XIV	Int. std added, mg	NMR	NF XIV
1	43.8	100.4	98.8	48.3	100.4	100.3
2	38.8	100.6	99.2	52.5	98.7	100.7
3	42.8	99.1	99.7	53.7	99.9	100.6
4	47.4	101.1	100.5	40.1	98.9	100.1
5	46.1	101.1	99.1	70.7	100.6	100.6
6	45.8	100.4	99.6	63.1	99.4	99.6
7	45.9	100.9	100.4	39.1	99.7	100.0
8	50.0	99.1	100.7	48.0	99.0	99.6
9	41.4	99.7	99.1	44.0	101.3	99.6
10	63.0	101.5	99.0	59.5	98.2	100.0
Av.[a]		100.39	99.61		99.61	100.05
Range		99.1–101.5	98.8–100.8		98.2–101.3	99.0–100.7
SD		0.84	0.69		0.96	0.54
RSD, %		0.84	0.70		0.96	0.54

[a] Statistics are at the 1σ level.

Table 2. Recovery of methoxyflurane added to 2 commercial lots by NMR spectroscopic method

	Lot 1				Lot 2			
		Methoxyflurane				Methoxyflurane		
Sample No.	Int. std added, mg	Added, mg	Rec., mg	Rec., %	Int. std added, mg	Added, mg	Rec., mg	Rec., %
1	46.4	113.3	112.3	99.1	49.6	99.7	101.5	101.8
2	44.6	106.7	105.5	98.9	50.9	103.1	104.3	101.2
3	44.5	171.5	171.5	100.0	51.6	103.8	100.1	96.4
4	46.1	179.5	182.9	101.9	46.9	100.4	99.5	99.1
5	41.0	102.2	104.5	102.2	44.2	92.9	91.6	98.6
6	44.8	111.5	112.7	101.1	43.5	108.5	109.3	100.7
7	45.0	110.0	108.6	98.7	54.8	98.2	97.9	99.7
8	41.0	98.6	97.9	99.3	38.6	99.1	100.1	101.0
Av.[a]				100.15				99.81
Range				98.7–102.2				96.4–101.8
SD				1.4				1.8
RSD, %				1.4				1.8

[a] Statistics are at the 1σ level.

tive analysis of 2 lots of methoxyflurane by the proposed and NF XIV methods. The average values obtained by using these 2 methods differed by less than 1%.

The recovery of methoxyflurane added to 2 commercial samples is presented in Table 2. These results indicate that the proposed method is precise for the concentration range studied.

In general, the NMR method was faster and simpler than the official GLC method. After the sample and internal standard are dissolved in the appropriate solvent, no further preparatory manipulations are required.

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AOAC

Potentiometric and Atomic Absorption Spectrometric Determination of Sulfonamides in Pharmaceutical Preparations

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Potentiometric and atomic absorption spectrometric (AAS) methods are described for the determination of various sulfonamides in tablets, suspensions, and injections, based on reactions with silver and copper ions. The methods involve direct titration at pH 8, using either a graphite electrode in which silver and copper sulfides have been precipitated, or ion selective electrodes to monitor the potential change. AAS measurements of the excess metal ions after metal-sulfonamide reaction are also reported. Results compare favorably with those obtained using USP XIX procedures. The methods described are selective, simple, and precise.

Determination of sulfonamides in pharmaceutical preparations by direct titration with sodium nitrite, in acidic media at low temperature, has been reported by the *U.S. Pharmacopeia* (1), the *British Pharmacopoeia* (2), and the American Pharmaceutical Association Foundation (3). The equivalence point is detected either electrochemically (dead stop end point) or visually (starch-iodide external indicator). Potentiometric titration using a platinum electrode (4) and visual titration using internal indicators (5) have also been suggested. Various other methods including nonaqueous titration (2, 6, 7), and bromometry with visual (8) and coulometric (9) detection of the equivalence point have also been described. However, these methods either are inapplicable to the pharmaceutical preparations or lack selectivity. Moreover, the stability of the titrants used is affected by acidity, temperature, dissolved oxygen, and atmospheric carbon dioxide.

Although the reaction of the sulfonamide group with heavy metal ions is specific and selective, there have been few reports on the determination of sulfonamides by reaction with metals. Wide application of these methods is hampered by the partial solubility of some metal sulfonamides and the lack of suitable indicators for direct titrimetry. However, direct and indirect titrimetric and gravimetric procedures using silver ions (10–12), and reaction with silver ions followed by addition of potassium nickel cyanide to the filtrate and titration of the released nickel with EDTA (13) have been proposed. Precipitation of copper, lead, and cadmium sulfonamides and titration of the excess metal ions with EDTA has also been suggested (14, 15). Direct titration with divalent metal ions has not, so far, been reported. However, most of these methods necessitate a time-consuming filtration step, isolation of the metal sulfonamides precipitate in absolutely pure form before subsequent measurement, and extraction of the sulfonamides from pharmaceutical preparations.

The present investigation describes potentiometric procedures for determining various sulfonamides in pharmaceutical preparations by direct titration with silver or copper ions, using a modified graphite electrode sensitive for both ions or solid state silver and copper ion selective electrodes. Indirect atomic absorption spectrometry (AAS) without prior filtration is also described.

Experimental

Reagents

All reagents were analytical grade, and deionized water was used throughout.

The sulfonamides were certified pharmaceutical samples ≥99.5% pure. Standard silver and copper nitrate solutions (0.01M), stock standards of silver (5 mg/mL) and copper (2 mg/mL) nitrate solutions in 0.02M nitric acid, thymol blue indicator (0.1% w/v in 96% ethanol), and 0.1M sodium hydroxide solution were used.

Apparatus

The potentiometric measurements were carried out with a Radiometer pH meter (pH M 22r), using a graphite-(Ag_2S-CuS) electrode (see below), silver ion selective electrode (Orion 94-16A), or copper ion selective electrode (Orion 94-22A) in conjunction with a double junction reference electrode (Orion 92-02) with 10% KNO_3 in the outer compartment.

AAS measurements were conducted using a Unicam 1900 spectrometer equipped with a deuterium lamp (SP 1960) for background correction and a digital readout unit. Absorbances

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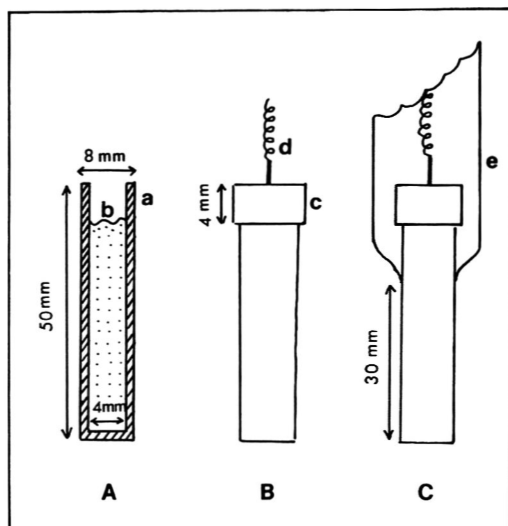

Figure 1. Schematic diagram of preparation of modified graphite electrode: A, graphite rod (a) containing Ag_2S-CuS coprecipitate slurry (b). B, copper sheet (c) attached to copper wire (d). C, rod inserted in polyvinyl chloride tube (e).

of silver and copper were measured at 328.1 and 324.8 nm, respectively, using a laminar-flow burner with an acetylene (1.4–1.8 L/min) and air (4.5–5.5 L/min) mixture.

Electrode Preparation

Prepare slurry of silver and copper sulfides coprecipitate by dissolving 0.65 g copper nitrate and 0.35 g silver nitrate in 5 mL deionized water, followed by addition of 1 g sodium sulfide in 3 mL deionized water. Filter precipitate, and wash several times with deionized water until precipitate is free of sulfide and metal ions. Cut a piece of spectral pure graphite rod (50 × 8 mm od) and make a hole in the rod (~45 mm × 4 mm id). Introduce metal sulfide coprecipitate slurry into hole (Figure 1A) and place graphite rod in centrifuge cup with open end up. Centrifuge 5 min at 4000 rpm, and repeat addition of coprecipitate slurry and centrifugation until hole is completely filled. Ensure electrical connection by wrapping open end of graphite rod with small sheet of pure copper metal (~3 × 0.4 cm) attached to copper wire (Figure 1B). Insert rod in polyvinyl chloride sleeve so that ca 3 cm rod protrudes as measuring surface (Figure 1C). Soak graphite electrode ca 4 h in deionized water. Stir during soaking period. Electrode is then ready for use. When not in use, store electrode in deionized water. Life span of electrode is at least 8 months.

Sample Preparation

To prepare commercial samples, grind 20 tablets, mix contents of 5 ampules, or shake syrup. Accurately weigh 1 g pulverized dried pure sulfonamide powder or amount of preparations equivalent to 1 g sulfonamide. Transfer to 100 mL volumetric flask, and add 10 mL deionized water and 5 drops of 0.1% thymol blue indicator. Add 0.1M NaOH dropwise until solution is blue. Shake flask well until sample dissolves completely, and dilute to volume with deionized water.

Potentiometric Measurements

Transfer 5.0 mL aliquot of sample solution to 100 mL beaker, add 15 mL deionized water, and immerse graphite–(Ag_2S–CuS) electrode in conjunction with double junction reference electrode in solution. Slowly titrate with 0.01M solution of either silver nitrate or copper nitrate. Solid state silver and copper ion selective electrodes may also be used with silver and copper titrants, respectively. As end point is approached (indicated by increase in potential jump), add titrant in equal increments (0.02 mL) with continuous stirring. End point is calculated from maximum slope $\Delta E/\Delta V$: 1 mL 0.01M $AgNO_3$ or 2 mL 0.01M $Cu(NO_3)_2$ is equivalent to 0.79 mg —SO_2—NH—.

AAS Measurements

Transfer 10.0 mL aliquot of sample solution to 100 mL volumetric flask. Add 10.0 mL stock silver nitrate (5 mg/mL) or copper nitrate (2 mg/mL) solution, shake well, and let stand 10 min. Dilute to volume with deionized water, shake, and centrifuge. Transfer 5.0 mL aliquot of supernate to 100 mL volumetric flask, dilute to volume with 0.02M HNO_3, and shake. Aspirate in air–acetylene flame and measure absorbance at 328.1 and 324.8 nm for silver and copper, respectively. Carry out blank experiment and compare with calibration graphs prepared by transferring aliquots of stock silver and copper solutions (0.10–1.00 mL) to 100 mL volumetric flasks. Dilute solutions to volume with 0.02M HNO_3, and aspirate in flame as described above.

$$\text{Sulfonamide (\%)} = 0.927 \times M \times (Ag_1 - Ag_2)/W$$
$$= 3.148 \times M \times (Cu_1 - Cu_2)/W$$

where M is molecular weight of sulfonamide sample; Ag_1 and Cu_1 are silver and copper contents (mg) of blank; Ag_2 and Cu_2 are silver and copper contents (mg) of supernate after sulfon-

Table 1. Potentiometric and AAS determinations of sulfonamide powders

	Average recovery, % [a]						
		Reaction with silver			Reaction with copper		
Sample	USP XIX	Graphite electrode	Ag–ISE	AAS	Graphite electrode	Cu–ISE	AAS
Sulfadiazine	99.8	99.6	99.7	99.4	99.8	99.7	99.0
Sulfadimidine	99.8	99.8	99.5	99.0	99.7	99.6	99.4
Sulfamerazine	99.8	99.5	99.6	99.4	99.6	99.7	99.1
Sulfathiazole	99.7	99.6	99.7	99.3	99.7	99.8	99.6
Sulfamethoxypyridazine	99.7	99.8	99.8	99.5	99.8	99.9	99.3
Sulfamethoxydiazine	99.8	99.9	99.7	99.5	99.7	99.7	99.4
Sulfamethoxazole	99.7	99.8	99.8	99.7	99.9	99.7	99.4
Sulfamethoxine	99.5	99.7	99.6	99.4	99.8	99.7	99.6
Sulfapyridine	99.9	99.8	99.8	99.6	99.9	99.8	99.6
Mean SD	0.4	0.1	0.2	0.4	0.2	0.2	0.5

[a] Average of 5 determinations.

amide reaction; and W is sample weight (mg).

Results and Discussion

A preliminary investigation of the solubility of different metal sulfonamides in aqueous solutions at various pH levels indicated that both silver and copper ions form extremely insoluble precipitates with many structurally different sulfonamides at pH 7.4–8.6. The exact stoichiometry at this pH range, substantiated by addition of excess metal ions to the sulfonamide solution followed by AAS measurement of the metals in the supernate, was 1:1 and 1:2 for silver:sulfonamide and copper:sulfonamide, respectively. The mean standard deviation (SD) was 0.4%. Elemental microanalysis of the precipitates conformed with the molecular formulas $R—SO_2NAg—R$ and $(R—SO_2NR)_2Cu.2H_2O$.

Potentiometric Measurements

A modified graphite electrode prepared by coprecipitation of both silver and copper sulfide

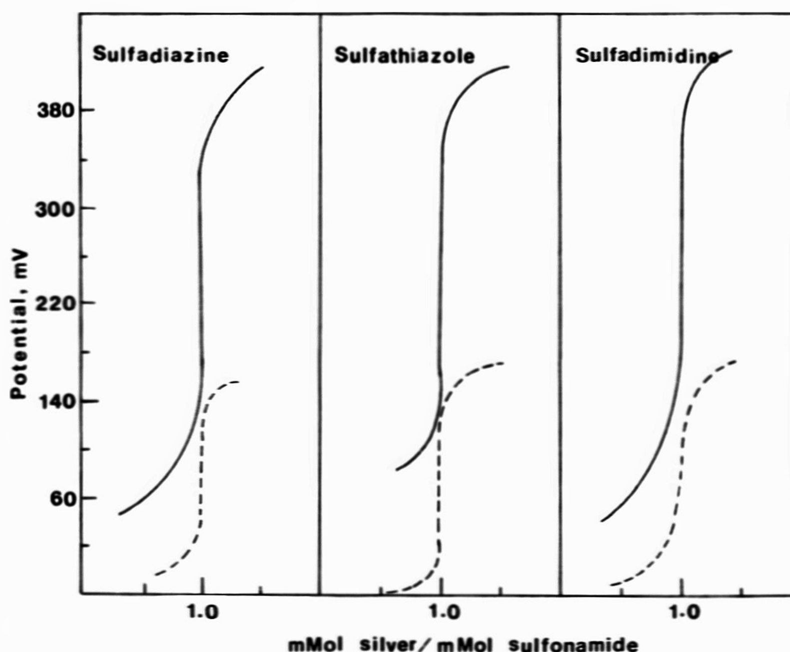

Figure 2. Typical potentiometric titration curves of some sulfonamides with silver nitrate, using (—) graphite electrode and (---) silver ion selective electrode.

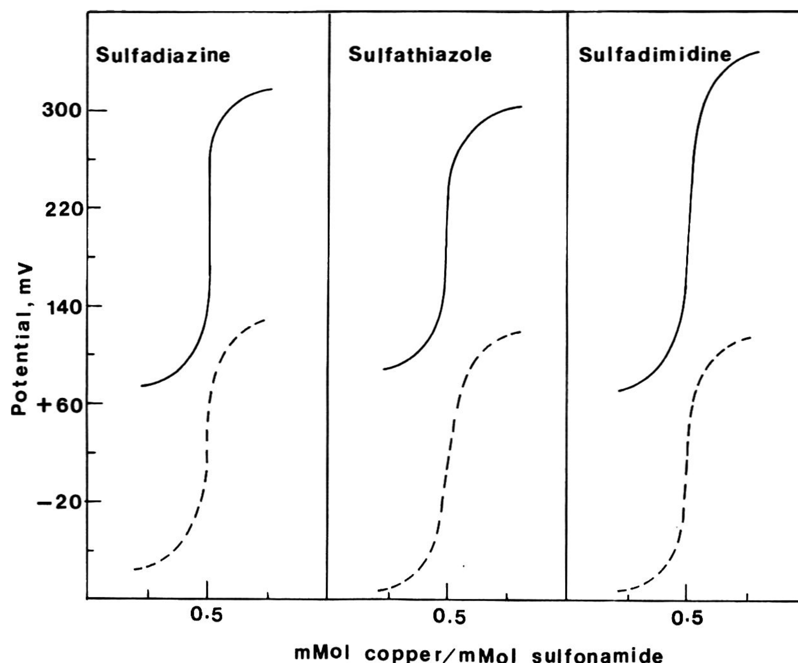

Figure 3. Typical potentiometric titration curves of some sulfonamides with copper nitrate, using (—) graphite electrode and (- - -) copper ion selective electrode.

within a graphite rod was used to monitor the potentiometric titration of various sulfonamides at pH 8 with either silver or copper nitrate. Average recoveries for certified sulfonamide standards in the concentration range 50 μmol–0.3 mmol (equivalent to 10–50 mg) were 99.7% (mean SD 0.1%) and 99.8% (mean SD 0.2%) with silver and copper nitrate titrants, respectively. Titration of the same samples under the same conditions, using the solid state silver and copper ion selective electrodes, gave comparable results (average recovery 99.7%, mean SD 0.2%) (Table 1).

However, the modified graphite electrode offered many advantages over the ion specific electrodes. Apart from the low cost and simple preparation procedure, the magnitude of the potential break at the equivalence point using silver nitrate as a titrant was a factor of 3 greater than that obtained using the solid state silver electrode (Figure 2), probably due to the absence of an inner reference electrode and internal filling solution. Using copper nitrate titrant, the magnitude of the potential break was as great as that obtained using the solid state copper electrode (Figure 3). The graphite electrode showed Nernstian response toward both silver and copper ions with cationic slopes of 60 and 27 mV/concentration decade, respectively, over the metal concentration range 10^{-1}–10^{-4}M. Different procedures for electrode preparation, electrochemical characteristics, and applications will be described elsewhere.

AAS Measurements

Determination of sulfonamides by reaction with silver and copper ions was also followed by AAS. Aliquots of the supernatant solutions containing the excess metal ions, after metal sulfonamide precipitation, were diluted to bring the final metal content within the linear range of calibration graphs prepared under similar conditions with the same background. The results (Table 1) show an average recovery of 99.4% and a mean SD $\leq$0.3% for both silver and copper. However, this procedure can be used with other sulfonamides whose silver and copper salts are slightly dissociated and the presence of excess metal ions shifts the equilibrium towards complete precipitation.

Accuracy and Selectivity

Mean average recoveries of the proposed potentiometric methods and those obtained by the USP XIX procedure (average recovery 99.7%, mean SD 0.4%) agreed well. However, the pre-

Table 2. Potentiometric and AAS determinations of sulfonamides in some pharmaceutical preparations

			Average recovery, % of label[a]						
				Reaction with silver			Reaction with copper		
Product	Source	Labeled amount	USP XIX	Graphite electrode	Ag–ISE	AAS	Graphite electrode	Cu–ISE	AAS
Diazovit, susp. (sulfadiazine)	Alex., Egypt	5.5 g/ 100 mL	101.0	100.8	100.4	102.6	101.2	100.3	101.3
Mecozine, tablet (sulfadimethoxine)	Memphis, Egypt	0.5 g/ tab.	95.3	95.3	94.9	96.4	95.2	95.3	95.9
Sulfadimidine, tablet	El-Nasr, Egypt	0.5 g/ tab.	96.9	97.3	97.0	97.8	97.1	96.8	96.5
Sulfamerazine, tablet	Kahira, Egypt	0.5 g/ tab.	99.9	99.8	99.7	100.4	99.7	99.6	98.9
Bactrim, tablet (sulfamethoxazole)	Roche, Switz.	0.4 g/ tab.	97.4	98.0	97.7	96.9	97.8	97.9	97.5
Bayrena, ampule (sulfamethoxydiazine)	Bayer, Germany	0.5 g/ amp.	96.9	97.1	97.3	96.2	97.0	97.2	97.4
Longactin, tablet (sulfamethoxypyridazine)	Alex., Egypt	0.5 g/ tab.	100.1	100.0	99.9	102.1	99.8	99.9	99.2
Sulfapyridine, tablet	CID, Egypt	0.5 g/ tab.	99.3	99.2	99.3	99.7	99.1	99.4	100.1
Mean SD			2.5	1.2	1.5	2.9	1.5	1.5	3.0

[a] Average of 5 determinations.

cision and standard deviations of the present methods were better. These data were further checked by matching with those obtained by combustion of sulfonamides in an oxygen-filled flask (16), followed by titration of the resultant sulfate ions with barium perchlorate, using thorin (*o*-[(2-hydroxy-3,6-disulfo-1-naphthyl)-azo] benzenearsonic acid, disodium salt) indicator. Results were identical to those obtained by the present potentiometric methods.

The selectivity and convenience of the present procedures over the official ones were demonstrated by routine quality control analysis of some sulfapyridine powders. Using the USP XIX nitrite procedure, results were within the acceptable range, although 2% biased on the positive side (average recovery 102%). However, elemental analysis of the sulfur content of these samples gave results consistently 6% biased on the negative side (average recovery 94%). Examination of these samples by thin layer chromatography (TLC) revealed contamination with the precursor aminopyridine. Potentiometric titration of the samples with either silver or copper nitrate, using the modified graphite electrode or the ion selective electrodes, gave results (average recovery 94.4%) that agreed with the sulfur content analyses. The present methods thus overcome the problems associated with the determination of sulfonamides in the presence of amines where nitrosation, acid–base, and bromometric titrations are not useful.

Analysis of Pharmaceutical Preparations

The influence of some excipients and diluents commonly used in the preparation of tablets, syrups, injections, drops, and ointments was investigated by the proposed procedures. Magnesium stearate, talc powder, gum arabic, carboxymethylcellulose, cocoa butter, vanillin, Tween-80, polyvinylpyrrolidone, glucose, lactose, sucrose, acacia, ethylene glycol, and glycerol in the range 100–300 mg were mixed with 25 mg pure sulfadiazine, and assayed by both potentiometric and AAS techniques, using silver and copper reactions. There was no matrix effect due to the presence of any of these substances.

Average recoveries and mean standard deviations for replicate determinations of sulfonamides in commercial preparations, using the proposed procedures, are shown in Table 2. With the graphite electrode, titration with silver and copper showed average recoveries of 98.4% of the nominal values (mean SD 1.2%) and 98.3% (mean SD 1.5%), respectively. The mean average recovery obtained by using the ion selective electrodes was 98.3% (mean SD 1.5%). Less precise results were obtained by the most widely

accepted USP XIX procedure (average recovery 98.4%, mean SD 2.5%). The AAS methods showed an average recovery of 98.7% and a mean SD of 3%.

The present procedures are selective and simple. Moreover, frequent standardization of the titrant, titration at low temperature, electrode cleaning after each measurement, and extraction of the sulfonamides from the pharmaceutical preparations are avoided. The methods described have been used in quality control at El Nasr Pharmaceutical Chemical Co. in parallel with Pharmacopeia methods for the past 2 years and are of considerable value.

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X-Ray Powder Diffraction Data for Nine Medicinal 2-Imidazolines

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X-ray powder diffraction data for nine 2-imidazolines have been obtained by the powder diffractometer and by the photographic Debye-Scherrer techniques. The data for individual 2-imidazolines are tabulated in terms of lattice spacings (*d* in Å) and the relative intensities of lines. The best method of packing for powder diffraction was investigated. It was advantageous to use chromium Kα radiation in the Debye-Scherrer technique.

Hanawalt et al. (1) introduced the powder diffraction technique for identifying polycrystalline materials. They described a system for classifying data based on values of d, the interplanar spacing, and I/I_1, the relative intensities of the lines, based on the strongest line as 100. This information may be used to identify a crystalline form of an organic compound, even when it is present in a mixture, by comparison of its diffraction pattern with those of known samples, such as those in the Joint Committee on Powder Diffraction Standards *Powder Diffraction File* (2), in which X-ray powder patterns are classified according to the spacings of the 3 lines of strongest relative intensity.

Development of Method

Diffraction patterns for the nine 2-imidazolines were recorded by the powder diffraction and Debye-Scherrer techniques. Samples supplied by the manufacturers included antazoline hydrochloride, clonidine hydrochloride, naphazoline nitrate, oxymetazoline hydrochloride, phentolamine mesylate, tolazoline hydrochloride, tramazoline hydrochloride, tymazoline hydrochloride, and xylometazoline hydrochloride. The melting points of the compounds agreed with those quoted in the literature.

In both methods a ground sample was taken, but a grinding mill was not used because prolonged grinding of soft crystalline substances, such as organic compounds, causes lattice distortions and consequent line broadening. Particle sizes were measured on a microscope screen, but generally the smoothness of the lines (not spotty) on the X-ray diffraction photographs was taken as an indication that the particles were approximately the correct size (~5 μm).

The X-ray diffraction photographs of the nine 2-imidazolines were obtained by using a 114.83 mm diameter powder camera, using 3 different X-ray radiations for each individual sample, copper Kα (wavelength 1.542 Å) with a nickel filter, cobalt Kα (wavelength 1.790 Å) with an iron filter, and chromium Kα (wavelength 2.291 Å) with a vanadium filter. A small quantity of each ground sample was mounted in a glass tube. Exposures were 8 h for copper radiation, ca 16 h for cobalt radiation, and 32 h for chromium radiation, using a fine collimator.

The X-ray powder diffractometer patterns were recorded by mounting ca 2 g ground sample into a window in an aluminum specimen holder and then exposing it to the X-ray beam ca 45 min, using copper Kα radiation. It was essential, for the most satisfactory results, that the number of crystallites contributing to each reflection was sufficiently large to generate signals of reproducible intensity and that preferred orientation of the crystallites was held to a minimum. Since the former requirement had been satisfied, we investigated the way the method of packing affected the diffraction patterns to determine which method gave the most reproducible results, because there is a close relationship between method of packing and preferred orientation.

Three basic methods of packing have been reported: (*1*) the McCreery method (loading the powder from the back) (3); (*2*) loading the powder from the front (3, 4); and (*3*) the Byström-Asklund method of loading the powder from the edge (5). Other methods of packing are variations on these 3 methods (6).

The front packing method is the crudest way of preparing the sample. This technique yielded results for tolazoline hydrochloride, naphazoline nitrate, clonidine hydrochloride, tramazoline hydrochloride, and antazoline hydrochloride as consistent as the other 2 methods of packing for the relative intensities. For phentolamine mesylate, oxymetazoline hydrochloride, xylometazoline hydrochloride, and tymazoline hydrochloride, however, the results were unsatisfactory. Using the front loading technique, the

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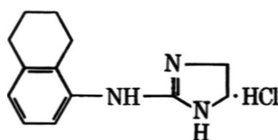
Table 1. X-ray diffraction data for tramazoline hydrochloride[a]



$C_{13}H_{17}N_3 . HCl$
CAS-1082-57-1 parent
CAS-3715-90-0 HCl

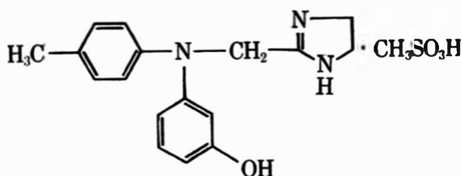
Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	I/I_1	d (Å)	I/I_1	d (Å)	I/I_1	d (Å)	I/I_1
10.60	33	10.60	60	10.60	62	10.60	58
7.35	**63**	**7.34**	**91**	**7.37**	**92**	**7.35**	**90**
6.96	13	6.96	22	6.97	26	6.96	16
6.22	50	6.22	60	6.22	71	6.22	67
5.38	16	5.38	16	5.38	18	5.39	14
5.22	7	5.22	10	5.25	12	5.22	9
4.61	13	4.61	9	4.62	13	4.61	11
4.45	73*	—	—	4.42	68 sh	4.45	64 sh
4.39	**100**	**4.42**	**100**	**4.42**	**100**	**4.39**	**100**
4.27	7	—	4	—	—	—	4
4.19	18	4.19	24	4.19	28	4.18	20
3.97	**74**	**3.97**	**85**	**3.97**	**86**	**3.97**	**83**
3.76	25	3.75	33	3.76	37	3.76	25
3.68	9	—	9	—	11	—	9
3.62	17	3.62	20	3.62	14	3.63	18
3.43	17	3.43	21	3.43	26	3.43	19
3.36	40	3.35	47	3.37	52	3.36	42
3.30	32*	—	43 sh	—	43 sh	—	30*
3.26	50	3.26	67	3.26	69	3.26	56
3.15	10	3.15	16	3.16	14	3.15	12
3.03	9	3.01	10	3.01	11	3.02	10
3.00	10	—	12	—	12	—	12
2.88	13	—	17	—	20	—	20
2.84	14	2.85	18	2.85	19	2.84	18
2.82	15	—	19	—	18	—	17
2.75	9	2.74	14	2.75	13	2.74	12
2.76	14	2.76	18	2.72	18	2.70	14
2.61	9	2.60	19	2.61	18	2.60	7
2.47	7	2.47	8	2.47	9	2.46	8
2.44	7	2.43	8	2.43	10	2.44	10
2.42	8	2.42	9	2.42	9	2.42	10
2.40	7	2.39	8	2.39	7	2.40	7
2.28	8	2.25	11	2.27	10	2.28	8

[a] * = peaks attached to strong line; sh = shoulder attached to strong line; — = measurement not possible. The 3 most intense lines are shown in boldface type.

first 2 or 3 lines at low θ angles for the drugs mentioned were relatively more intense than were those obtained by the other 2 methods of sample packing. The values for *d* spacings were in good agreement with those obtained by the other 2 methods of sample loading, in which preferred orientation is reduced to a minimum.

There was good agreement in the values for interplanar spacings obtained by using the powder diffractometer (McCreery and Byström-Asklund methods of preparing the sample) and the photographic powder technique. In the latter method, more compounds gave 2 or 3 very intense lines at low angles than loading the powder from the front (method 2) in the goniometer technique. This indicates that most of the compounds examined were exhibiting preferred orientation. It was proposed that intense lines at low angles should not be included when selecting the 3 most intense lines for identification unless both techniques are in complete agreement when the order of relative intensities is considered. Users of powder data should be aware that there are often some discrepancies between diffractometer and camera data for the same substance, as a close study of the tables will show.

Table 2. X-ray diffraction data for phentolamine mesylate [a]



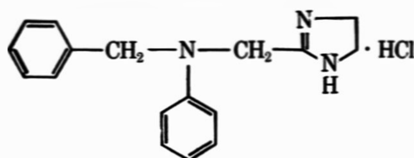
$C_{17}H_{19}N_3O.CH_3SO_3H$

CAS-50-60-2 parent
CAS-73-05-2 HCl
CAS-65-28-1 CH_3SO_3H

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	I/I₁	d (Å)	I/I₁	d (Å)	I/I₁	d (Å)	I/I₁
12.80	**55**	**12.80**	**90**	**12.80**	**92**	**12.80**	**90**
10.20	13	10.20	23	10.20	20	10.20	18
8.85	3	—	3	—	6	—	4
7.56	28	7.56	41	7.57	47	7.57	44
7.00	3	—	5	—	5	—	3
6.42	3	—	4	—	5	—	3
6.01	24	6.03	25	6.01	24	6.03	19
5.75	13	5.75	20	5.73	20	5.75	15
5.28	17	5.31	27	5.29	25	5.28	19
4.78	25	4.73	33	4.72	34	4.76	27
4.69	28	4.70	36	4.68	31	4.70	27
4.35	**79**	**4.38**	**96**	**4.37**	**93**	**4.36**	**96**
4.20	59 sh	—	—	—	—	—	40 sh
4.15	**100**	**4.17**	**100**	**4.17**	**100**	**4.14**	**100**
4.10	25 sh	—	—	—	—	—	25 sh
4.02	13 sh	—	—	—	—	—	11 sh
3.82	22	3.80	32	3.80	24	3.81	19
3.72	36	3.72	55	3.71	44	3.72	34
3.63	13	3.63	23	3.60	17	3.62	13
3.49	3	—	5	—	5	—	4
3.41	3	—	6	—	7	—	6
3.28	7	3.22	8	3.28	5	3.30	6
3.22	19	3.18	27	3.20	24	3.22	24
3.08	5	—	8	—	8	—	8
3.02	6	—	6	—	8	—	7
2.98	5	—	5	—	5	—	6
2.87	3	—	6	—	5	—	5
2.80	3	—	11	—	11	—	10
2.77	10	2.75	17	2.73	16	2.75	15
2.69	5	—	6	—	7	—	6
2.57	4	—	5	—	5	—	6

[a] See footnote *a*, Table 1.

Table 3. X-ray diffraction data for antazoline hydrochloride [a]

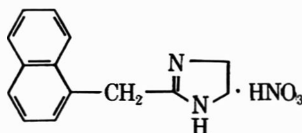


$C_{17}H_{19}N_3$.HCl
CAS-91-75-8 parent
CAS-2508-72-7 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	I/I₁	d (Å)	I/I₁	d (Å)	I/I₁	d (Å)	I/I₁
12.50	37	12.50	63	12.50	76	12.50	73
10.50	5	—	7	—	7	—	16
9.22	21	9.21	26	9.21	29	9.24	32
7.20	7	—	7	—	10	—	9
6.08	3	—	5	—	5	—	9
5.75	**51**	**5.54**	**51**	**5.54**	**63**	**5.72**	**43**
5.32	38	5.32	41	5.30	47	5.29	64
5.17	12	—	13	—	10	—	—
5.05	7	—	7	—	7	—	—
4.83	25	4.82	24	4.79	32	4.79	36
4.62	13	—	12	-	15	-	27
4.45	**58**	**4.45**	**54**	**4.46**	66	**4.44**	55
4.36	18	—	—	—	21	—	—
4.25	14	—	—	—	16	—	—
4.16	21	4.15	24	4.13	27	4.12	25
4.10	11	—	14	—	16	—	—
3.98	29	3.97	30	3.96	36	3.97	27
3.82	53[a]	3.82	52 sh	3.81	57 sh	3.80	36[a]
3.75	**100**	**3.74**	**100**	**3.72**	**100**	**3.75**	**100**
3.58	19	3.59	22	3.53	22	3.52	20
3.41	41	3.41	42	3.40	46	3.40	48
3.31	34	3.30	36	3.31	40	3.35	43
3.06	14	3.06	16	3.09	17	3.01	16
2.93	20	2.93	22	2.97	25	2.97	20
2.67	11	2.67	13	2.68	14	2.67	12
2.50	10	2.50	12	2.56	13	2.47	12
2.44	9	2.44	8	2.47	9	—	10

[a] See footnote *a*, Table 1.

Table 4. X-ray diffraction data for naphazoline nitrate [a]

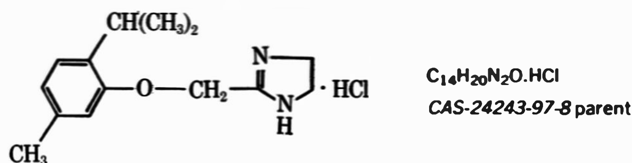


$C_{14}H_{14}N_2.HNO_3$
CAS-835-31-4 parent
CAS-550-99-2 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁
7.97	25	7.92	35	7.97	35	7.94	32
7.22	51	7.21	63	7.22	64	7.19	69
6.57	9	6.57	12	6.55	13	6.52	18
6.09	4	6.06	11	6.04	10	6.07	13
6.00	6						
5.56	43	5.57	45	5.57	52	5.54	47
5.24	**56**	**5.24**	**61**	**5.25**	**65**	**5.23**	**63**
4.61	**55**	**4.62**	**58**	**4.62**	**61**	**4.61**	**51**
4.43	9	—	9	—	7	—	11
4.29	19	4.29	26	4.30	25	4.28	24
3.99	47	3.95	57	3.95	54	3.99	47
3.84	**100**	**3.84**	**100**	**3.84**	**100**	**3.83**	**100**
3.77	48 sh	3.77	57[a]	3.77	53[a]	3.78	50[a]
3.76	36 sh	—	—	—	—	—	—
3.64	19	—	23	3.66	17	—	14
3.59	26	3.59	36	3.58	37	3.58	28
3.48	34	3.49	43	3.48	41	3.48	32
3.21	17	3.21	23	3.21	21	3.22	21
3.01	13	3.01	21	3.02	21	3.01	21
2.82	4	—	7	—	8	—	7
2.77	4	—	7	—	7	—	7
2.63	7	2.64	9	2.63	10	—	7
2.29	8	2.29	8	2.30	10	—	8

[a] See footnote *a*, Table 1.

Table 5. X-ray diffraction data for tymazoline hydrochloride [a]

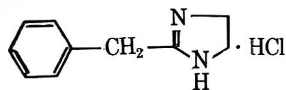


$C_{14}H_{20}N_2O.HCl$
CAS-24243-97-8 parent

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	I/I1	d (Å)	I/I1	d (Å)	I/I1	d (Å)	I/I1
12.10	65	12.10	92	12.10	91	12.10	93
9.30	24•	—	18 sh	—	19 sh	9.29	19•
8.85	**76**	**8.79**	**76**	**8.85**	**81**	**8.79**	**74**
7.70	58	7.66	72	7.69	48	7.63	51
7.38	31•	7.35	40•	7.37	25•	7.37	25•
6.80	19	6.76	13	6.79	15	6.79	16
6.51	5	—	5	—	—	—	—
6.17	4	—	4	—	—	—	—
5.72	39	5.68	44	5.71	29	5.70	25
5.25	48	5.23	47	5.24	45	5.22	43
5.19	12	—	13	—	13	—	13
4.85	16	4.84	13	4.84	16	—	14
4.71	30	4.70	31	4.71	25	4.70	20
4.61	20	—	18	4.61	21	4.62	20
4.48	5	—	6	—	—	—	—
4.18	57	4.18	63	4.18	42	4.18	38
4.03	**63**	**4.03**	**69**	**4.03**	**50**	**4.00**	**63**
3.88	51	3.84	68	3.83	58	—	36
3.82	35						
3.70	67•	3.70	45•	3.70	50•	3.70	34•
3.60	78•	—	84•	3.60	86•	3.60	40•
3.56	**100**	**3.56**	**100**	**3.56**	**100**	**3.55**	**100**
3.47	32•	—	24•	—	22•	—	24•
3.38	20	—	21	—	20	3.38	21
3.30	18	—	22	3.29	19	3.29	23
3.15	24	3.15	17	3.15	18	3.15	16
3.05	17	3.04	19	3.05	17	3.04	19
3.95	19	—	19	—	14	—	19
2.90	16	—	22	—	24	—	20
2.79	11	2.79	11	2.79	14	2.78	14
2.68	8	2.62	10	2.68	10	2.67	11
2.33	14	—	12	—	8	2.33	12
2.32	16	—	14	2.32	7	2.31	10
2.23	6	—	8	2.23	6	2.23	9
2.10	7	2.11	7	2.10	9	2.10	10

[a] See footnote *a*, Table 1.

Table 6. X-ray diffraction data for tolazoline hydrochloride [a]



$C_{10}H_{12}N_2.HCl$
CAS-59-98-3 parent
CAS-59-97-2 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	*I*/*I*1	*d* (Å)	*I*/*I*1	*d* (Å)	*I*/*I*1	*d* (Å)	*I*/*I*1
8.72	**35**	**8.72**	**39**	**8.69**	**42**	**8.71**	**38**
7.51	13	7.53	18	7.52	23	7.52	19
5.96	21	5.97	23	5.94	24	5.96	23
5.61	4	5.55	5	5.57	5	5.61	5
5.30	6	5.31	8	5.34	7	5.31	8
5.08	11	5.09	13	5.06	13	5.10	11
4.85	19	4.86	21	4.84	23	4.85	22
4.48	25	4.47	29	4.49	25	4.49	27
4.44	13	—	—	—	5	—	5
4.35	5	—	—	—	9	—	8
4.23	4 sh	—	—	—	—	—	6 sh
4.03	**29**	**4.00**	**31**	**4.03**	**27**	**4.01**	**28**
3.97	23	4.00	29	3.98	20	4.00	22
3.75	7	—	—	—	—	—	—
3.66	**100**	**3.66**	**100**	**3.65**	**100**	**3.66**	**100**
3.28	19	3.27	18	3.28	20	3.28	16
3.10	17	3.09	19	3.09	20	3.10	17
3.01	7	3.00	6	3.00	9	3.01	6
2.92	7	2.91	8	2.91	9	2.92	9
2.85	9	2.84	10	2.84	10	2.85	11
2.79	11	2.78	13	2.78	11	2.79	10
2.69	5	2.69	6	2.68	8	2.66	7
2.60	6	2.63	7	2.63	6	2.60	6
2.53	7	2.60	8	2.60	9	2.54	8
2.49	4	2.48	4	2.47	5	2.49	3

[a] See footnote *a*, Table 1.

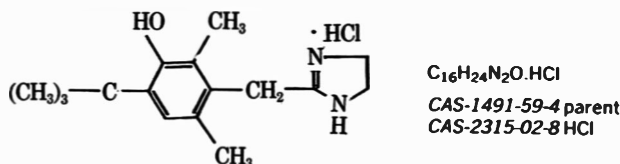
Relative humidity also affects the values of the intensities of the lines. Packed samples left exposed to air for some days produced different relative line intensities in the diffraction pattern compared with the line intensities obtained immediately after packing. It appeared that moisture had been absorbed, and it is therefore advisable to place the ground sample in an oven at the appropriate temperature, then pack it in the sample holder, and run the diffraction pattern immediately after packing.

In the patterns obtained by the powder diffractometer, each centimeter was equal to 1° of 2θ on the chart paper output. A glass vernier ruler was used for measuring distances between the lines on the films, and the lattice spacings (d in Å) were calculated using the previously quoted wavelengths and the Bragg equation $d = \lambda/2\sin\theta$. Values for d spacings can be obtained by using currently available tables of interplanar spacings as a function of θ or 2θ. The values obtained for interplanar spacings by the 2 techniques using the different radiations did not show a deviation greater than ±0.07 Å. In the photographic technique, the optical density traces were obtained from the films by means of a microdensitometer, while in the diffractometer technique the diffraction patterns were recorded directly on chart paper. The relative intensities (I/I_1) were measured simply in terms of peak height (I) above background, relative to peak height above background for the strongest line (I_1) in each pattern taken as 100.

Results and Discussion

Tables 1–9 show the data obtained for the nine 2-imidazolines in terms of the lattice spacings and the relative intensities of the lines. Four combinations of equipment were used to obtain diffraction patterns. The powder diffractometer was applied with CuKα radiation only; the data given are the averages from McCreery and Byström-Asklund loadings. The photographic technique was used in conjunction with 3 ra-

Table 7. X-ray diffraction data for oxymetazoline hydrochloride[a]



$C_{16}H_{24}N_2O.HCl$
CAS-1491-59-4 parent
CAS-2315-02-8 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁
13.10	42	13.10	66	13.10	77	13.10	73
8.01	41 sh	—	—	—	—	—	38 sh
7.87	**73**	**7.85**	**74**	**7.85**	76	**7.99**	**74**
7.11	**100**	**7.10**	**100**	**7.09**	**100**	**7.10**	**100**
6.84	31	—	—	—	—	—	30
6.69	61	6.70	53	6.68	61	6.66	52
6.50	36	6.48	31	6.47	34	6.45	30
6.10	33	6.08	27	6.09	34	6.09	24
5.97	12[a]	—	10 sh	5.92	14[a]	5.96	10[a]
5.66	15[a]	—	11 sh	5.67	15[a]	5.66	10[a]
5.48	**83**	**5.47**	79	**5.47**	93	**5.46**	**77**
5.36	17[a]	—	14 sh	5.31	14[a]	5.36	12[a]
4.89	26	4.88	12	4.88	30	4.89	24
4.77	23	4.76	21	4.74	27	4.74	24
4.42	68	4.42	58	4.41	64	4.41	52
4.29	38[a]	4.29	36[a]	4.31	41[a]	4.29	34[a]
4.16	44	4.17	31	4.17	38	4.17	31
4.08	21	—	17	—	—	—	15
4.02	23	—	18	—	—	—	18
3.91	58	3.90	49	3.90	52	3.91	43
3.79	54	3.78	41	3.78	48	3.79	44
3.64	35	3.64	29	3.64	30	3.62	36
3.50	45	3.50	39	3.49	45	3.50	34
3.40	42	3.40	34	3.39	34	3.39	33
3.14	15	—	13	—	14	—	13
3.12	15	—	12	—	14	—	11
2.98	17	—	14	—	16	—	16
2.93	13	—	9	—	13	—	13
2.79	16	2.79	12	2.79	15	2.78	14
2.65	12	2.64	11	2.64	12	2.65	11
2.56	8	—	7	—	8	—	7
2.56	7	—	5	—	7	—	6
2.40	10	—	8	—	12	—	9
2.13	8	—	9	—	10	—	9

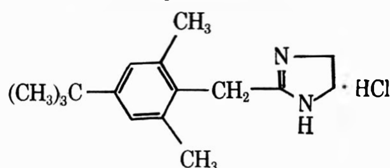
[a] See footnote *a*, Table 1.

diations, CrKα, CoKα, CuKα. The best results in terms of resolution and separation were obtained by the powder diffractometer technique and the photographic technique using CrKα radiation. Poor resolution and separation was observed in the patterns obtained by the photographic technique using CoKα and CuKα radiations. Some peaks appeared as shoulders on stronger lines and these were not considered in the selection of the 3 strongest lines in the pattern. Interplanar spacing values for these shoulders are given in the tables. These shoulders were resolved into separate peaks by the powder diffractometer and by the photographic technique using CrKα radiation.

When the powder diffractometer technique was applied to tramazoline hydrochloride using the 3 different methods of packing, a peak occurred attached to the strongest line in the patterns, and the same peak appeared as a shoulder in the photographic method. Moreover, the second strongest line in the powder diffractometer technique appeared as the third strongest line in the photographic method, and vice versa.

For phentolamine mesylate, the 3 strongest

Table 8. X-ray diffraction data for xylometazoline hydrochloride [a]



$C_{16}H_{24}N_2.HCl$
CAS-525-36-3 parent
CAS-1218-35-5 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁
13.90	55	13.90	69	13.90	83	13.90	71
7.43	54	7.43	58	7.42	67	7.40	61
7.03	8	—	6	—	7	—	6
6.06	**89**	**6.06**	**87**	**6.03**	**92**	**6.01**	**84**
5.24	**76**	**5.24**	**77**	**5.23**	**83**	**5.23**	**72**
5.02	6	—	6	—	7	—	7
4.65	19	—	10	—	9	—	18
4.44	68	4.44	59	4.43	71	4.42	55
4.34	26[a]	—	—	—	22 sh	—	16 sh
4.27	37	4.26	28	4.26	29	4.24	21
4.04	38	4.04	27	4.10	32	4.10	30
3.95	31	3.93	24	3.92	25	3.92	23
3.87	14	—	10	—	11	—	7
3.74	**100**	**3.74**	**100**	**3.74**	**100**	**3.73**	**100**
3.52	24	3.51	22	3.52	25	3.52	23
3.46	19	3.44	17	3.43	16	3.44	16
3.28	25	3.25	21	3.26	25	3.24	19
3.10	25	3.06	25	3.05	30	3.06	23
3.02	15	—	15	—	14	—	14
2.89	12	—	11	—	10	—	10
2.81	11	—	10	—	9	—	9
2.71	8	—	9	—	10	—	8
2.62	8	—	9	—	7	—	7
2.49	6	—	7	—	8	—	6
2.34	9	—	10	—	9	—	8

[a] See footnote *a*, Table 1.

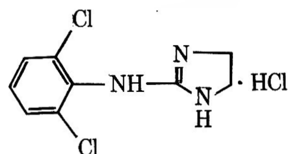
lines were identical in the 2 methods, but in proportion to the strength of other lines the third intense line appeared much stronger in the photographic method, also in the diffractometer method when the powder was loaded from the front. The line at 12.80 Å was selected as one of the 3 strong lines in spite of poor agreement between diffractometer and camera intensities, because it remained the third most intense line in the diffractometer results where preferred orientation had been reduced to a minimum. The 3 small shoulders which were observed on the most intense line in the photographic method using CrKα and CoKα radiation were used.

When antazoline hydrochloride was investigated by the photographic technique, the first line at low Bragg angles was very intense and for the reasons indicated previously was not included in the 3 strongest lines. If the strong line at low Bragg angles was ignored, the 3 most intense lines were the same for the powder diffractometer and the photographic technique. A peak attached to the strongest line was observed in both methods using the different radiations and the different methods of packing.

For naphazoline nitrate, the 3 strongest lines were identical in the 2 techniques, and there was also good agreement in intensities of the other lines. A prominent peak was observed attached to the strongest line on the microdensitometer traces using the 3 radiations in the photographic method. When the diffractometer powder technique was applied using the 3 methods of loading the sample, the same peak was observed only as 2 shoulders of very low intensity.

For the remaining compounds, tolazoline hydrochloride, oxymetazoline hydrochloride, xylometazolite hydrochloride, clonidine hydrochloride, and tymazoline hydrochloride,

Table 9. X-ray diffraction data for clonidine hydrochloride[a]



$C_9H_9Cl_2N_3 \cdot HCl$
CAS-4205-90-7 parent
CAS-4205-91-8 HCl

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁	*d* (Å)	*I*/*I*₁
8.88	26	8.88	46	8.87	50	8.88	49
6.99	23	7.03	21	7.02	26	6.99	24
6.70	35	6.73	36	6.70	39	6.70	37
5.98	15	5.98	26	5.99	32	5.98	32
5.21	26	5.23	31	5.22	39	5.20	39
4.52	8		11		16		14
4.46	12	4.50	11	4.51	16	4.50	11
3.97	24	3.98	30	3.98	36	3.97	34
3.88	9	3.85	19	3.88	22		23
3.81	10		24	3.82	24	3.88	25
3.58	**86**	**3.59**	**96**	**3.59**	**94**	**3.58**	**93**
3.42	19[a]	—	29 sh	—	27 sh	—	26 sh
3.36	**100**	**3.36**	**100**	**3.36**	**100**	**3.36**	**100**
3.27	**49**	**3.27**	**51**	**3.27**	**57**	**3.25**	**56**
—	6	—	10	—	9	—	8
3.05	30	3.05	40	3.05	45	3.04	44
—	8	—	16	—	15	—	16
2.88	15	2.88	24	2.87	29	2.87	29
2.63	11	2.63	14	2.64	18	2.63	18
—	5	—	5	—	9	—	9
2.48	6	—	6	—	8	2.48	8
2.43	12	2.42	11	2.42	14	2.42	13
—	7	—	6	—	8	—	8
—	7	—	7	—	9	—	9
—	7	—	5	—	7	—	10
—	8	—	8	—	11	—	10
—	6	—	9	—	10	—	11
—	8	—	7	—	7	—	8
—	7	—	8	—	8	—	7
2.20	11	2.18	10	2.18	10	2.20	6

[a] See footnote, *a*, Table 1.

relative intensities were in agreement for both techniques.

Conclusion

X-ray powder diffraction patterns provide a means of identification of a specific polymorphic modification of an organic compound, but care must be used in sample preparation. In this work, data for the photographic Debye-Scherrer method using 3 different radiations and for the diffractometer technique using 3 different methods of packing are presented. No fundamental discrepancies were found in the *d* spacing for the strongest lines using the 2 different methods. The variations in intensities of the strongest lines in the diffraction patterns have been explained. A consideration of the resolving power of the 2 methods indicates that resolution might be improved by using a diffractometer with a CrKα source.

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Nuclear Magnetic Resonance Spectroscopic Method for Determination of Penicillamine in Capsules

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A rapid and specific nuclear magnetic resonance (NMR) spectroscopic method for the determination of penicillamine in capsules is presented. The sample is directly dissolved in D_2O and its spectrum recorded on a 90 MHz instrument. The 2 singlets appearing at 1.58–1.64 ppm, due to the nonequivalent gem-dimethyl groups, were integrated and compared with the integral obtained for the phenyl proton signals of sodium saccharin, which is the internal standard. The results obtained by the proposed method closely agreed with those found by the method of USP XX.

Penicillamine (3-mercaptovaline; β,β-dimethylcysteine) is a chelating agent which forms soluble complexes with many heavy metals. Therapeutically, it is primarily used for the removal of copper in patients suffering from Wilson's disease. It has also been found useful in the treatment of heavy metal poisoning, cystinuria, and rheumatoid arthritis. Commercial preparations of penicillamine consist solely of the D-isomer, inasmuch as its L-enantiomer displays significantly greater toxicity.

The official assay procedure for penicillamine in bulk form (1) and in capsules (2) involves titration with mercuric acetate in the presence of a diphenylcarbazone indicator. Variations of this procedure have included electrometric detection of the end points (3, 4). These and other volumetric determinations (5, 6) are considered relatively nonspecific, because various sulfhydryl compounds are found to interfere. Penicillamine has also been determined by colorimetry (7) and high pressure liquid chromatography (8). Vollmer and Lee (9) compared one colorimetric method with 3 volumetric methods.

This paper describes a simple and rapid nuclear magnetic resonance (NMR) spectroscopic method for determining penicillamine in capsules, which at the same time allows its identification. The only other reported application of this technique to the analysis of penicillamine appears to be the work of Cockerill et al. (10), dealing with the determination of its enantiomeric purity.

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Experimental

Apparatus

A 90 MHz Varian Model EM-390 NMR spectrometer was used throughout the study.

Reagents

(**a**) *Penicillamine*.—USP Reference Standard, U.S. Pharmacopeial Convention, Inc., Rockville, MD 20852.

(**b**) *Penicillamine disulfide*.—Pfaltz and Bauer, Inc., Stamford, CT 06902.

(**c**) *Sodium saccharin*.—Sherwin Williams Chemicals, Cleveland, OH 44101.

(**d**) *Deuterium oxide* (D_2O).—Merck and Co., Inc., Rahway, NJ 07065.

(**e**) *Sodium 2,2-dimethyl-2-silapentane sulfonate* (*DSS*).—NMR Specialties, New Kensington, PA 15068.

Determination

Form composite by emptying and mixing contents of $\geq$10 capsules and determine average capsule content weight. Transfer accurately weighed portion of the powder equivalent to 250 mg penicillamine to 15 mL glass-stopper centrifuge tube. Add ca 500 mg sodium saccharin of known purity, and 2.5 mL D_2O. Dissolve with aid of vortex mixer, centrifuge, and transfer ca 0.4 mL of the supernatant to NMR tube. Obtain spectrum, using spin rate of ca 70 cps. Integrate the 2 penicillamine singlets appearing at 1.58–1.64 ppm with respect to DSS, as well as the saccharin multiplet at 7.4–7.9 ppm. In each case, obtain average of at least 5 integrations. Calculate penicillamine content according to formula:

$$\text{Penicillamine, mg/capsule} = (I_p/I_s) \times (EW_p/EW_s) \times S \times (AC/P)$$

where I_p = integral height of penicillamine; I_s = integral height of sodium saccharin; EW_p = equivalent weight, 24.87, of penicillamine; EW_s = equivalent weight, 51.29, of sodium saccharin; S = weight of anhydrous sodium saccharin

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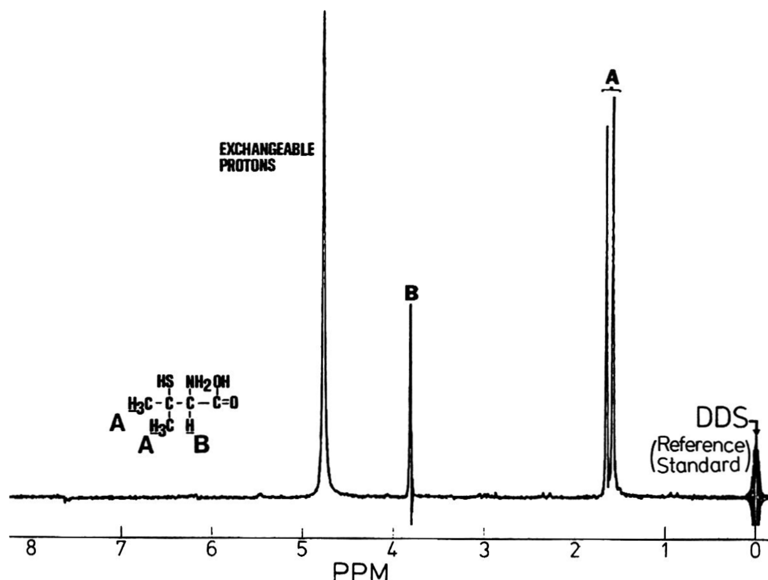

Figure 1. Proton NMR spectrum of penicillamine in D_2O. DDS = sodium 2,2-dimethyl-2-silapentane sulfonate.

added; AC = average weight of capsule content; P = weight of sample taken.

Results and Discussion

The proton NMR spectrum of penicillamine in D_2O (Figure 1) exhibited 2 singlets at 1.58 and 1.64 ppm due to its 2 nonequivalent methyl groups, and a singlet at 3.82 ppm due to the methine proton. All other protons, i.e., SH, NH_2, and COOH, were exchangeable. Chemical shifts are given with respect to DSS.

Penicillamine is ideally suited to analysis by NMR spectroscopy, in part because of its low equivalent weight (149.21 MW/6 protons = 24.87), and in part because of its high dosage content (250 mg/capsule). D_2O was chosen as the NMR solvent for a number of reasons: first, penicillamine is readily soluble in D_2O, thus contributing to the sensitivity of the method; second, D_2O use precludes interference from any exchangeable protons; and finally, the effect of the capsule excipient, stearic acid, on the NMR spectrum of penicillamine is eliminated, because it is insoluble in D_2O.

Sodium saccharin offers several advantages as an internal standard. It is readily available in a highly pure form, it possesses a reasonably low equivalent weight (205.15 MW/4 protons = 51.29), and its phenyl protons resonate downfield (7.4–7.9 ppm), away from the signals produced by penicillamine, capsule excipients, and spinning side bands.

The proposed method permits the direct analysis of the sample. Both penicillamine and the internal standard readily dissolved in D_2O. The slight opalescence produced by stearic acid was easily removed by centrifugation.

Table 1. Recovery of penicillamine by NMR spectroscopy

Sample	Penicillamine added, mg	Penicillamine found, mg	Rec., %
1	224.9	224.2	99.7
2	226.1	227.7	100.7
3	255.2	249.3	97.7
4	252.5	255.5	101.2
5	277.9	278.4	100.2
6	275.3	278.9	101.3
Mean			100.13
Range			97.7–101.3
SD			1.34
RSD, %			1.3

Table 2. Determination of penicillamine (% of declared) in commercial capsules by NMR spectroscopic and USP XX titrimetric methods

Sample	USP method			NMR method		
	Detn 1	Detn 2	Av.	Detn 1	Detn 2	Av.
1	98.9	99.4	99.2	99.0	99.1	99.1
2	96.7	96.8	96.7	94.8	97.4	96.1
3	99.8	99.1	99.5	98.6	101.1	99.8
Mean			98.47			98.33
Range			96.7–99.5			96.1–99.8
SD			1.54			1.97
RSD, %			1.6			2.0

Recovery data were obtained by simulating commercial samples with amounts of penicillamine corresponding to 90–110% of declared (225–275 mg/capsule). The results presented in Table 1 show an average recovery of 100.1%.

Three lots of penicillamine capsules were assayed in duplicate by the official and the proposed methods. The results are compared in Table 2, and are in good agreement. Figure 2 illustrates the NMR spectrum of a commercial sample of penicillamine under typical conditions of analysis.

Penicillamine can undergo oxidative transformation to penicillamine disulfide, a compound with an NMR spectrum strongly resembling that of the parent compound (see Figure 3). The disulfide methyl groups produce 2 singlets, one appearing slightly upfield from the penicillamine singlet at 1.58 ppm, and the other overlapping with the penicillamine singlet at 1.64 ppm. Although small amounts of this product may remain undetected by NMR spectroscopy, concentrations greater than 2% will be detected, and concentrations of 5% or more can be estimated by measuring the heights of the signals generated by the corresponding methyl groups of penicillamine and its disulfide. An expanded spectrum is used for this purpose. In order to estimate the amount of penicillamine disulfide in the mixture, a baseline is constructed across the corresponding singlets appearing at about 1.58 ppm for penicillamine and about 1.55 ppm for penicillamine disulfide (see Figure 4). The peak heights are then measured from this baseline and entered in the following formula:

$$\% \text{ Penicillamine disulfide} = PH_d/(PH_p + PH_d)$$

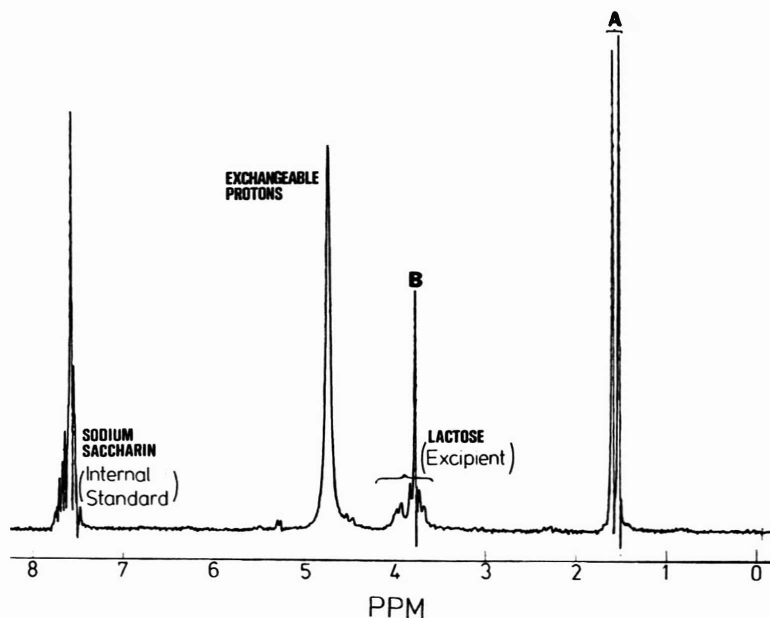

Figure 2. Proton NMR spectrum of a commercial sample of penicillamine under typical conditions of analysis: A, singlets due to methyl groups; B, singlet due to methine proton.

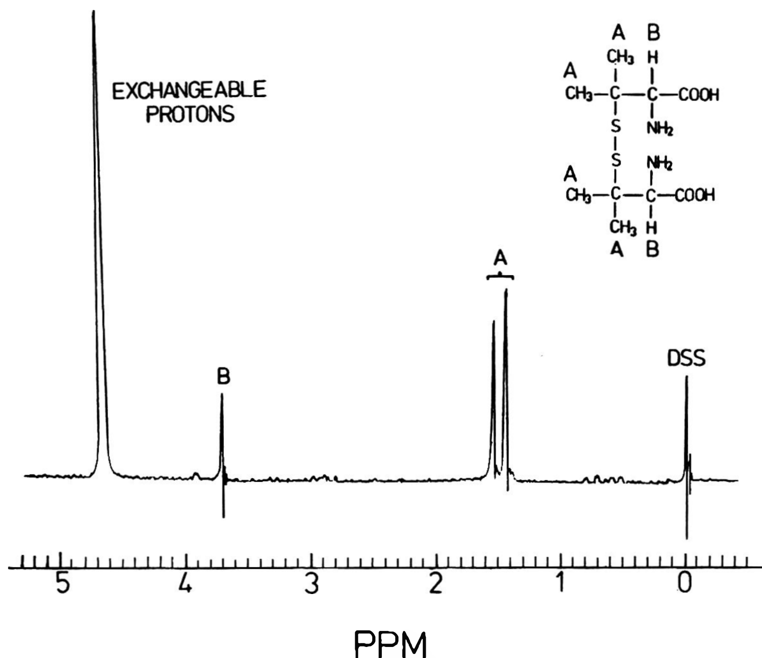

Figure 3. Proton NMR spectrum of penicillamine disulfide in D_2O. DDS = sodium 2,2-dimethyl-2-sila-pentane sulfonate.

where PH_d = peak height of penicillamine disulfide and PH_p = peak height of penicillamine. Estimation of penicillamine disulfide by direct comparison of peak heights under the described experimental conditions is valid since the equivalent weights for this compound and for penicillamine are almost equal (24.87 vs 24.70). A recovery study on a penicillamine USP Refer-

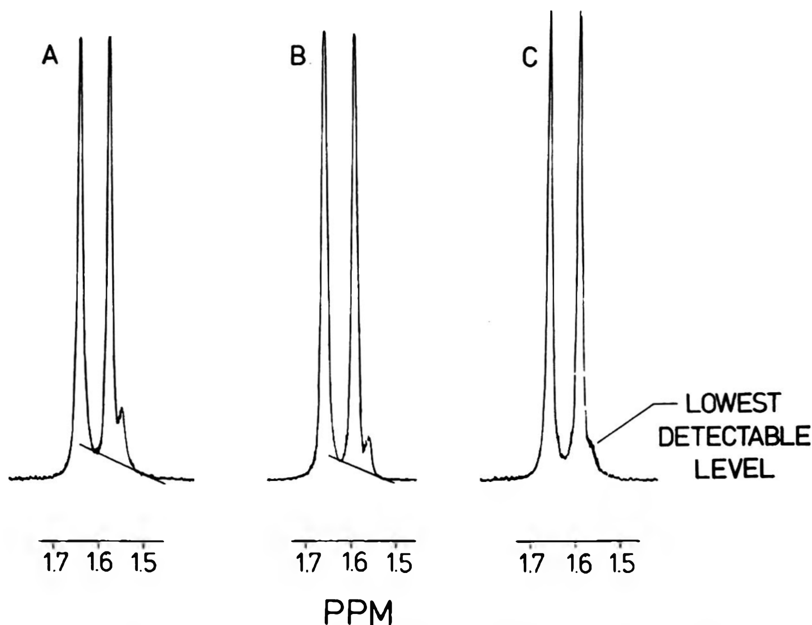

Figure 4. Expanded proton NMR spectra of penicillamine containing known amounts of penicillamine disulfide: A, spike at 10.7%; B, spike at 7.9%; C, spike at 2.5%.

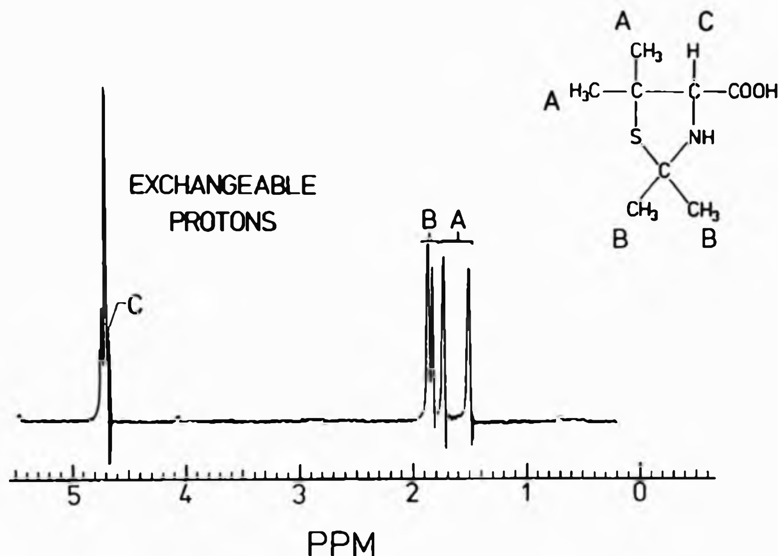

Figure 5. Proton NMR spectrum of penicillamine—acetone adduct in D_2O.

erence Standard sample spiked with 10.7 and 7.9% of penicillamine disulfide yielded values of 10.7 and 7.5%, respectively. The lowest detectable level was approximately 2.5% (see Figure 4C).

Penicillamine reacts quite readily with acidified acetone to form an adduct (2,2,5,5-tetramethyl-4-thiazolidine carboxylic acid) (11) whose NMR spectrum (Figure 5) exhibits 4 singlets at 1.52, 1.73, 1.84, and 1.90 ppm. This adduct also forms slowly when acetone is added to a solution of penicillamine in D_2O. By using deuterated acetone and observing the gradual growth of the singlets at 1.52 and 1.73 ppm, it was determined that these 2 peaks were due to the penicillamine methyl groups, whereas those at 1.84 and 1.90 ppm were due to the acetone methyl groups. Because the disulfide is unable to form the adduct, it was hoped that the utilization of this derivative would offer an improved separation between the methyl peaks of penicillamine and penicillamine disulfide, thus facilitating the determination of these compounds in the presence of each other. Unfortunately, the desired resolution was not attained. This approach, however, does offer an additional and very specific identification test for penicillamine.

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Spectrophotometric Determination of Procaine Hydrochloride in Presence of 4-Aminobenzoic Acid, Using a Combined Polynomial Method

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A method is presented for the determination of procaine hydrochloride without prior separation from degradation products, using the combined polynomial method. Thus, p_w ($= 3p_2 + 4p_3$) calculated over the wavelength range 272-316 nm at 4 nm intervals, using 12-point orthogonal polynomials, was linearly related to concentration (0.3-1.3 mg/100 mL), reproducible (rel. std dev. 0.88%), and independent of degradation products. Mean percent recovery for 6 mixtures of procaine hydrochloride and degradation products was 100.49 ± 1.33. The relationship log $c\%$ vs time for a solution in pH 10 buffer was linear with a slope of −0.171/day.

Several methods have been reported for the determination of procaine hydrochloride. Nonaqueous titrimetric methods (1-4) can be applied if the extent of degradation is less than 10%. Diazometric methods have been applied by several workers, using different indicators (5-7). The bromometric method gives incorrect results when 4-aminobenzoic acid, the main degradation product of procaine hydrochloride, is present (8, 9). Other methods, such as argentimetric (10), conductometric (11, 12), complexometric (13), and thermometric (14) titrations, have been reported. Spectrophotometric (15), colorimetric (16-20), and spectrofluorometric (21, 22) methods have been recommended. Chromatographic methods (23-26) have been applied for determining procaine hydrochloride in the presence of degradation products.

The official method (27) specifies titration with sodium nitrite, with the end point determined electrometrically. This method is specific for the aromatic amino group and therefore does not differentiate procaine and 4-aminobenzoic acid.

The present work deals with the spectrophotometric determination of procaine hydrochloride in the presence of its main degradation product, 4-aminobenzoic acid, without separation, using the combined polynomial method of Wahbi and Ebel (28).

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Development of Method

Procaine hydrochloride in pH 10 buffer has a maximum absorption at 290 nm. The degradation product, which was prepared according to a known procedure (29), has a maximum absorption at 266 nm in the same solvent (Figure 1). The log A vs λ curves for 4-aminobenzoic acid and the degradation products in pH 10 buffer were superimposable over the wavelength range 250-310 nm (Figure 2). This may prove that the chemical nature of the main degradation product is 4-aminobenzoic acid. Furthermore, the ratio p_2/p_0 calculated at $\lambda_m = 265$ nm, the optimum wavelength range in the p_2 convoluted curve of the degradation product (Figure 3) derived by using 12-point orthogonal polynomials at 4 nm intervals, was −2.3501. The same ratio calculated for 4-aminobenzoic acid over the same set of wavelengths was −2.363. The deviation of the former ratio from the latter is about −0.5%. This proves that the degradation product of procaine hydrochloride obtained by using the adopted method is mainly 4-aminobenzoic acid.

Choice of Optimum Conditions

According to general rules (28, 30-32), the quadratic, P_2, and cubic, P_3, polynomials (31) have been chosen to construct the required combined polynomial, P_w. The quadratic polynomial P_2 contributes greatly to the absorption curve of procaine hydrochloride (Figure 1) and therefore its coefficient should afford a precise estimate of concentration, C. Accordingly, p_2 and p_3 have been calculated from the absorption spectra of procaine hydrochloride, X (0.001%), and the degradation products, Z, at a concentration equivalent to 0.001% procaine hydrochloride in pH 10 buffer by using 12-point orthogonal polynomials at 2 and 4 nm intervals. The signs and values of the integers a and b have been varied until $p_w(Z)$ is negligibly small relative to $\alpha_w C_x$ where $\alpha_w = p_w(1\%, 1\text{ cm})$ for procaine hydrochloride. This requirement can be achieved by calculating the ratio $p_w(Z)/p_w(X)$, which measures the expected percentage error

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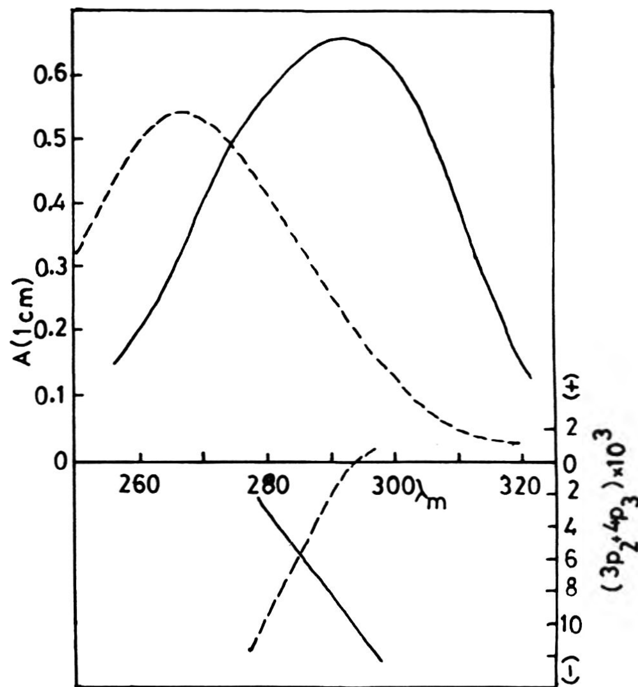

Figure 1. Absorption curves for 0.001% procaine hydrochloride (——) and its degradation products (- - -) in pH 10 buffer, and the corresponding ($3p_2 + 4p_3$) convoluted curves derived by using 12-point orthogonal polynomials at 4 nm intervals.

in the determination of procaine hydrochloride (X), due to the presence of the degradation products (Z). In this connection, for $p_w = 3p_2 + 4p_3$ calculated over the wavelength range 272–316 nm at 4 nm intervals and using 12-point orthogonal polynomials, $p_w(Z) \times 10^3$ equals $+0.0281$, where $p_w(X) \times 10^3 = -10.274$ and the expected percentage error, $p_w(Z)/p_w(X) = -0.27\%$. Accordingly, the p_w convoluted curves for the analyzed compound (X) and the irrelevant absorption have been plotted. Unfortunately, however, at the set of wavelengths and integers ($a = 3$ and $b = 4$) finally chosen, p_w occurred on a slope in the p_w-convoluted curve of procaine hydrochloride. This means that the calculated p_w will be prone to overall shifts in the spectrophotometer's wavelength scale. For that reason, an experiment was designed to estimate the effect of a ±0.5 nm shift in the wavelength scale on the calculated p_w for procaine hydrochloride. Thus, a solution of 0.001% procaine hydrochloride in pH 10 buffer solution was measured at 3 sets of wavelengths with $\lambda_m^0 - 0.5$ nm, λ_m^0, $\lambda_m^0 + 0.5$ nm, where $\lambda_m^0 = 294$ nm. Then, $p_w = (3p_2 + 4p_3)$ was calculated for each set of 12 absorbances. The sensitivity of p_w at $\lambda_m = 294$ nm for $+0.5$ and -0.5 nm shifts was $+2.76$ and -3.07%, respectively (Table 1). The deviation shown in Table 1 is not serious if we take into consideration that measurement of standard and sample usually takes about 20 min using the double beam spectrophotometer.

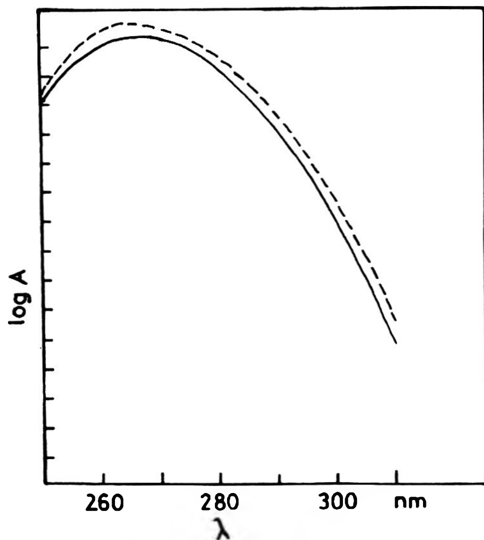

Figure 2. Log A vs λ curves for 4-aminobenzoic acid (——) and the alkaline-induced degradation product of procaine hydrochloride (- - -) in pH 10 buffer.

After selecting the polynomial, wavelength

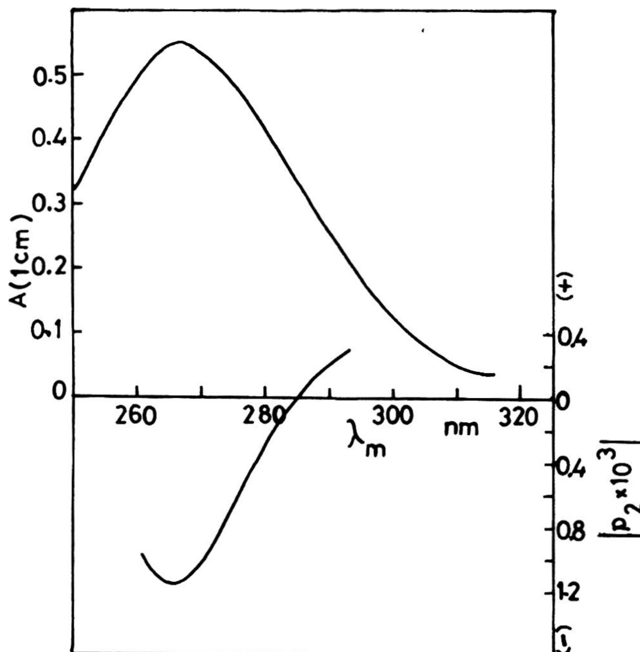

Figure 3. Absorption curve for degradation product corresponding to 0.001% procaine hydrochloride in pH 10 buffer and the corresponding P_2 convoluted curve derived by using 12-point orthogonal polynomials at 4 nm intervals.

range, intervals, and values of a and b, the combined polynomial was constructed as shown in Table 2.

The respective $p_w = 3p_2 + 4p_3$ can be obtained directly by forming the product $\Sigma_{i=0}^{11} A_i P_{wi}$ and dividing by D. The combined polynomial given in Table 2 fulfills the following requirements:

$$\text{(i)} \sum_{i=0}^{11} P_{wi} = 0$$

$$\text{(ii)} \sum_{i=0}^{11} P_{wi} \times P_{ui} = 0,$$

where P_u is a polynomial belonging to 12 points but not P_2 or P_3,

$$\text{(iii)} \sum_{i=0}^{11} P_{wi} \times P_{2i}/D = 3,$$

Table 1. Sensitivity of $p_w = (3p_2 + 4p_3)$, calculated over ranges shown at 4 nm intervals, to ±0.5 nm shift in the wavelength scale

Statistic	λ^0_m 293.5 nm (271.5–315.5 nm)	λ^0_m 294 nm (272–316 nm)	λ^0_m 294.5 nm (272.5–316.5 nm)
$p_w \times 10^3$	−9.959	−10.274	−10.558
Deviation, %	−3.07	0	+2.76

$$\text{(iv)} \sum_{i=0}^{11} P_{wi} \times P_{3i}/D = 4$$

Linearity of P_w to Concentration

Seven solutions of procaine hydrochloride covering a concentration range 0.3–1.3 mg/100 mL pH 10 buffer were prepared by accurate dilution of a strong solution of procaine hydrochloride (0.1%) prepared in distilled water. A(1 cm) of each solution was then measured at 4 nm intervals between 272 and 316 nm, inclusive, against pH 10 buffer solution. The coefficient p_w of the combined polynomial, P_w, was then calculated for each set of 12 absorbances. Graphs of $p_w = (3p_2 + 4p_3)$ and A_{max} when plotted against concentration gave reasonable linearity. The percentage fit for each parameter to concentration was 99.98 and 99.99, respectively.

Linear equations have been calculated using regression analysis. Thus,

$$p_w \times 10^3 = 0.0918 - 10.857c$$

$$A_{290} = -0.0022 + 0.6861c$$

where c is the concentration in mg/100 mL.

Reproducibility of p_w

Separate determinations of p_w were made for different concentrations of procaine hydro-

Table 2. Computation of the combined polynomial, P_w, and the corresponding divisor for determination of procaine hydrochloride in the presence of degradation products

$$P_{wi} = \frac{(aP_{ji}N_k/F) + (bP_{ki}N_j/F)}{N_jN_k/F}$$

$a = 3, b = 4, N_2 = 12012, N_3 = 5148, F = 1716, j = 2, k = 3$

i	P_2	aP_2	$\frac{aP_2N_3}{1716}$	P_3	bP_3	$\frac{bP_3N_2}{1716}$	P_w $\frac{aP_2N_3 + bP_3N_2}{1716}$
0	+55	+165	+495	+33	+132	+924	+1419
1	+25	+75	+225	−3	−12	−84	+141
2	+1	+3	+9	−21	−84	−588	−579
3	−17	−51	−153	−25	−100	−700	−853
4	−29	−87	−261	−19	−76	−532	−793
5	−35	−105	−315	−7	−28	−196	−511
6	−35	−105	−315	+7	+28	+196	−119
7	−29	−87	−261	+19	+76	+532	+271
8	−17	−51	−153	+25	+100	+700	+547
9	+1	+3	+9	+21	+84	+588	+597
10	+25	+75	+225	+3	+12	+84	+309
11	+55	+165	+495	−33	−132	−924	−429
							Divisor (D) = 36036

$\therefore \Sigma_{i=0}^{11} P_{wi}A_i/D = (3p_2 + 4p_3)$.

chloride in pH 10 buffer solution. p_w(1%, 1 cm) was calculated for each solution; the relative standard deviation was 0.88% indicating reasonable reproducibility.

Experimental

Instruments and Reagents

(a) *Spectrophotometer.*—Double beam, Beckman DU 24.

(b) *Buffer solution.*—pH 10 (Clark & Lubs' borate buffer). Into a 1 L volumetric flask, measure 500 mL of a solution that is 0.1M in each of KCl and H_3BO_3, and 439 mL 0.1N NaOH. Dilute to volume with distilled water.

Determination

Accurately weigh ca 40.0 mg procaine hydrochloride. Transfer sample to a 250 mL volumetric flask, using distilled water, dilute to volume with distilled water, and mix well. Pipet 5 mL of this solution into a 100 mL volumetric flask and dilute to volume with pH 10 buffer. Measure absorbances of this solution over the wavelength range 272–316 nm at 4 nm intervals against a blank. Similarly prepare and measure a reference standard. Calculate the coefficient, p_w, from each set of absorbances from the expression $[A_0(+1419) + A_1(+141) + A_2(-579) + A_3(-853) + A_4(-793) + A_5(-511) + A_6(-119) + A_7(+271) + A_8(+547) + A_9(+597) + A_{10}(+309) + A_{11}(-429)]/36036$, where subscripts 0,1,2, . . . 11 stand for 272 nm, 276 nm, 280 nm . . . , 316 nm, respectively.

Results and Discussion

Six mixtures of procaine hydrochloride and degradation products in pH 10 buffer were prepared and assayed using the combined polynomial method. The results obtained are shown in Table 3. The mean percent recovery was 100.49 ± 1.33. Errors in the present method can be attributed to: (*1*) wavelength setting errors, (*2*) non-zero coefficient that may have been contributed by the added degradation products to the assay coefficient, and (*3*) overall shifts in the wavelength scale which affect coefficients sited on slopes in the corresponding p_w-convoluted curve (Figure 1).

Several unsuccessful trials have been carried out on the application of the single polynomial method (23, 30) for determining procaine hydrochloride in the presence of the degradation products, using p_2 belonging to 12-point orthogonal polynomials at 4 nm intervals.

As an example, the mean percent recovery was 95.19 ± 2.06 using p_2 calculated over the same set of wavelengths, i.e., 272–316 nm, at 4 nm intervals (Table 3). The poor results obtained using p_2 alone were due to contribution of p_2 from the degradation products, which was canceled by the method using $3p_2 + 4p_3$.

For the purpose of comparison, percentage recoveries were calculated according to A_{max} and unacceptably high results were obtained. The error in each result decreases with the increase in the concentration of procaine hydrochloride relative to the concentration of the degradation

Table 3. Spectrophotometric determination (rec., %) of procaine hydrochloride in the presence of degradation products, using different methods

Exp.	Added, mg[a]	Method			
		A_{290}	p_2	$p_w = (3p_2 + 4p_3)$	Modified Vierordt
1	0.48	111.80	92.42	99.53	100.31
2	0.64	109.74	93.61	99.42	100.30
3	0.80	106.79	94.68	100.05	99.71
4	0.96	104.99	95.55	99.92	99.53
5	1.12	105.46	97.92	102.92	100.84
6	1.28	103.68	96.96	101.11	99.48
Mean			95.19	100.49	100.03
Std dev.			2.06	1.33	0.54

[a] Each contains degradation products corresponding to 0.16 mg procaine hydrochloride.

products. The latter was kept constant in the 6 mixtures.

The modified Vierordt's method (33) was used to determine procaine hydrochloride (λ_1 = 266 nm, λ_2 = 290 nm) in the same 6 mixtures. The mean percent recovery was 100.03 ± 0.54. However, the presence of linear irrelevant absorption, as may originate from differences between batches of the "sample" and the "reference standard" of procaine hydrochloride, will certainly lead to erroneous results in this method.

On the other hand, the results obtained using the combined polynomial method will not be affected by interferences contributing to coefficients other than those involved in p_w. In other words, the calculation of $(3p_2 + 4p_3)$ will correct for constant, linear, quartic, quintic, etc., components of an irrelevant absorption.

The maximum concentration of 4-aminobenzoic acid at which procaine could still be assayed accurately by using the combined polynomial method has been determined by preparing 7 mixtures of both compounds at various concentration levels. Table 4 shows that procaine can be determined down to 65% with respect to 4-aminobenzoic acid.

Table 4. Recovery of procaine hydrochloride in presence of 4-aminobenzoic acid using the combined polynomial method

Exp.	Added, mg/100 mL		Rec., %
	Procaine HCl	4-Amino-benzoic acid	
1	1.4	0.2	100.5
2	1.2	0.4	100.3
3	1.0	0.6	98.5
4	0.8	0.8	99.3
5[a]	0.6	1.0	99.5
6	0.4	1.2	113.9
7	0.2	1.4	152.3

[a] Maximum concentration of 4-aminobenzoic acid at which procaine hydrochloride could still be assayed accurately.

Degradation of Procaine Hydrochloride

A solution of 0.010 g procaine hydrochloride in 10 mL distilled water was diluted to 100 mL with pH 10 buffer, and kept at room temperature. Aliquots equivalent to 1 mg were diluted with pH 10 buffer to 100 mL at zero time and every day over a period of 5 days. The absorbances of these solutions were measured at the wavelengths 272–316 nm, at 4 nm intervals. The pH 10 alkaline buffer was used to accelerate the decomposition of procaine. The coefficient, p_w, was calculated for each set of measurements and the concentration of procaine hydrochloride was computed. The plot of log c% against time was linear with a slope of −0.171/day and a percentage fit of 99.95. It could therefore be concluded that the proposed method for determining procaine hydrochloride based on calculating $(3p_2 + 4p_3)$ at the mentioned set of wavelengths is specific for the intact molecule, independent of degradation products.

The present method is superior to the official diazometric method (27) because the latter does not differentiate between procaine and the degradation product, 4-aminobenzoic acid. Five different weights (from 80 to 400 mg) of procaine hydrochloride have been assayed using the official method. The mean percent recovery was 100.35 ± 0.125. A solution of the alkaline-induced degradation products of procaine hydrochloride was prepared. Different volumes of this solution corresponding to 100–200 mg procaine hydrochloride were analyzed by the diazometric method. The mean percent recovery was 100.22 ± 0.48 when the equivalent weight of procaine hydrochloride was used.

The present method can only be applied to the determination of procaine hydrochloride in the presence of degradation products when it is not admixed in other formulations.

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PESTICIDE FORMULATIONS

Infrared Spectrophotometric Method for Methazole Formulations: Collaborative Study

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An infrared method was developed for the analysis of Probe 75% wettable powder formulation for methazole content. The procedure involves an extraction of the powder by agitation with acetone. The suspension is centrifuged, and the clear supernatant liquid is measured for absorbance at 755 cm^{-1}. A collaborative study was conducted with 10 laboratories participating. One laboratory omitted the centrifugation, which resulted in a low assay. Statistical analysis of the data showed no significant variances. The coefficient of variation for 10 laboratories analyzing 2 samples in duplicate was 1.29%. The method has been adopted interim official first action.

Probe® 75% wettable powder (WP) is an herbicide effective against several species of grasses and broadleaf weeds. The active ingredient is methazole, 2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione, applied as a wettable powder formulation.

A collaborative study was conducted of an infrared spectrophotometric method. Ten laboratories received 2 samples of Probe 75% WP, an analytical reference standard of methazole, and a copy of the method.

Methazole (2-(3,4-Dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione)

Infrared Spectrophotometric Method

(Applicable to wettable powder contg methazole as only active ingredient)

Apparatus and Reagent

(a) *Infrared spectrophotometer.*—Capable of measuring A from 700 to 900 cm^{-1}, with matched 0.5 mm NaCl or KBr cells.

(b) *Methazole std soln.*—Weigh, to nearest mg, 0.48–0.52 g ref. std methazole (available from Velsicol Chemical Corp., 341 E Ohio St, Chicago, IL 60611) into 4 oz polyethylene screw-cap bottle, pipet in 50.0 mL acetone, and mech. shake 15 min to dissolve.

(c) *Acetone.*—Anal. reagent grade (Mallinckrodt, or equiv.).

Determination

Weigh, to nearest mg, 0.63–0.67 g sample into 4 oz polyethylene screw-cap bottle, pipet in 50.0 mL acetone, and mech. shake 1 h. Centrfg. 30 min to obtain clear supernate.

Fill both cells of spectrophtr with acetone, and place in instrument. Optimize gain; set 100% adjust to give 95–98% T at 755 cm^{-1}. Set slit in program or manual mode for optimum sensitivity and resolution. Fill sample cell with std soln, and scan region from 860 to 700 cm^{-1} (A'). Using same conditions, fill same cell with sample soln and scan twice (A). Measure A and A' at 755 cm^{-1}, using min. at 845 cm^{-1} as baseline.

Calculation

$$\% \text{ Methazole} = (W' \times A \times P \times 100)/(W \times A')$$

where W and W' = g sample and std, resp.; and P = % purity of std.

Results and Discussion

Eighteen collaborators were contacted, of which 10 completed the study. Most collaborators had no problem implementing the method as written. Collaborator 10 substituted a gravity (settling) step in place of centrifugation, and the resulting data were statistically low; this emphasizes the need for centrifugation. Some collaborators used reciprocating shakers in place of a wrist-action shaker, with no apparent effect.

One collaborator investigated the use of chloroform in place of acetone as solvent. Re-

This report of the Associate Referee was presented at the 94th Annual Meeting of the AOAC, Oct. 20–23, 1980, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee A. The Association will vote on adoption of the method as official first action at the 95th Annual Meeting, Oct. 19–22, 1981, at Washington, DC.

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Table 1. Results from collaborative study of methazole

Coll.	Sample A, %	Sample B, %
1	74.3, 74.9	74.1, 75.0
2	73.6, 72.8	73.9, 73.8
3	74.4, 74.8	74.9, 74.6
4	74.8, 76.2	75.2, 75.7
5	74.8, 75.5	74.4, 74.9
6	75.5, 75.9	74.7, 75.8
7	73.5, 73.8	72.9, 73.4
8	73.3, 73.0	73.6, 73.2
9	74.2, 75.5	75.7, 75.6
10[a]	(68.1, 69.6)	(70.4, 69.9)
Av.	74.4	74.5
S_d	1.30	
S_r	0.40	
S_b	0.87	
$S_x = \sqrt{S_r^2 + S_b^2}$	0.96	
CV, %	1.29	

[a] Rejected as outlier.

sults indicated poor repeatability between duplicates vs the acetone results at the same laboratory, and verified results obtained by this investigator's laboratory.

Table 1 gives all results as received. Data from Collaborator 10 were omitted from analysis on the basis of the Dixon test and failure to conduct the analysis as prescribed. The standard deviations are calculated by Youden's method for closely matched pairs (W. J. Youden & E. H. Steiner (1975) *Statistical Manual of AOAC*, AOAC, Arlington, VA). Applying Steiner's method for more than one sample and 2 replicates, the repeatability standard deviation is 0.53, and reproducibility standard deviation is 1.00. When these are compared with Youden's values, S_r and S_x, they demonstrate the similarities and the differences obtained by the 2 statistical approaches.

A 2-way sample plot (Figure 1) shows the elliptical distribution of data; the major axis closely approximates a 45° angle.

Recommendation

The Associate Referee recommends that the infrared method for methazole in 75% wettable powder formulation be adopted interim official first action.

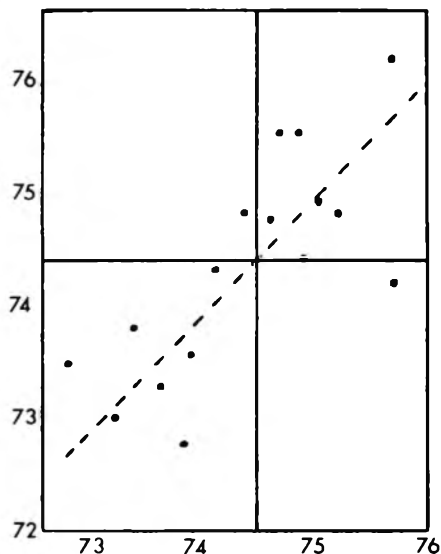

Figure 1. Two-sample plot of methazole collaborative results.

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PESTICIDE RESIDUES

Improved Multiresidue Gas Chromatographic Determination of Organophosphorus, Organonitrogen, and Organohalogen Pesticides in Produce, Using Flame Photometric and Electrolytic Conductivity Detectors

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The multiresidue procedure of Luke et al., which uses extraction with acetone and partition with petroleum ether and methylene chloride, was simplified and shortened by eliminating the Florisil cleanup. Double concentration with petroleum ether in the Kuderna-Danish evaporator following the initial concentration removed the last traces of methylene chloride. The extract was then injected into a gas chromatograph, using a Hall electrolytic conductivity detector for organohalogen, organonitrogen, and organosulfur pesticides or a flame photometric detector for organophosphorus pesticides. Recoveries of 79 pesticides are presented.

The various pesticides that are used on fresh fruits and vegetables present a challenge to the residue analyst. An analysis by the multiresidue procedure (1, 2) of Mills et al. (3) can no longer be considered sufficient for the more widely used pesticides. Changing patterns of pesticide use require additional analyses for water-soluble organophosphorus compounds (4), triazines (5), and methyl carbamates (6). However, the scope and intensity of pesticide monitoring are usually determined by the budget resources of the monitoring laboratory. Few laboratories can afford to do in-depth sample analysis. In addition, the perishable nature of produce and the speed at which it is distributed limit the time that is available for the examination of a sample.

In 1975, Luke et al. (7) described a procedure that was potentially capable of determining almost all nonpolar as well as most polar pesticides. Although only 31 recoveries were reported, they were representative of water-soluble organophosphorus compounds, triazines, and methyl carbamates, as well as pesticides that can be determined by the Mills et al. procedure (3). The analytical scheme is based on the extraction of all nonpolar and most polar pesticides from the sample. Florisil cleanup is delayed until after the organophosphorus and organonitrogen residues have been determined by gas chromatography (GC) because the Florisil cleanup removes the more polar pesticides, many of which are organophosphorus and organonitrogen compounds. The specificity of the thermionic detector (TID) to organophosphorus and organonitrogen compounds minimizes the need for sample cleanup for the determination of these compounds. Sample cleanup is necessary only for the determination of organochlorine residues, which are detected by the relatively nonspecific electron capture detector (EC).

Recent commercial design improvements (8) in electroconductivity detection (HECD) (9) have resulted in detectors with subnanogram sensitivity toward organosulfur, organonitrogen, or organohalogen compounds. The specificity of the HECD detectors for either nitrogen (HECD-N), sulfur (HECD-S), or halogen (HECD-X), as well as the flame photometric detector for phosphorus (FPD-P), virtually eliminates the need for cleanup procedures that are dictated by the use of nonspecific detectors such as the EC. As a result, the applicability of a GC method for a pesticide residue is dependent only on its extraction and its chromatography. The scope of many analytical procedures is presently limited by cleanup procedures which remove some pesticides in addition to interfering plant extractives.

This paper describes changes made in the Luke et al. procedure to use the HECDs. The Florisil cleanup has been eliminated. Water-soluble organophosphorus compounds, triazines, methyl carbamates, and pesticides that are determined by the multiresidue procedure (1-3) are determined faster and with less effort than by previous methods. Additional recovery data are included to demonstrate the greater number of

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pesticides detected as well as to indicate the potential of the method to detect and quantitate most pesticides.

METHOD

Reagents and Apparatus

As described in Luke et al. (7) except as follows:

1. Tracor Model 700A HECD, or equivalent, for halogen, nitrogen, or sulfur.
2. Flame photometric detector with filter for phosphorus.
3. GC columns with the following conditions: *A*: 1.2 m × 2 mm id, 2% DEGS, column temperature 180°C, helium flow rate 60 mL/min; *B*: 1.2 m × 2 mm id, 2% DEGS/0.5% H_3PO_4, column temperature 180°C, helium flow rate 25–30 mL/min; *C*: 30.5 × 2 mm id, 2% DEGS/0.5% H_3PO_4, column temperature 120°C, helium flow rate 25–30 mL/min; *D*: 1.2 m × 2 mm id, 2% OV-101, column temperature 200°C, helium flow rate 30–60 mL/min; *E*: 76.2 × 2 mm id, 4% SE-30/6.5% OV-210, column temperature 200°C, helium flow rate 60 mL/min.

The solid support for the DEGS columns is 80–100 mesh Chromosorb W (AW); 100–120 mesh and 80–100 mesh Chromosorb W (HP), respectively, is used for columns *D* and *E*. If possible, columns are packed so that injections are made into the column packing and not into the glass wool.

Procedure

Follow procedure of Luke et al. (7) through partitioning with methylene chloride. (Extract 100 g sample with 200 mL acetone, and filter. Partition 80 mL filtrate with 100 mL petroleum ether and 100 mL methylene chloride. Repeat partitioning with 2 additional 100 mL portions of methylene chloride.) Concentrate organic solvents in Kuderna-Danish concentrators on steam bath to 1 mL. Add 100 mL petroleum ether to concentrator and reconcentrate solution to 1 mL. Repeat reconcentration with 50 mL petroleum ether and then with ca 20 mL acetone. Cool and adjust volume to 7 mL. Inject ca 2 μL into the following GC detector-column systems: HECD-X, *B* and *D*; HECD-N, *B* and *D*; HECD-S, *C*; FPD-P, *A*, *D*, and *E*.

Results and Discussion

In addition to eliminating the Florisil cleanup, acetone was replaced by petroleum ether for reconcentration. The acetone reconcentration step in the Luke et al. procedure (7) reduces the amount of methylene chloride in the sample solution because it affects the performance of the TID detector. However, because of the increased sensitivity of the HECD-X, the residual methylene chloride background must be reduced even further. The residual methylene chloride also has a deleterious effect on the HECD-N and HECD-S scrubbers. Substituting petroleum ether with equal volumes of acetone will not adequately reduce the methylene chloride level. The final reconcentration with acetone minimizes the precipitation of polar residues such as Monitor, which could occur if the final sample solvent is petroleum ether. In addition, acetone reduces the initial interaction of polar residues with the column support when the sample solution is injected into the GC column.

Advantages of Proposed Modification

Table 1 lists additional recovery data for the pesticides determined by the original Luke et al. procedure as well as by the modified method, i.e., a total of 82 recoveries for 79 pesticides. Recovery values are included for 3 triazine, 2 methyl carbamate, 11 miscellaneous organonitrogen, 16 organochlorine, 1 organobromine, 1 organosulfur, and 44 organophosphorus compounds, of which 3 are water-soluble. Twenty-one of the recoveries are for pesticides that have been determined by the Mills procedure (2, Table 201-A). The 82 recoveries should also be applicable to the proposed modification because the basic extraction process is intact. The recoveries support the premise of Luke et al. (7) that the sample extract can contain any of the pesticides that are currently being used with the exception of ionic compounds. Because the time and effort to prepare a sample for GC is a constant, analysis of the extract provides a means for in-depth analyses.

The GC column-detector systems described here are currently being used in the authors' laboratory and illustrate our approach to maximize the number of pesticides that can be determined in produce, to simplify the interpretation of the chromatograms, and to minimize the actual time that a sample remains in the laboratory. If fewer gas chromatographs are available, samples must be set aside until a second GC system is available to confirm the identity of the residues found. The parameters of each GC column have been adjusted so that a chromatographic run is completed in 10–15 min. Aliquots of the same sample extracts are simultaneously injected into each of the GC systems. This procedure simplifies the interpretation of the chromatograms because residue peaks which are

Table 1. Recoveries obtained using the Luke et al. multiresidue procedure

Compound	Fort. level, ppm	Rec., %	Product	Detector
ametryn	0.103	103	cucumber	FPD-S[a]
azinphos-ethyl (Ethyl Guthion)	1.00	102	tomato	FPD-P
BHC, alpha	1.00	92	apricot puree	EC
BHC, beta	1.00	110	apricot puree	EC
bromophos	1.00	92	parsley	
bromopropylate (Acarol)	1.00	108	cucumber	HECD-X
captafol (Difolatan)	0.78	90[b]	tomato	EC
captan	1.04	105[b]	tomato	EC
carbophenothion sulfone	1.94	116	cucumber	FPD-P
chlorbenside	0.18	98	tomato	EC[a]
chlorfenvinphos	0.324	97	bell pepper	FPD-P
chlorothalonil (Daconil 2787)	1.02	81	cucumber	EC
chlorpyrifos (Dursban)	0.10	99	green beans	FPD-P[a]
chlorthiophos	0.0918	95	tomato	FPD-P
DDE	1.00	90	apricot puree	EC
DDVP	0.105	90	tomato	FPD-P
DEF	0.69	105	lettuce	FPD-P
demeton-*S*-sulfone	5.7	115	pepper	FPD-P
dialifor	1.32	115	potato	FPD-P
dichloran (Botran)	1.00	80	lettuce	EC
dicofol (Kelthane)	1.04	101	orange	EC
dicrotophos (Bidrin)	0.094	105	green beans	FPD-P[a]
dieldrin	0.10	85	lettuce	EC
dimethoate oxygen analog	1.51	90	grapes	FPD-P[a]
endosulfan II	0.07	100	green beans	EC
endosulfan sulfate	0.10	100	green beans	EC
endrin	0.059	108	tomato	EC
EPN	1.05	105	green beans	FPD-P
ETU (ethylene thiourea)	0.612	48	cucumber	HECD-N[a]
fenamiphos (Nemacur)	0.436	97	bell pepper	FPD-P
fenitrothion (Sumithion)	1.00	88	blueberry	FPD-P
fensulfothion (Dasanit)	1.0	107	rutabaga	FPD-P
fenthion	0.14	97	green beans	FPD-P[a]
folpet	1.08	96	lettuce	EC
folpet	0.151	95	bell pepper	EC
fonofos (Dyfonate)	1.00	92	parsley	FPD-P
heptachlor epoxide	1.0	92	tomato	EC
leptophos (Phosvel)	0.102	113	potato	FPD-P
linuron	0.50	91	lettuce	N/P[a]
malathion oxygen analog	1.52	112	potato	FPD-P[a]
mephosfolan (Cytrolane)	0.17	106	tomato	FPD-P
methidathion (Supracide)	0.862	93	orange	FPD-P[a]
methomyl	0.988	95	lettuce	HECD-N[a]
methyl carbophenothion (Methyl Trithion)	0.56	118	tomato	FPD-P
metribuzin (Sencor)	0.19	96	tomato	FPD-S[a]
mirex	0.152	106	tomato	EC
naled	3.79	97	strawberry	FPD-P[a]
oxydemeton-methyl (Metasystox R)	0.12	88	grapes	FPD-P
oxydemeton-methyl sulfone	0.113	103	grapes	FPD-P[a]
oxythioquinox (Morestan)	0.054	107	orange	FPD-S[a]
parathion oxygen analog	1.50	105	tomato	FPD-P
PCNB	0.015	112	tomato	EC
permethrin	0.102	108	tomato	HECD-X
phenthoate	0.118	94	cucumber	FPD-S[a]
phenthoate	0.0117	110	tomato	FPD-P
phorate sulfone	0.105	93	lettuce	FPD-P[a]
phorate sulfoxide	0.114	116	lettuce	FPD-P[a]
phosalone	1.80	92	grapes	FPD-P
phosmet (Imidan)	0.25	108	tomato	FPD-P
phosphamidon	1.00	91	apple puree	
phoxim	0.10	110	tomato	FPD-P
phoxim oxygen analog	0.10	105	tomato	FPD-P
profenofos (Curacron)	0.10	102	tomato	FPD-P
prometryn	0.108	104	cucumber	FPD-P[a]
pronamide (Kerb)	1.04	89	cantaloupe	N/P[a]
propargite (Omite)	1.85	107	orange	FPD-S
propham (IPC)	0.208	79	lettuce	N/P

Table 1. (cont'd)

Compound	Fort level. ppm	Rec., %	Product	Detector
propoxur (Baygon)	1.25	98	cantaloupe	N/P
pyrazophos	0.66	107	tomato	FPD-P
ronnel	0.10	104	pears	FPD-P
sulprofos (Bolstar)	0.105	105	green beans	FPD-P
sulprofos sulfone	0.10	105	green beans	FPD-P
sulprofos sulfoxide	0.105	106	green beans	FPD-P
TDE	1.00	94	apricot puree	EC
Tedion	0.10	82	apple puree	EC
Tedion	0.054	106	peas	HECD-X
tetrachlorvinphos (Gardona)	1.08	113	green beans	FPD-P[a]
thiabendazole	1.07	103	orange	FPD-S[a]
thionazin (Zinophos)	0.608	100	tomato	FPD-P
triazophos (Hostathion)	0.134	106	tomato	FPD-P
trichlorfon (Dylox)	0.110	110	orange	FPD-P[a]
vinclozolin (Ronilan)	0.0914	98	strawberry	HECD-X

[a] DEGS column used in quantitation.
[b] Additional Florisil elution required.

identified on one system are immediately confirmed on a second system. For example, simazine can be identified by the HECD-N and HECD-X detectors for both the OV-101 and DEGS columns. Thus, identifying an unknown peak as a residue peak as opposed to an extract peak is simplified. A peak that appears on more than one elemental detector is likely to be a chemical residue. The responses that are obtained by the HECDs, and to an extent the FPD-P, are proportional to a compound's elemental composition. The relative elemental composition of an unknown residue peak can be used to pinpoint its identity when one or more retention values are listed in a reference table. The operation of a multiple system of 8 gas chromatographs does not require a comparable number of analysts to perform the determinations. The simplicity of the chromatograms obtained by the element-specific detectors plus the technique of simultaneously injecting the same sample solution into each of the GC systems allows the GC system to be operated by one analyst.

The elimination of the Florisil cleanup has simplified the logistics of residue analysis. Samples already analyzed for organophosphorus and organonitrogen residues need not be set aside until manpower is available to carry out the Florisil cleanup. The times required for compositing, extracting, partitioning, and solvent reconcentrating are about 5, 7, 15, and 20 min, respectively, for a total of about ¾ h. This sample preparation time can be reduced as more samples are treated together. The simplicity of the proposed modification of the Luke et al. procedure allows 6 persons to complete the analysis of more than 30 samples/8 h day for several weeks.

For some of the more polar organochlorine compounds, such as chlorothalonil (Daconil 2787), captan, captafol (Difolatan), and folpet, the elimination of the Florisil cleanup results in a simpler and more reliable analysis. The recovery of these polar pesticides from the Florisil column depends on the activity of the Florisil that is used. The Luke et al. procedure (7) completely recovered chlorothalonil in its elution system when standards only were used. Table 1 lists a recovery of 81% for chlorothalonil. In recent work by the authors, chlorothalonil could not be recovered in the Florisil eluate. An additional elution with ethyl ether was required to obtain a quantitative recovery.

Interpretation of the Gas Chromatograms

Table 2 lists the columns, the types of compounds that may be determined, and the references for the GC retention tables. These data can be used to help identify residue peaks. The interpretation of the chromatograms is at times difficult because of the incompleteness of the GC retention tables. Pesticides listed in one table are not always listed in another and in some cases they are not listed at all. Although the references listed for the OV-101 and SE-30/OV-210 columns are for the DC-200 and DC-200/QF-1 columns, respectively, experienced residue analysts will not have any difficulty identifying the older, nonpolar pesticides. Identification of peaks in a DEGS chromatogram could be more difficult because of reported differences in the DEGS stationary phases that are sold by com-

Table 2. References for retention data of GC columns

Column	Liquid phase	Pesticide type	Ref. for retention data
A	DEGS	organochlorine/ triazines	10 or 2, Table 333-C
B	DEGS/ H_3PO_4	organochlorine	2, Table 331-C
C	DEGS	special compounds (methomyl, ETU)	
D	OV-101	organophosphorus	2, Table 333-A (for DC-200) or 10
		organochlorine/ triazines	2, Table 331-A (for DC-200) or 11
E	SE-30/ OV-210	organophosphorus	2, Table 333-B (for DC-200/ QF-1) or 10
		organochlorine/ triazines	2, Table 331-B (for DC-200/ QF-1) or 12

mercial sources (13). We have observed that relative retention times also vary greatly with column temperature and age of the column but the same general order of elution is maintained. Table 3 lists retention data for a DEGS column at 180°C, which residue laboratories could use as a guide to build a retention table for their DEGS column. We suggest that laboratories start with the compounds Monitor and acephate.

GC determinations of polar residues such as dimethoate oxygen analog (DMOA), Monitor, and acephate can be particularly difficult. For example, a laboratory that analyzes a sample that contains illegal levels of DMOA may not find any DMOA when the sample extract is injected into a nonpolar phase GC column. A DC-200/TID GC system that requires 2 ng parathion to produce a half-scale recorder deflection requires 40 ng DMOA to produce a similar response, despite the fact that DMOA elutes in one-third the time required by parathion (2, Table 333-A). Polar residues chromatograph poorly, if at all, on nonpolar GC columns. We have found DEGS to be the best liquid phase for GC determinations of polar residues. Despite the problem of nonuniform DEGS, and the variation of relative retention times with temperature and column age, we consider it a primary GC liquid phase in the determination of pesticide residues.

The 30.5 mm DEGS column was developed for the determination of methomyl. In our initial attempts to chromatograph methomyl on a 1.8 m DEGS column at 180°C, we were able to obtain a peak for microgram amounts of standard, but a detector sensitivity at which nanogram amounts of compound are normally determined was required. Apparently methomyl decomposed on the GC column and the observed peak was due to the residual methomyl. By decreasing the column length as well as the operating temperature, the desired sensitivity was obtained for methomyl. However, quantitation may become erratic with repeated sample extract injections. Ongoing work indicates that this GC column could also be used for the related compounds oxamyl and aldicarb.

Broccoli, brussels sprouts, cauliflower, onions, and peas give significant peaks with the HECD-N and FPD-P detectors at a sensitivity range of about 0.1-5 ng residue. Peppers (except bell) produce a peak with the HECD-N detector and radishes with the FDP-P detector. Peas also produce a peak with the HECD-X detector. Other products generally give chromatograms that are relatively free of peaks. Our experience covers most vegetables, but for fruits is limited to melons, strawberries, grapes, and citrus fruits. In general, samples listed for both the HECD-N and HECD-X are not analyzed using these detectors because of the complex chromatograms that these samples exhibit. The background for the FPD-P detector is due to sulfur (large amounts of sulfur cannot be effectively filtered). This sulfur background normally does not present a problem for the determination of organophosphate compounds. The organochlorine peak found in peas has been identified by Marumo et al. (14). It is the only natural organochlorine compound that we have encountered thus far.

The absence of a sample cleanup step requires that background peaks be kept to a minimum. In addition to prewashing the glass wool (7), the reagent grade anhydrous sodium sulfate and the sharkskin filter paper must be prewashed to remove traces of organonitrogen compounds. Polychlorinated biphenyls also have been found in some of the filter paper used. At one time an increase in an organophosphate background peak that occurred near DMOA was observed. The peak was tris(2-chloroethyl) phosphate, a commonly used fire retardant which was coated on the glass wool filters of the laboratory's air conditioning system. The sudden increase in background was caused by a recent filter change. The glass wool type of filter has been replaced with a filter made of uncoated nylon fibers.

In addition to the pesticide residues that are being determined, the chromatograms also reflect the sample extractives that are present in the

Table 3. Relative retention data for a 2% DEGS GC column [a]

RRT [b]	Compound	Molecular formula
0.08	tributyl phosphate	$C_{12}H_{27}PO_4$
0.10	ethoprop (Mocap)	$C_8H_{19}O_2PS_2$
0.14	diazinon	$C_{12}H_{21}N_2O_3PS$
0.14	sulfotepp	$C_8H_{20}O_5P_2S_2$
0.14 [c]	demeton-*O*	$C_8H_{19}O_3PS_2$
0.15	phorate	$C_7H_{17}O_2PS_3$
0.15	terbufos (Counter)	$C_9H_{21}O_2PS_3$
0.15 [c]	mevinphos, alpha (Phosdrin)	$C_7H_{13}O_6P$
0.19 [c]	mevinphos, beta (Phosdrin)	$C_7H_{13}O_6P$
0.19	merphos	$C_{12}H_{27}PS_3$
0.21	fonofos (Dyfonate)	$C_{10}H_{15}OPS_2$
0.23	disulfoton (Disystox)	$C_8H_{19}O_2PS_3$
0.23	diazinon oxygen analog	$C_{12}H_{21}N_2O_4P$
0.24	TEPP (tetraethyl pyrophosphate)	$C_8H_{20}O_7P_2$
0.24 [c]	demeton-*O*	$C_8H_{19}O_3PS_2$
0.24	demeton-*S*	$C_8H_{19}O_3PS_2$
0.26	etrimfos	$C_{10}H_{17}N_2O_4PS$
0.26	dichlofenthion	$C_{10}H_{13}Cl_2O_3PS$
0.27	naled	$C_4H_7Br_2Cl_2O_4P$
0.29	methamidophos (Monitor)	$C_2H_8NO_3PS$
0.31	Aspon	$C_{12}H_{28}O_5P_2S_2$
0.38 [d]	dioxathion	$C_{12}H_{26}O_6P_2S_4$
0.39	pirimiphos-methyl	$C_{11}H_{20}N_3O_3PS$
0.40	ronnel	$C_8H_8Cl_3O_3PS$
0.40	chlorpyrifos (Dursban)	$C_9H_{11}Cl_3NO_3PS$
0.40	Dowco 214	$C_7H_7Cl_3NO_3PS$
0.40	salithion	$C_8H_9O_3PS$
0.41	pirimiphos-ethyl	$C_{13}H_{24}N_3O_3PS$
0.44	tris(chloropropyl) phosphate	$C_9H_{18}Cl_3O_4P$
0.50	Schraden	$C_8H_{24}N_4O_3P$
0.50	DEF	$C_{12}H_{27}OPS_3$
0.53	bromophos-ethyl	$C_{10}H_{12}BrCl_2O_3PS$
0.54	ronnel oxygen analog	$C_8H_8Cl_3O_4P$
0.57	trichlorfon	$C_4H_8Cl_3O_4P$
0.58 [c]	phosphamidon	$C_{10}H_9ClNO_5P$
0.60	bromophos	$C_8H_8BrCl_2O_3PS$
0.61	dicrotophos (Bidrin)	$C_8H_{16}NO_5P$
0.61	phoxim	$C_{12}H_{15}N_2O_3PS$
0.64	acephate (Orthene)	$C_4H_{10}NO_3PS$
0.76	malathion	$C_{10}H_{19}O_6PS_2$
0.82	fenthion	$C_{10}H_{15}O_3PS_2$
0.91 [c]	phosphamidon	$C_8H_9ClNO_5P$
0.92	malathion oxygen analog	$C_{10}H_{19}O_7PS$
1.00	parathion	$C_{10}H_{14}NO_5PS$
1.03	tris(2-chloroethyl) phosphate	$C_6H_{12}Cl_3O_4P$
1.07	profenofos (Curacron)	$C_{11}H_{15}BrClO_3PS$
1.09	dimethoate oxygen analog	$C_5H_{12}NO_4PS$
1.10	fenitrothion (Sumithion)	$C_9H_{12}NO_5PS$
1.17	phenthoate	$C_{12}H_{17}O_4PS_2$
1.17	iodofenphos	$C_8H_8Cl_2IO_3PS$
1.18	methyl parathion	$C_8H_{10}NO_5PS$
1.22	phoxim oxygen analog	$C_{12}H_{15}N_2O_4P$
1.23	parathion oxygen analog	$C_{10}H_{14}NO_6P$
1.23	mecarbam	$C_9H_{20}NO_5PS$
1.23 [c]	chlorthiophos	$C_{11}H_{12}Cl_2O_3PS_2$
1.32	phorate sulfoxide	$C_7H_{17}O_3PS_3$
1.34 [c]	chlorthiophos	$C_{11}H_{12}Cl_2O_3PS_2$
1.36	dimethoate	$C_5H_{12}NO_3PS_2$
1.37	ethion	$C_9H_{22}O_4P_2S_4$
1.41	methyl parathion oxygen analog	$C_8H_{10}NO_6P$
1.48	crufomate (Ruelene)	$C_{12}H_{19}NClO_3P$
1.61 [c]	chlorthiophos	$C_{11}H_{12}Cl_2O_3PS_2$
1.63	monocrotophos (Azodrin)	$C_7H_{14}NO_5P$
1.66	diamidfos (Nellite)	$C_8H_{13}N_2O_2P$
1.75	tetrachlorvinphos (Gardona)	$C_{10}H_9Cl_4O_4P$
1.78	sulprofos (Bolstar)	$C_{12}H_{19}O_2PS_2$
1.81	crotoxyphos (Ciodrin)	$C_{12}H_{19}O_6P$
1.86	carbophenothion (Trithion)	$C_{11}H_{16}ClO_2PS_3$
1.89	demeton-O sulfone	$C_8H_{19}O_5PS_2$
2.18	carbophenothion oxygen analog	$C_{11}H_{16}ClO_3PS_2$
2.25	methyl carbophenothion	$C_9H_{12}ClO_2PS_3$
2.43	methidathion (Supracide)	$C_6H_{11}O_3N_2PS_3$
2.95	phenkapton	$C_{11}H_{15}Cl_2O_2PS_3$
3.49	mephosfolan (Cytrolane)	$C_8H_{16}O_3NPS_2$
3.62	oxydemeton-methyl (Metasystox R)	$C_7H_{17}O_4PS$
4.43	phosfolan (Cyolane)	$C_7H_{14}NO_3PS_2$
5.14	cyanofenphos (Surecide)	$C_{15}H_{14}NO_2PS$
5.26	triphenylphosphate	$C_{18}H_{15}O_4P$
5.42	oxydemeton-methyl sulfone	$C_7H_{17}O_6PS$
5.92	tris(dichloropropyl) phosphate	$C_9H_{15}ClO_4P$
6.53	fensulfothion	$C_{11}H_{17}O_4PS_2$
7.18	EPN	$C_{14}H_{14}NO_4PS$
7.18	triazophos (Hostathion)	$C_{12}H_{16}N_3O_3PS$
10.7	phosalone	$C_{12}H_{15}ClNO_4PS_2$
11.9	famphur	$C_{10}H_{16}O_5NPS_2$
14	phosmet (Imidan)	$C_{11}H_{12}O_4NPS_2$
16.4	sulprofos sulfoxide	$C_{12}H_{19}O_3PS_2$
17.5	azinphos-ethyl (Ethyl Guthion)	$C_{12}H_{16}N_3O_3PS_2$
20	azinphos-methyl (Guthion)	$C_{10}H_{12}N_3O_3PS_2$
29	sulprofos sulfone	$C_{12}H_{19}O_4PS_2$

[a] DEGS, 80–100 mesh Chromosorb W (AW) 1.22 m × 2 mm id with helium flow 80 mL/min at 180°C column temperature and 190°C injection temperature.
[b] Retention time relative to parathion at 180°C.
[c] One of two or more related peaks.
[d] Peak height response varies with injections.

sample solution as well as contaminants that are introduced by the analytical procedure and by the laboratory environment. Both the Luke et al. procedure and the proposed modification produce relatively simple chromatograms which allow the determination of 0.01 ppm residue. Several of the recovery values in Table 1 are based on a fortification level of 0.1 ppm.

Quantitation of Polar Residues

Luke et al. (7) reported that the uncleaned sample solution had a small but noticeably detrimental effect on the performance of the GC column. Peak broadening with increased tailing of peaks occurred after 20 sample injections. The GC column was restored by renewing the glass wool plug at the head of the column into which the injections are normally made. Injecting the sample extract into the GC column support and not into the glass wool plug will further mini-

mize the effect of the sample extract on the GC. When the performance of the GC column deteriorates to an unacceptable level from repeated sample injections, it can be restored by replacing the column material (the section darkened by the sample injections).

In addition to peak broadening and tailing, partial decomposition of pesticides during gas chromatography has been observed because sample residues have been deposited at the start of the GC column. Peaks corresponding to TDE and methoxychlor olefin have been observed for DDT and methoxychlor, respectively. Decreasing sensitivity has been observed for methomyl, captan, and captafol after repeated sample injections. The effect of pesticide decomposition on the GC column to the analytical scheme, however, is minimal and can be reduced further by more frequent replacement of the column packing. The presence of the decomposition peaks can sometimes be used as confirmation of the parent compounds. On the other hand, injections of uncleaned sample extracts have a beneficial effect on the GC determinations of many polar residues. Peak shapes as well as pesticide response often improve with injections of sample extract. Sometimes the peak height responses of a residue will be reproducible for multiple sample injections but erratic for injections of the corresponding standard. The sample solution seems to have a protective effect for the residue. This erratic behavior is usually not observed when a DEGS column is used. In these situations, a lower but more accurate value can be obtained by the use of the DEGS column; the unprotected standard solution is not affected by it. To remedy the problem, the GC standard is made up in the solution of a similar sample in which residues have not been found. Using this standard-in-sample extract on a column other than DEGS will yield a value that is more comparable to that obtained by the DEGS column. Examples of compounds for which this effect has been observed are monocrotophos (Azodrin), captan, DMOA, ametryn, phenthoate, phoxim, phoxim oxygen analog, prometryn, and metribuzin (Sencor). Thus the cleanup of sample extracts for the determination of polar residues is not necessary and may be undesirable.

Experience with Hall Detectors

Our laboratory purchased the first commercial version of the HECD, Tracor Model 310. However, we were not satisfied with the day-to-day performance of the instrument. The Tracor Model 700A HECD is a superior instrument that requires only periodic maintenance once it is properly installed. However, we have had problems with the operation of new Model 700A HECD detectors. This, in part, may have been because we have had Tracor's earlier production models. Also, we bought the Model 700A HECD with the expectation of the 10–20 pg pesticide sensitivity described by Hall (9); Tracor's specification for its lower detection limit is 10^{-13} g chlorine. Tracor's lower detection limit is about 10 times larger than that of Hall. The test solution supplied with the Model 700A HECD gives 4 ng heptachlor for a test injection. This level was too high to meet our needs. We have had the full cooperation of Tracor in improving our Model 700A HECDs. With the installation of our first 700A HECD (the halogen detector), we obtained poor sensitivity for 1 ng amounts of pesticide, and the peaks in the chromatograms tailed excessively. We had noted that the installer had turned on the gas chromatograph before the furnace had arrived at its operating temperature. Apparently condensation of column bleed had occurred and was responsible for the poor detector response. After changing the reaction tube that goes through the furnace, we obtained the detector sensitivity and the chromatography reported by Hall (9). For example, we obtained half-scale deflection for 0.4 ng heptachlor epoxide at our normal operating detector sensitivity. The problem of column-bleed condensation and contamination of the reaction tube is not recognized by Tracor in their present instrument manual. As a precaution, the GC column temperature should be lowered before the furnace is turned off and should not be raised until the furnace is at its operating temperature. Tracor's manual does state that high loads of halogen- or nitrogen-containing liquid phases should not be used because they yield acidic or basic products that will "poison" the conducting liquid of the HECD. They recommend that loads of less than 2% be used when phases such as QF-1, OV-210, and OV-225 are used. We have tried to use an old OV-210 column with the HECD-X detector but it exhibited a significant increase in background at 100°C. When the OV-210 column was replaced with an OV-101 column, the original detector sensitivity could not be obtained. Sensitivity was regained when the reaction tube was replaced. As a result, we do not feel that halogen-containing phases should be used with HECD systems.

We have traced the problem source of one of our HECDs to one of its gas flow control valves, which appeared to be contaminated by a halo-

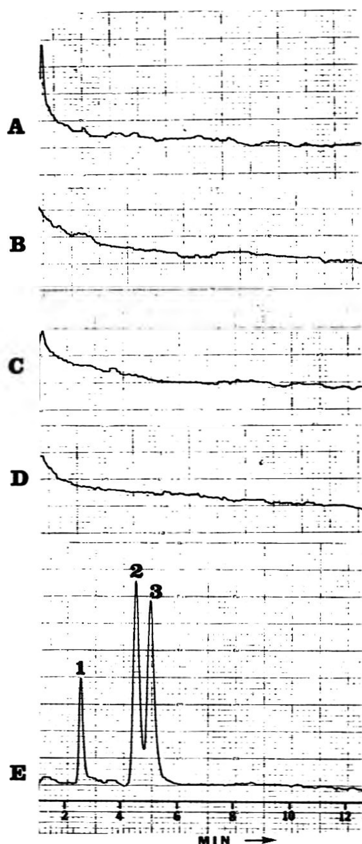

Figure 1. HECD (halogen mode) chromatograms of uncleaned extracts (8 mg). 2% OV-101 column at 200°C and 20 mL/min carrier gas flow. A, lettuce; B, potato; C, plum; D, summer squash; E, standard pesticide mixture. 1, aldrin 0.2 ng; 2, dieldrin 0.6 ng; 3, endrin 0.6 ng. The vent period is not included in the chromatograms.

genated lubricant or by a residual halogenated solvent (possibly used to clean the valve's components during its manufacture).

For HECDs, the column effluent into the detector must be vented for about 0.5 min after a sample is injected. Failure to vent will render the HECDs inoperative. The recovery time for the HECD-X when nonhalogenated solvents have not been vented is about 15 min. With halogenated solvents the recovery time is more than 1 h. The HECD-N is less tolerant to nonventing. If the detector is unvented, the recovery time will be several hours. A recovery time of less than 1 h can be obtained by insulating the exposed scrubber coil of the detector and thereby raising its temperature. After about 0.5 h, removal of the insulation should result in a baseline with relatively little noise.

The HECD takes the column effluent together with a reaction gas through a furnace and into a solvent of which the conductance is measured. Hall (9) stated that the detector is not affected by gas flows of 5–500 mL/min. With Tracor's current design of the detector, Model 700A HECD-X, we have observed an increase in the noise level at total gas flows of about 150 mL/min. The HECDs use reaction gas flows of 50–100 mL/min and as a result are limited to the low column flow

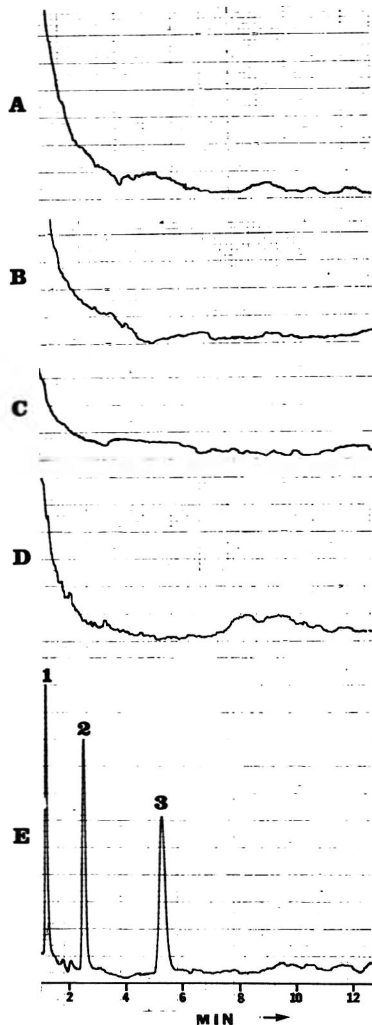

Figure 2. HECD (nitrogen mode) chromatograms of uncleaned extracts (8 mg). 2% OV-101 column at 200°C and 20 mL/min carrier gas flow. A, lettuce; B, potato; C, plum; D, summer squash; E, standard pesticide mixture. 1, simazine 0.8 ng; 2, parathion 9 ng; 3, TOK 12 ng. The vent period is not included in the chromatograms.

rates which are permitted by the 2 mm id column tubing.

Figures 1 and 2 are chromatograms obtained using the HECD-X and HECD-N during routine sample analysis.

Summary

The proposed procedure provides a simple extraction and efficient partitioning for the rapid analysis of samples. The total time and number of pesticides detected are dependent on the extent of the determinative steps. Although the recovery data are limited to the 31 recoveries of Luke et al. (7) and the 82 recoveries of Table 1, the extract should contain most nonionic compounds. The recoveries for bromopropylate (Acarol), chlorthiophos, profenofos (Curacron), captafol, chlorpyrifos (Dursban), fonofos (Dyfonate), permethrin, phenthoate, phorate sulfoxide, phorate sulfone, pyrazophos, vinclozolin (Ronilan), and triazophos were made after the compounds were found during routine residue analysis with the GC systems that are described. An increased scope of pesticide coverage could be obtained by increasing the number of GC systems and by analyzing the extract by high pressure liquid chromatography.

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Gas-Liquid Chromatographic and Gas-Liquid Chromatographic-Mass Spectrometric Determination of Fenvalerate and Permethrin Residues in Grasshoppers and Duck Tissue Samples

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A procedure is described for determining fenvalerate and permethrin residues in grasshoppers and duck tissues. Samples are Soxhlet-extracted with hexane and cleaned up by gel permeation chromatography with an in-line alumina column. Samples are analyzed by gas-liquid chromatography with electron capture detection, and confirmed by gas-liquid chromatography-mass spectrometry. The average recovery from fortified tissues was 97%.

Analytical procedures have been published for determining residues of the synthetic pyrethroids, fenvalerate [cyano(3-phenoxyphenyl)-methyl 4-chloro-α-(1-methylethyl)benzeneacetate] and permethrin [(3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate] in biological samples by gas-liquid chromatography with electron capture detection (GLC-ECD). These procedures usually require a 2-step procedure for cleanup. Wszolek et al. (1) determined residues of fenvalerate in milk and in cow feces, using acetonitrile partitioning; fecal samples required a final isolation on a Florisil column. Oehler (2) described a method for determining permethrin residues in bovine tissues in which fat and muscle samples were cleaned up by high pressure liquid chromatography on a bonded phase column, followed by an additional cleanup on Florisil. Fujie and Fullmer (3) presented a procedure for determining residues of permethrin in plants, animals, and soils, using an automated gel permeation chromatographic (GPC) system. The method used a 27 cm column of SX-3 Bio-Beads and a hexane–ethyl acetate (1 + 3) eluting solvent. Samples containing oils and lipids were further cleaned up on a Florisil column.

We initially attempted to analyze biological samples for residues of fenvalerate by using the GPC procedure similar to that described by Fujie and Fullmer but without final Florisil column cleanup. However, the residues could not be confirmed because extracts contained co-extracted material that particularly interfered with the analysis by gas-liquid chromatography–mass spectrometry (GLC-MS). Consequently, a study was undertaken to develop a single-step cleanup procedure for both fenvalerate and permethrin residues in biological samples.

For a single-step cleanup of grasshoppers or avian tissue, we used an in-line alumina column modification to the GPC system (4) methylene chloride–cyclohexane (15 + 85) solvent. We analyzed the fortified samples for fenvalerate or permethrin by using GLC-ECD and GLC-MS.

METHOD

Apparatus

(a) *Gel permeation chromatograph.*—AutoPrep 1001 GPC (ABC Laboratories) equipped with 60 × 2.5 cm id column packed with 80 g Bio-Beads SX-3 resin, 200–400 mesh, compressed to bed length of about 47 cm.

(b) *Gas-liquid chromatograph.*—Hewlett-Packard Model 5713A with a ^{63}Ni electron capture detector, automatic sampler, and Spectra-Physics Autolab System I computing integrator, recorder attenuation 4. Fenvalerate: 180 × 0.4 cm id glass column packed with 3% SP-2100 on 80–100 mesh Supelcoport, operated at 240°C; GLC attenuation 8. Permethrin: 180 × 0.2 cm id glass column packed with 3% SP-2330 on 100–120 mesh Supelcoport, operated at 220°C; GLC attenuation 4.

Other GLC conditions: injection port 250°C; detector 300°C; and carrier gas flow 60 mL/min of 5% methane in argon—identical for both compounds.

(c) *Gas-liquid chromatograph–mass spectrometer.*—Finnigan Model 4000 with Model 6000 data system, equipped with 183 × 0.2 cm id glass column containing 1% OV-1 on 80–100 mesh Supelcoport. GLC conditions: injection port 230°C; glass jet separator 235°C; helium carrier gas 30 mL/min; column temperature for fenvalerate analysis 215°C; column temperature for permethrin analysis 210°C. MS conditions:

Mention of a pesticide and/or a proprietary product does not constitute a recommendation for use by the U.S. Fish and Wildlife Service.

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Table 1. Average recovery of fenvalerate and permethrin from fortified samples

Compound	Fortification, ppm	Av. recovery,[a] % Grasshopper	Mallard carcass
Fenvalerate	0.5	97 (4.6)	97 (2.3)
	1.0	94 (4.0)	96 (1.1)
Permethrin	0.5	100 (4.0)	93 (2.3)
	1.0	100 (1.1)	100 (2.0)

[a] Standard deviation in parentheses; $N = 3$.

electron energy 70 eV; electron multiplier 1800 V, scan time 3 s. Mass range was divided into 3 subranges, 120-300, 301-400, 401-450, and integration times were 2, 10, and 12 ms/atomic mass unit, respectively.

(d) *Rotary evaporator.*—Model Buchi Rotavapor-R water bath at 30°C.

(e) *Powder funnels.*—Glass, 80 mm diameter, 16 mm od stem.

(f) *GPC receiver flask.*—Flat-bottom, 300 mL, 24/40 ₮ neck joint.

(g) *Homogenizer.*—Virtis, stainless steel cups, 150 mL.

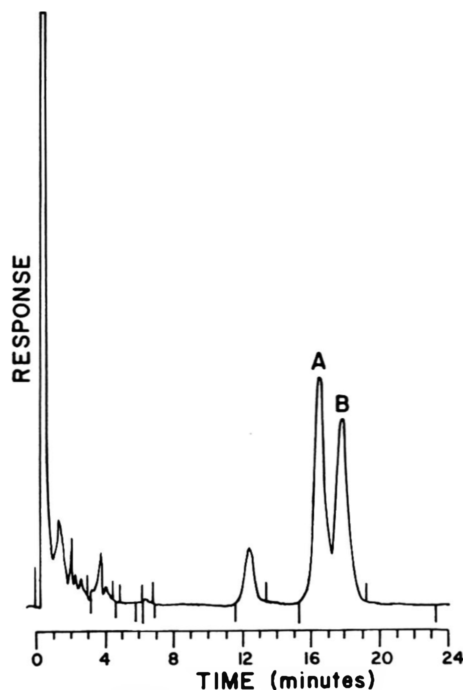

Figure 1. Gas-liquid chromatogram of 2.0 ng fenvalerate, peaks A and B, recovered from 2.0 mg fortified grasshopper.

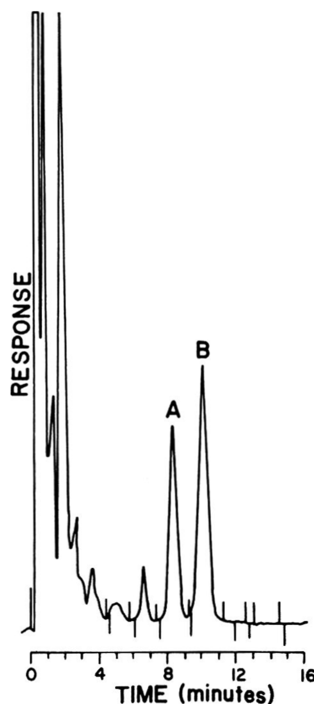

Figure 2. Gas-liquid chromatogram of 2.0 ng permethrin recovered from 2.0 mg fortified mallard carcass. A, cis isomer; B, trans isomer.

Reagents

(a) *Pesticide grade solvents.*—Hexane, methylene chloride, cyclohexane (Burdick & Jackson).

(b) *GPC eluting solvent.*—Methylene chloride-cyclohexane (15 + 85, v/v)

(c) *Alumina.*—Neutral, Brockman Activity I, 80-200 mesh. Dry at 205°C overnight, add 5% water, shake 3 h, and let stand overnight in sealed jar.

(d) *Standards.*—Fenvalerate (Pydrin, Shell 99.3%) and permethrin (Ambush, Imperial Chemistry Industry, 40.2% cis and 51.4% trans isomers). Compounds were dissolved separately in isooctane at 1.0 μg/mL for GLC and 10 μg/mL for spiking. Standards were obtained from U. S. Environmental Protection Agency, Beltsville, MD.

Preparation and Extraction

Blend 10 g portion of homogenized mallard carcass (*Anas platyrhynchos*) or grasshoppers (order Orthoptera) with 5 and 10 μg of each compound, separately, in Virtis cup. Add 200 g Na_2SO_4, mix, transfer to glass thimble, and Soxhlet-extract 7 h with hexane. Transfer extract

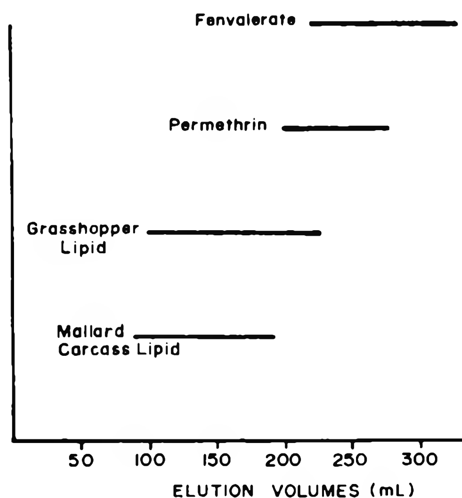

Figure 3. Elution volumes of compounds and lipids from Bio-Beads SX-3, 80 g, eluted with methylene chloride-cyclohexane (15 + 85).

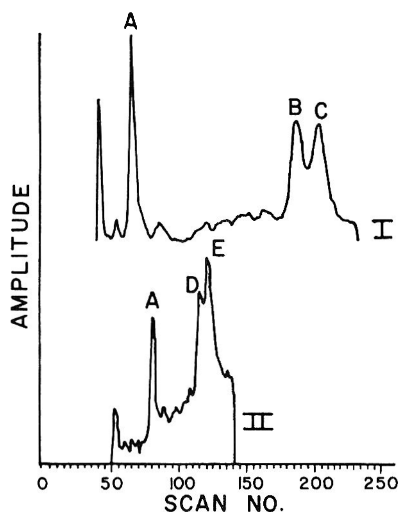

Figure 4. Total ion chromatogram I, ca 500 mg mallard carcass fortified with fenvalerate (peaks B and C); II, ca 500 mg grasshopper homogenate fortified with permethrin (peaks D and E). Peak A is di-2-ethyl-hexyl phthalate.

to 500 mL Phillips beaker and evaporate to dryness under gentle stream of air in 30°C water bath. Reconstitute extract in GPC eluting solvent, transfer to 10 mL centrifuge tube, and dilute to 10 mL.

Cleanup and Analysis

Place plug of glass wool in stem of funnel and pour in 1.5–2.0 g deactivated alumina. Set funnel in neck of receiving flask and place GPC sample collection tube into funnel. Inject sample with syringe into 5 mL capacity sample loop of GPC AutoPrep and elute with methylene chloride–cyclohexane (15 + 85) at pumping rate of 5 mL/min. Set operating times to dump 200 mL, collect 140 mL, and wash 15 mL. When GPC

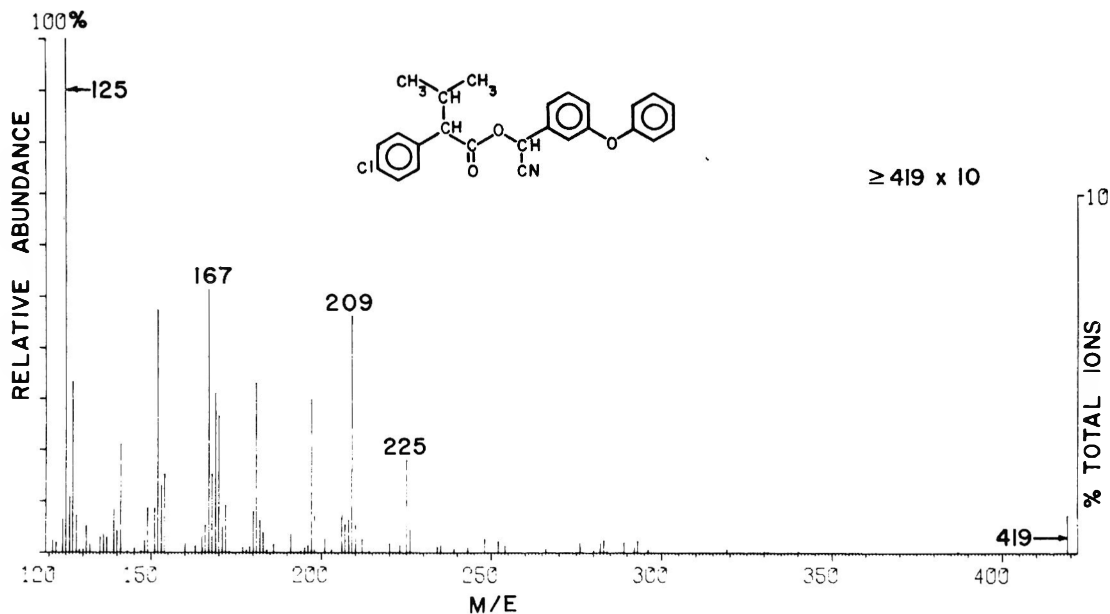

Figure 5. Mass spectrum of fenvalerate peak B, Figure 4.

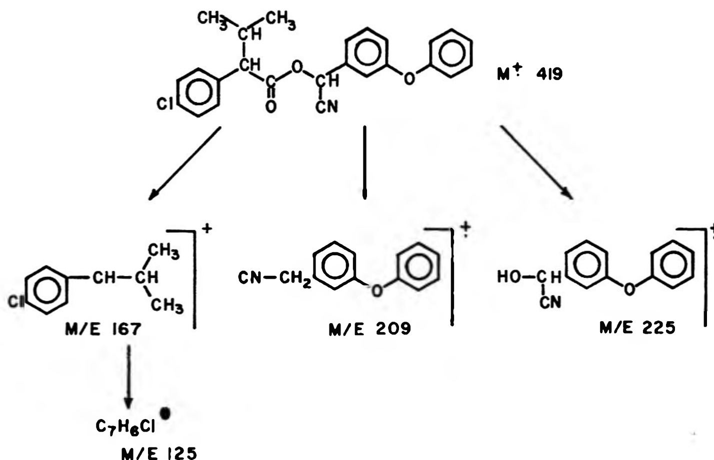

Figure 6. Possible fragmentation of fenvalerate molecule.

cycle is completed, rinse alumina column 3 times with 5 mL eluting solvent and let drain. Concentrate sample on rotary evaporator to 4 mL, transfer to centrifuge tube, and adjust to 10 mL with cyclohexane.

Inject 4.0 μL sample onto appropriate GLC column. Integrate total area of both isomers and compare with standard. Concentrate sample to ca 0.1 mL and analyze by GLC-MS for confirmation.

Results and Discussion

The recovery from grasshopper and mallard carcass samples fortified with fenvalerate or permethrin ranged from 93 to 100% (Table 1). Figure 1 shows a chromatogram of 2.0 ng fenvalerate recovered from fortified grasshoppers. Each peak, labeled A and B, represents a pair of enantiomers as described by Lee et al. (5). Figure 2 shows a chromatogram of permethrin recovered from fortified duck tissue. The cis and trans isomers of permethrin were completely separated on the 3% SP-2330 column (3).

Polychlorinated biphenyls (PCBs) and the common organochlorine pesticides, if present in the sample, will not interfere in the analysis. The retention time of the last eluting peak from 40 ng Aroclor 1260 was 3–5 min before the syn-

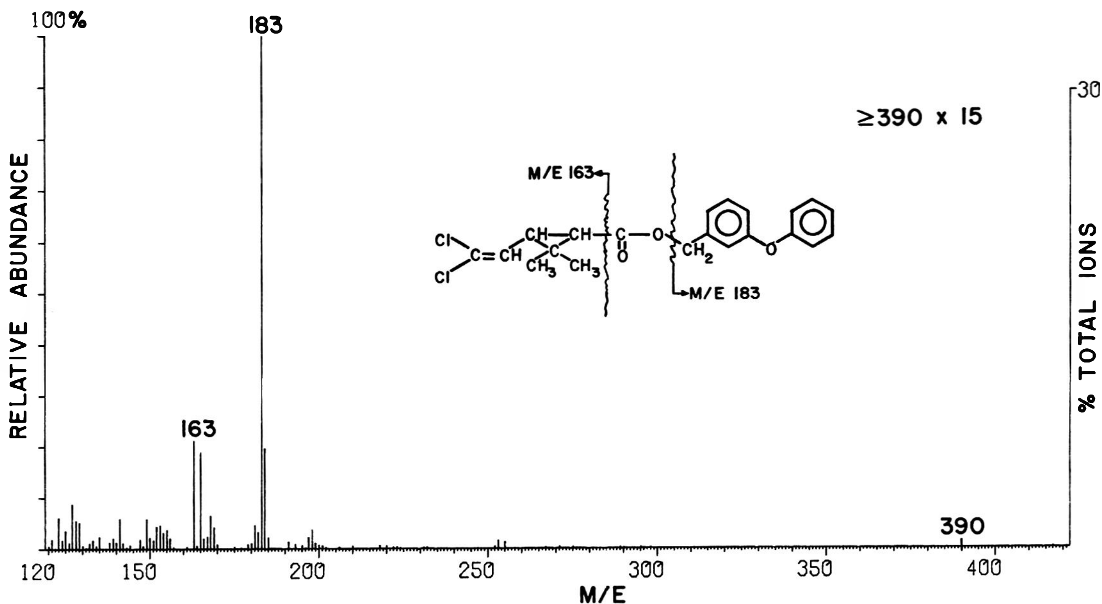

Figure 7. Mass spectrum of permethrin peak D, Figure 4.

thetic pyrethroids eluted on their respective GLC columns. If desired, these synthetic pyrethroids could be separated from PCBs by using the SilicAR column as described by Kaiser et al. (6). The compounds are eluted in fraction 4 along with dieldrin and endrin.

The elution profiles of these compounds and matrices from the GPC column without the in-line alumina column are shown in Figure 3. Data were determined by automatically fractionating 50 μg standards or 0.50 g lipids into 10 mL fractions as described by Griffitt and Craun (7). Good separation was achieved by the GPC procedure except for an overlap of the grasshopper lipids and permethrin. This was evident by a dark green extract. However, this pigment was completely removed by the in-line alumina column.

The cleanup procedure produced extracts that were suitable for GLC-MS analysis as shown in Figure 4.

The mass spectrum of fenvalerate has a weak molecular ion at m/e 419; major ions were observed at m/e 225, 209, 167, and 125 (Figure 5). These ions can be explained by the fragmentation scheme shown in Figure 6. The mass spectra obtained from peaks B and C, Figure 4, are identical.

The mass spectrum of permethrin has a weak molecular ion at m/e 390 (Figure 7). Major ions at m/e 183 and 163 are readily explained by simple cleavage of the molecular ion. The geometric permethrin isomers, peaks D and E, Figure 4, have identical mass spectra.

Acknowledgments

We appreciate the technical assistance of Alexander Krynitsky and Marian Kreamer, and interpretation of mass spectra by Martha Gay.

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Simultaneous Electron Capture Detection of Chlorpyrifos and Its Major Metabolite, 3,5,6-Trichloro-2-Pyridinol, after Gel Permeation Chromatography

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Preparative gel permeation chromatography of ethyl acetate extracts of Southern Pea vines precedes the simultaneous quantitation by electron capture gas chromatography of chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl)-phosphorothioate] and its major metabolite 3,5,6-trichloro-2-pyridinol, which is analyzed as its *N,O*-bis(trimethylsilyl)acetamide derivative. The dark green extract of a pea vine sample (spiked with both the pesticide and its metabolite) is concentrated and chromatographed on a Bio-Beads S-X3 column. Fractions are monitored for cleanup effectiveness by measuring the absorbance at 490 nm and also by gas chromatographic analysis for chlorpyrifos and 3,5,6-trichloro-2-pyridinol recovery. Results showed that the pesticide and its metabolite are separated from the bulk of optically absorbent compounds. Recoveries from 25 g spiked pea vine samples, cleaned up on the Bio-Beads column, are 100% at levels of 0.2 ppm (5 μg) and above, and 62% at levels ranging from 0.01 (0.25 μg) to 0.2 ppm. This analytical procedure provides a fast, sensitive method for simultaneous quantitation of these compounds which were previously determined by 2 separate procedures.

Chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl)-phosphorothioate] is the active ingredient of the widely used nonsystemic insecticide and acaricide Lorsban (a trademark of The Dow Chemical Co.). In most of the original residue studies, quantitation of the pesticide and its oxygen analog by phosphorus flame photometric detection followed co-extraction and individual elution from the same silica gel column (1-4). Later studies incorporated analysis for the phenolic hydrolysis product of chlorpyrifos (3,5,6-trichloro-2-pyridinol), the insecticide's principal metabolite (5-7). Analysis for this third compound necessitated a separate procedure involving extraction, cleanup on alumina, and trimethylsilyl derivatization before quantitation by electron capture detection. Recently, investigators have analyzed for chlorpyrifos and 3,5,6-trichloro-2-pyridinol only (8-10), because the oxygen analog was rarely found and, if detected, constituted only 1% of the chlorpyrifos level (11). The detection techniques for the pesticide and its metabolite are sensitive and have existed for some time. However the lack of an effective concurrent cleanup has necessitated performing 2 separate procedures for adequate determination of both compounds. A simple procedure allowing co-analysis of the pesticide and its metabolite would represent a considerable savings of time and effort. Because the established procedures use silica for chlorpyrifos cleanup and alumina for 3,5,6-trichloro-2-pyridinol cleanup, an alternative to solid-liquid chromatography is necessary for effective concurrent cleanup of these 2 compounds.

Exclusion chromatography has emerged as one of the most important methodologies in biochemistry since the development of crosslinked dextran gels in 1959 (12). One of the earliest studies of this technique for pesticide cleanup was published in 1972 (13); since then, similar procedures have been used for cleanup and multi-residue analysis for most general classes of pesticides (14). Several such successful applications involved the use of Bio-Beads S-X3 (neutral, porous styrene-divinyl-benzene copolymer beads with a molecular weight exclusion limit of 2000) for gel permeation chromatography of chlorinated pesticides (15-17). Our study investigates the simultaneous electron capture quantitation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol in ethyl acetate extracts of Southern Pea vines, following co-elution from a gel permeation column and silylation of the metabolite.

METHOD

Reagents

(**a**) *Solvents.*—Pesticide-grade ethyl acetate and toluene (Mallinckrodt, Inc., St. Louis, MO 63134).

(**b**) *Silylation reagents.*—*N,O*-Bis(trimethylsilyl)acetamide (BSA) (PCR Inc., PO Box 1466, Gainesville, FL 32601); trimethylchlorosilane (Pierce Chemical Co., Rockford, IL).

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(c) *Sodium sulfate.*—Anhydrous (Mallinckrodt, Inc.).

(d) *Standards.*—Chlorpyrifos (Environmental Protection Agency); 3,5,6-trichloro-2-pyridinol (given by The Dow Chemical Co., Midland, MI).

(e) *Standard solutions.*—Stock solutions of 200 ng chlorpyrifos and 3,5,6-trichloro-2-pyridinol/mL ethyl acetate; dilute as needed.

Apparatus

(a) *Gas chromatograph.*—Automatically integrating, microprocessor-controlled, Hewlett-Packard Model 5840A gas chromatograph equipped with ^{63}Ni electron capture detector and 327 cm × 4 mm id glass column packed with 10% DC-200 on Gas-Chrom Q (Applied Science, PO Box 440, State College, PA 16801). Parameters: 95% argon–5% methane carrier gas flow 60 mL/min; injection volume 1 μL; detector 300°C; injection port 190°C; column oven initial hold 170°C for 8 min, program to 220°C at 30°/min, and hold for 15 min.

(b) *Gel permeation apparatus.*—AutoPrep Model 1001 (Analytical Biochemistry Laboratories, Inc., Columbia, MO), equipped with Gilson 3400-D column packed with 200–400 mesh Bio-Beads S-X3 (Bio-Rad Laboratories, 32nd and Griffin, Richmond, CA).

(c) *Spectrophotometer.*—Beckman Model DB equipped with quartz cells.

Calibration of Gel Permeation Column

Spike 25 g control sample of Southern Pea vines (peas intact) with 12.5 μg chlorpyrifos and 12.5 μg 3,5,6-trichloro-2-pyridinol. Extract sample with 100 mL ethyl acetate by Polytron homogenization (3 min at medium speed) followed by preparative centrifugation at 7000 × g for 10 min. Repeat extraction procedure twice and combine supernates. Remove water from combined extracts by passage through anhydrous sodium sulfate, and then reduce extract volume by rotary evaporation to less than 5 mL. Add 1.25 mL toluene and adjust volume to 5 mL with ethyl acetate. Apply sample to top of 53 × 2.5 cm Bio-Beads S-X3 column. Let sample enter column and then apply 5 mL ethyl acetate–toluene (3 + 1) in same manner, to further move sample into column. Attach column to gel permeation apparatus and elute with ethyl acetate–toluene (3 + 1) at 3 mL/min while collecting 2-min (6 mL) fractions. Monitor each fraction by (*1*) measurement of absorbance at 490 nm in spectrophotometer, and (*2*) gas chromatographic analysis for chlorpyrifos and silylation derivative of 3,5,6-trichloro-2-pyridinol.

Preparation of Samples

Spike representative 25 g pea vine samples with chlorpyrifos and 3,5,6-trichloro-2-pyridinol to various levels ranging from 0.01 to 1.00 ppm. Process samples on AutoPrep gel permeation apparatus that is set to discard first 165 mL eluate and to collect next 76 mL.

Derivatization of 3,5,6-Trichloro-2-Pyridinol

Reduce the 76 mL gel permeation fraction (which contains the chlorpyrifos and 3,5,6-trichloro-2-pyridinol) to 2–3 mL by rotary evaporation, and transfer to 10 mL test tube. Take sample completely to dryness and redissolve residue in 100 μL *N,O*-bis(trimethylsilyl)acetamide and 10 μL trimethylchlorosilane. Seal tube with Teflon-lined cap and heat at 65°C 1 h. Dilute to final volume of 1 mL with addition of 890 μL toluene, and seal container immediately.

Results and Discussion

The dark green ethyl acetate pea vine extract which is applied to the Bio-Beads column for

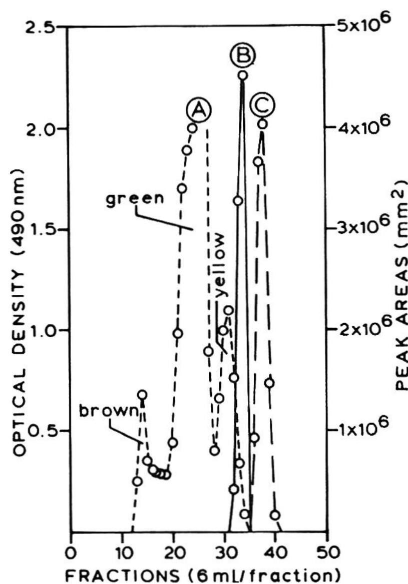

Figure 1. Bio-Beads S-X3 elution profile of 25 g Southern Pea vine sample spiked at 0.5 ppm chlorpyrifos and 3,5,6-trichloro-2-pyridinol: A, optical density (490 nm) vs fraction number; B, integrated area of derivatized 3,5,6-trichloro-2-pyridinol GLC peaks versus fraction number; C, integrated area of chlorpyrifos peaks vs fraction number.

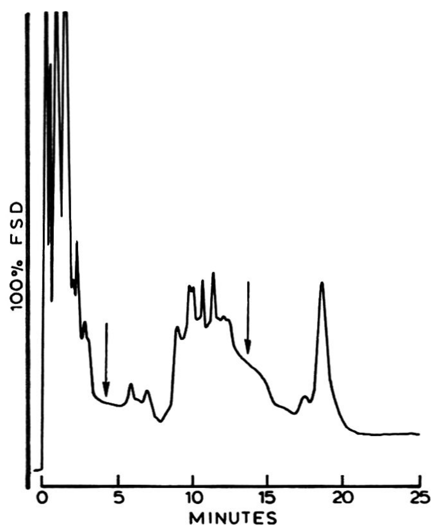

Figure 2. Gas chromatography (on 10% DC-200 with ^{63}Ni electron capture detection) of control 25 g Southern Pea vine ethyl acetate extract, cleaned up with Bio-Beads S-X3 gel permeation chromatography. Retention times for 3,5,6-trichloro-2-pyridinol (4.5 min) and chlorpyrifos (13.7 min) are marked with arrows.

calibration quickly develops into 3 distinct color layers, which elute in the order of brown, dark green, and bright yellow fractions. Figure 1 profiles (*1*) a plot of the absorbance at 490 nm vs fraction number, and (*2*) a plot of the integrated gas chromatographic areas for chlorpyrifos and 3,5,6-trichloro-2-pyridinol vs fraction number. The pesticide and its metabolite are well separated from the bulk of optically absorbent compounds. These 2 compounds elute with the latter part of the yellow fraction. Figure 1 also illustrates that chlorpyrifos and 3,5,6-trichloro-2-pyridinol could be completely separated if necessary.

Gas chromatography of the ethyl acetate extracts of control and spiked crop samples before gel permeation chromatography gives a continuous offscale detector response; after gel permeation and derivatization of the metabolite, the pesticide and metabolite are well separated from spiked crop samples on 10% DC-200, under the temperature programming conditions described in the method. Peak retention times are 4.5 min for the derivatized 3,5,6-trichloro-2-pyridinol and 13.7 min for chlorpyrifos. Peaks are integrated by microprocessor-mediated tangent skim, and no interferences are found in the control samples that would cause problems with the quantitation (Figure 2). A series of standards containing both pesticide and metabolite are treated for derivatization of the metabolite and chromatographed under GLC conditions identical to those described in the method. The standard curve constructed from these samples is linear to 1 ppm for both compounds with a minimum detectability of 0.1 ppb (2.5 pg injected) for chlorpyrifos and 0.5 ppb (12.5 pg injected) for the derivatized metabolite. Typical chromatograms of a 1 ppm standard and a 0.5 ppm sample of spiked pea vines are shown in Figure 3.

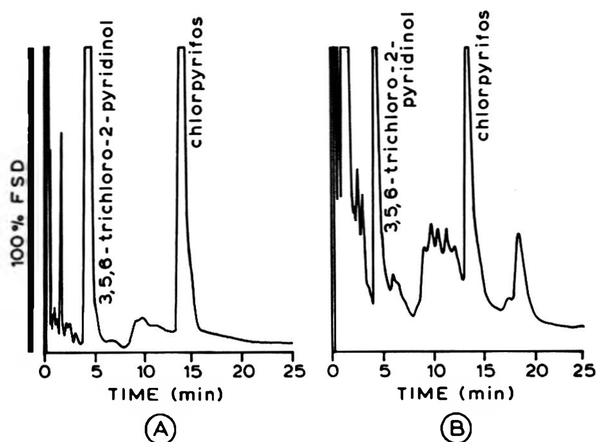

Figure 3. Gas chromatography (on 10% DC-200 with ^{63}Ni electron capture detection) of: A, 1 ppm chlorpyrifos, 3,5,6-trichloro-2-pyridinol standard; B, 25 g Southern Pea vine ethyl acetate extract, spiked at 0.5 ppm with both chlorpyrifos and 3,5,6-trichloro-2-pyridinol and cleaned up with Bio-Beads S-X3 gel permeation chromatography. Retention times are 13.7 and 4.5 min for chlorpyrifos and 3,5,6-trichloro-2-pyridinol, respectively.

Because the pesticide is present with the metabolite during the derivatization, it is necessary to determine if chlorpyrifos is affected. When the extract or standard is dried and the metabolite is silylated as described in the method, the chromatography of chlorpyrifos is unaffected. Chlorpyrifos standards treated according to the silylation procedure chromatograph identically to untreated standards.

Recoveries from representative Southern Pea vine samples (25 g) spiked to various levels with both pesticide and metabolite are 46–62% (0.01 ppm), 73–97% (0.05 ppm), 96–100% (0.2 ppm), 100-106% (0.5 ppm), and 100% (1 ppm) for chlorpyrifos and 43–74% (0.01 ppm), 49–53% (0.05 ppm), 95–100% (0.2 ppm), 89–95% (0.5 ppm), and 100% (1 ppm) for 3,5,6-trichloro-2-pyridinol. One of the major advantages afforded by the gel permeation cleanup is the ability to determine chlorpyrifos by electron capture detection, because the molecule contains 3 chlorines. This is a quantitative advantage over the phosphorus flame photometric detection which has commonly been used to detect the compound's one phosphorus atom. With this added sensitivity, the lower recovery data for the 0.01 ppm chlorpyrifos and 3,5,6-trichloro-2-pyridinol spikings do not sacrifice the accuracy of the residue quantitation. In this procedure, a 43% recovery of a 0.01 ppm spike (250 ng each of pesticide and metabolite) represents a 1 μL injection of 107.5 pg each of the 2 compounds. This amount is still 8 times greater than the minimum detectability of 3,5,6-trichloro-2-pyridinol and 43 times greater than the minimum detectability of chlorpyrifos. If part per billion residue levels were suspected, the indications from these data are that even with very low spiking recoveries the sensitivity of the method would be sufficient if larger injection volumes were used. A 20% recovery of a 1 ppb spiking (25 ng) would contain 25 pg in a 5 μL injection. This amount is above the limits of detection as mentioned for these 2 compounds.

Conclusion

The use of Bio-Beads S-X3 gel permeation chromatography provides a cleanup which results in a fast, sensitive, simultaneous residue analysis for chlorpyrifos and 3,5,6-trichloro-2-pyridinol. Isolation and quantitation of the 2 compounds by this method eliminates the necessity of performing 2 separate procedures, and thus represents a considerable savings in time, effort, and reagents.

Acknowledgment

The authors thank Tim Allen for his assistance in the development of this method.

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Extraction of Aquatic Herbicide Fluridone from Water and Determination by High Pressure Liquid Chromatography

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Fluridone is extracted from water onto a Sep-Pak C_{18} cartridge, and then eluted with a small volume of methanol. Fluridone is concentrated and then is measured by reverse phase high pressure liquid chromatography with UV detection at 254 nm. The method is sensitive to approximately 0.001 ppm and has resulted in recoveries averaging 103.1 ± 19.4, 94.8 ± 5.9, and 93.5 ± 8.5% for control water fortified with 0.001, 0.010, and 0.100 ppm (μg/mL) fluridone, respectively.

Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)pyridinone) is an experimental herbicide that provides control of troublesome vascular aquatic weeds in lakes and ponds with a single annual application (1-5). Consequently, a sensitive and rapid analytical method is desirable for determining the concentration of fluridone in water. Previously reported methods have included the derivatization of fluridone with phosphorus tribromide for measurement by electron capture gas chromatography (6, 7), or measurements of underivatized fluridone in water extracts by gas chromatography with nitrogen detection (8) and by high pressure liquid chromatography (HPLC) with UV detection (9). Fluridone concentrations as low as 5 ppb (5 μg/L) have also been measured by injection of a filtered water sample directly into an HPLC system (9).

This paper reports on the utility of Sep-Pak C_{18}® cartridges for the extraction of fluridone from water before determination by HPLC. The method was applied to determining the concentration of the herbicide in several treated bodies of water and the results are also reported here.

Experimental

Apparatus and Reagents

(a) *Solvents.*—HPLC grade methanol (Waters Associates) and HPLC grade water (J. T. Baker Chemical Co.) were used throughout.

(b) *Cartridges.*—Sep-Pak C_{18} cartridges were obtained from Waters Associates.

(c) *High pressure liquid chromatograph.*—Waters Model 6000A solvent delivery system, Waters Model 440 absorbance detector (254 nm fixed wavelength operated at 0.02 AUFS), Waters Model 710A intelligent sample processor, Houston Instrument Omni Scribe strip chart recorder, and μBondapak C_{18} column (3.9 mm id × 30 cm) with CO:PELL ODS guard column (Whatman, Inc.). Methanol-water (65 + 35) mobile phase was pumped through the column at 1.0 mL/min.

Cartridge Preparation

Prewash each cartridge by attaching to Luer tip on a 50 mL syringe and pumping 10 mL methanol through cartridge, followed by 20 mL HPLC grade water.

Extraction of Water Samples

Pump 50 mL aliquot of water through prewashed cartridge using 50 mL syringe, and discard eluate. Elute fluridone from adsorbent with 5 mL methanol, and collect eluate in 125 mL evaporating flask. Evaporate methanol using rotary vacuum evaporator and 40°C water bath. Add ca 10-20 mL methanol to assist evaporation of small amounts of water in flask. Dissolve residue in 2.0 mL methanol-water (65 + 35) and inject 200 μL into HPLC system under conditions described previously.

Direct Injection of Water Samples

For validation of cartridge cleanup, some water samples were also analyzed by direct injection HPLC method (9). An aliquot of water was filtered, and 1000 μL was injected into the chromatograph.

Results and Discussion

To evaluate the efficiency of Sep-Pak C_{18} cartridges for extracting fluridone from water, 6 replicate recovery samples were prepared by fortifying untreated pond water with fluridone at 0.001, 0.010, and 0.100 ppm (μg/mL). The Sep-Pak cartridge was very efficient at recovering trace levels of the herbicide from water. The recoveries (average ± standard deviation)

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Table 1. Fluridone concentration in treated lake and pond water

		Fluridone application				Fluridone concn (μg/mL)	
Body of water	Location	Formulation[a]	Rate (lb/A)	Method[b]	DAT[c]	Sep-Pak	Direct HPLC
Pond	Crystal River, FL	4AS	1.0	SA	7	0.037	0.040
		4AS	1.0	BAF	7	0.061	0.054
		5P	1.0	SA	7	0.011	0.010
		pretreatment control	0.0	—	—	NDR[d]	NDR
Lake	Haines City, FL	4AS	1.0	SA	14	0.004	0.005
		pretreatment control	0.0	—	—	NDR	NDR

[a] 4 lb/gal. aqueous suspension (4AS) and 5% clay pellet (5P).
[b] Surface application (SA) and bottom acre-foot (BAF) application (7).
[c] Days after treatment.
[d] No detectable residue.

were 103.1 ± 19.4, 94.8 ± 5.9, and 93.5 ± 8.5% at the 3 fortification levels, respectively.

The procedure as described is capable of determining fluridone concentrations in water as low as 1 ppb, which is approximately 100 times lower than the recommended application rates (1–4). It is probable that the sensitivity of the assay could be improved to sub-ppb levels by increasing the volume of water sample pumped through the cartridge, if desired.

The determination of fluridone in water from 3 ponds and a lake treated with the herbicide is summarized in Table 1. The results from the Sep-Pak/HPLC procedure compare favorably with the direct injection HPLC technique. Water samples collected before treatment indicated that no interfering compounds were present. The chromatograms shown in Figure 1 demonstrate the recovery of fluridone from pond water, using the Sep-Pak cartridge.

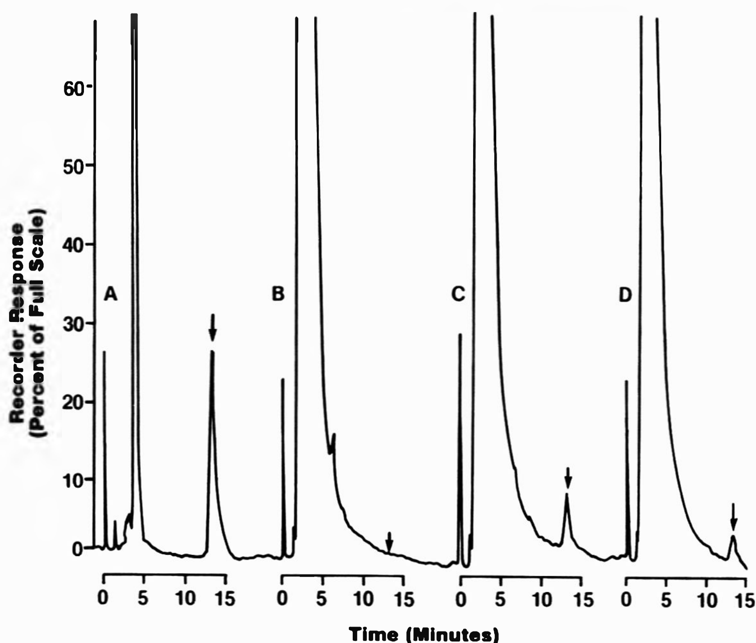

Figure 1. High pressure liquid chromatograms demonstrating the extraction of fluridone from pond water, using Sep-Pak C_{18} cartridge (arrows indicate retention time of fluridone): A, fluridone standard, 200 ng; B, untreated pond water; C, untreated pond water fortified with 0.010 ppm (μg/mL) fluridone, equivalent to 103% recovery; D, treated pond water containing 0.005 ppm (μg/mL) fluridone.

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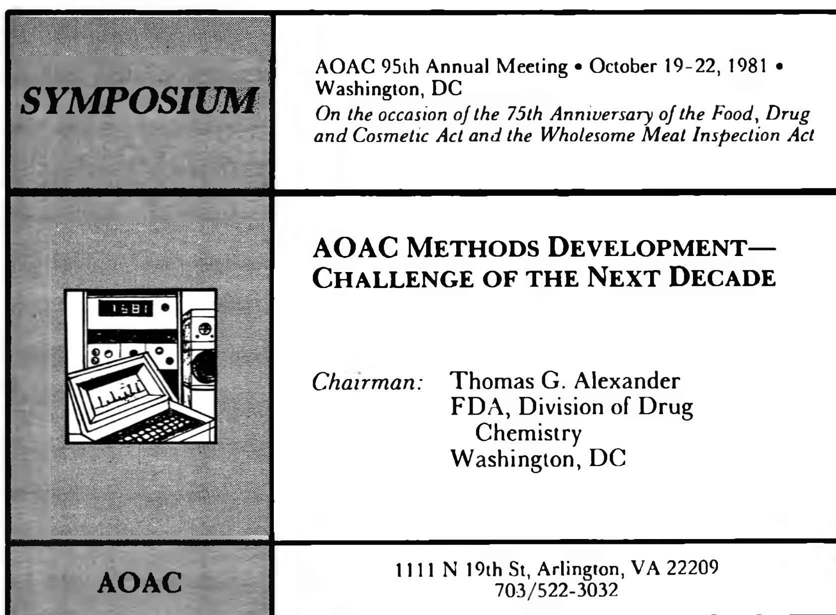

Gas-Liquid Chromatographic-Mass Spectrometric Confirmation of Endosulfan and Endosulfan Sulfate in Apples and Carrots

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A gas-liquid chromatography-mass spectrometric (GLC-MS) procedure is described for the confirmation of endosulfan I, endosulfan II, and endosulfan sulfate in apples and carrots. After extraction, cleanup, and determination by electron capture gas-liquid chromatography using current AOAC methodology, residues are confirmed by GLC-MS. The chemical ionization (CI) mode is used with methane as a reagent gas. Each residue is confirmed by a scan of only 4 regions of its mass spectrum rather than the full mass range. The 4 mass regions for the 2 endosulfan isomers are 274-280, 340-346, 368-374, and 404-412 atomic mass units (amu). For endosulfan sulfate, the mass regions are 286-294, 322-330, 384-392, and 420-428 amu. Four ions and their chlorine isotopic distributions are detected for each compound by this scanning technique. This method was developed by using carrots and apples to which had been added 0.1 ppm (50% of the current legal tolerance on carrots) of each of the 3 pesticides. The gas chromatographic retention times and the mass spectra of the 4 mass regions specified for the 3 pesticides were compared to those of reference standards injected under identical GLC-MS conditions and were used as the basis for confirming identity of the 3 compounds.

Endosulfan (1,4.5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite) is an organochlorine insecticide of the cyclodiene group and is used on a variety of food crops (1). Residues of endosulfan I, endosulfan II, and a metabolite, endosulfan sulfate (see Figure 1 for chemical structures), may be present on these crops. The methane chemical ionization (CI) spectrum of endosulfan I and limited region mass scans of endosulfan II and sulfate are shown in Figures 2 and 3. These spectra show an abundance of ions at high mass range.

Methodology using gas-liquid chromatography with electron capture detection (GLC-ECD) for determining residues of the 3 compounds in apples and cucumbers was collaboratively studied by Mitchell (2) and adopted by AOAC (3). The method (3) consists of extraction of the product with acetonitrile; cleanup of the extract by partition with petroleum ether; elution from a Florisil column with mixtures of hexane, methylene chloride, and acetonitrile; and determination of the residues by GLC-ECD.

The GLC-MS procedure presented here uses the sample extract from the GLC-ECD determinative step for confirming endosulfan residues. Positive confirmation was obtained on apples and carrots to which had been added 0.1 ppm of each of the 3 endosulfan compounds. These compounds were added as acetonitrile solutions to the ground apple and carrot samples before the extraction procedure.

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METHOD

Extraction and Determination

The analytical procedure used for extraction and determination is the AOAC official final action method for endosulfan, endosulfan sulfate, tetradifon, and tetrasul (3) with the following additions:

Apparatus

GLC-MS system.—Finnigan Model 3300F quadrupole mass spectrometer equipped with Finnigan 9500 gas chromatograph and Finnigan 6100 data system with Revision I software, or equivalent. Operate instrument in CI mode with gas chromatograph connected directly to mass spectrometer. Use methane as both carrier and ionization gas. *Column*:—Glass, 1.8 m × 2 mm id, containing 3% OV-101 on 80–100 mesh Chromosorb W (HP). *Operating conditions:*—Temperatures—injection port 250°C, oven 200°C, transfer line 250°C; adjust methane carrier flow to give pressure of 0.8 mm Hg in mass spectrometer source. *Mass spectrometer parameters:*—Electron energy 150 electron-volts, emission current 1.0 mamp, multiplier 2000 V. (Retention times for endosulfan I, endosulfan II, and endosulfan sulfate obtained with these parameters are ca 3.3, 4.6, and 6.2 min, respectively.)

Confirmation

(a) *Procedure.*—Concentrate portion of sample eluate II used in GLC-ECD determination to volume which contains 3–10 ng/μL for each residue encountered. Inject 1–10 μL of concen-

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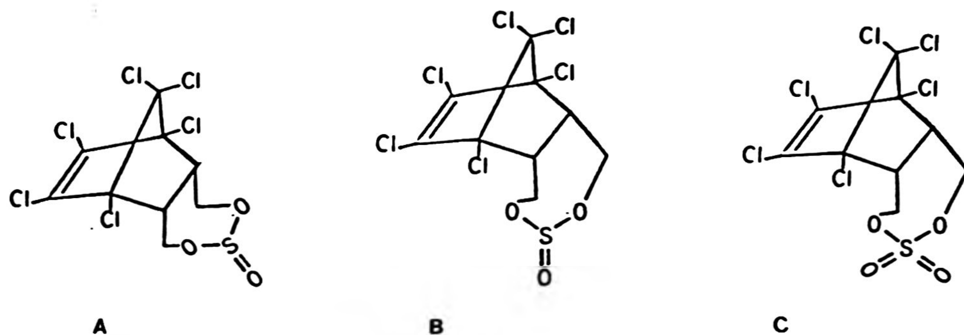

Figure 1. Chemical structure of A, endosulfan I; B, endosulfan II; and C, endosulfan sulfate.

trate into GLC-MS system. Divert solvent for 1 min and acquire data as indicated under (b) and/or (c) below. (Two injections are required if all 3 residues are present.) Inject the blank carried through this procedure.

Confirm residues by comparison of both their mass spectra and GLC retention times to those of reference standards injected under identical GLC-MS conditions.

(b) *Data acquisition for endosulfan I and endosulfan II.*—Mass ranges 274–280, 340–346, 368–374, 404–414 amu; integration time 16 ms/amu for each range; s/scan 0.8.

(c) *Data acquisition for endosulfan sulfate.*—Mass ranges 286–294, 322–330, 384–392, 420–428 amu; integration time 16 ms/amu for each range; s/scan 0.8.

Results and Discussion

The GLC-MS confirmation of chlorinated residues requires a high level of specificity and sensitivity. A full mass scan, i.e., from 100 to 500 amu for chemical ionization spectra, meets specificity demands but is usually not sensitive enough for low residue levels. The technique of selected ion recording (4, 5) offers increased

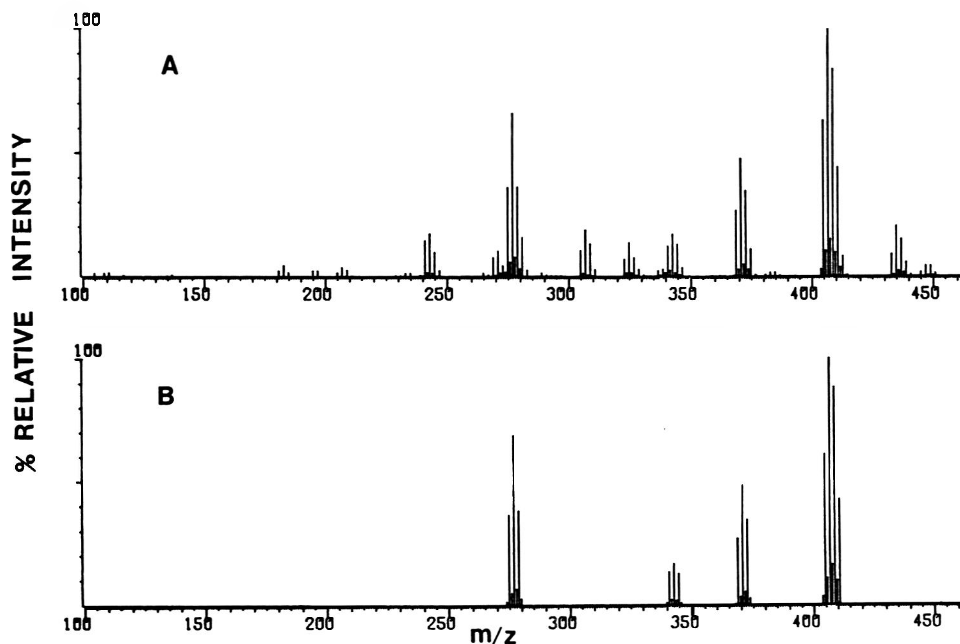

Figure 2. Methane CI spectra of A, endosulfan I standard material, full mass scan; and B, endosulfan I from a carrot sample showing only the 4 mass ranges scanned.

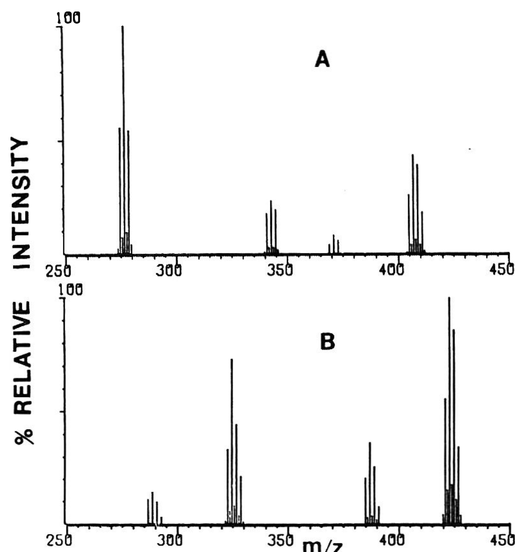

Figure 3. Methane CI spectra of the 4 mass ranges scanned for A, endosulfan II; and B, endosulfan sulfate.

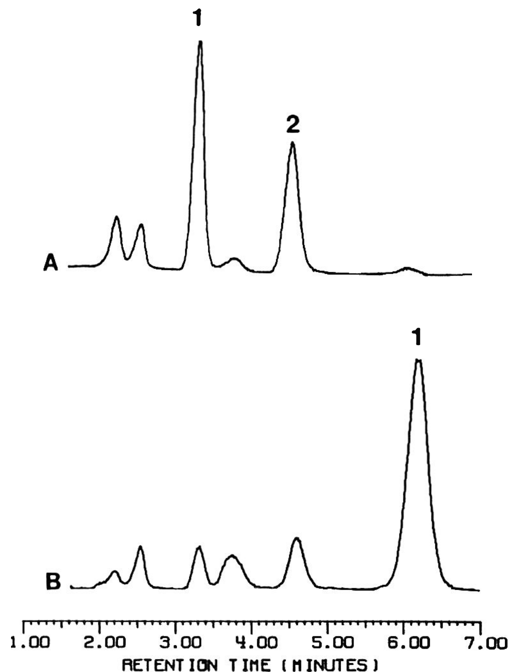

Figure 4. Typical separation obtained from 2 gas chromatograms of a spiked carrot sample produced from the total ion current, where A represents endosulfan I (1) and endosulfan II (2); and B represents endosulfan sulfate (1).

sensitivity but yields fewer data, thus providing less specificity. For compounds containing several chlorine atoms, such as endosulfan, it is desirable to record peaks for selected ions and their isotopes. These isotope clusters provide an additional measure of specificity.

Three scanning methods were investigated: full mass scans, 4 mass ranges, and selected ion recordings. All 3 scanning methods were under computer control, and no difficulties were encountered with any method using the present Finnigan Corp. Revision I software. Based on the investigations, scanning of 4 mass ranges at the specific retention times for each residue (see Figure 4) was selected as the method for confirmation. This method has a specificity comparable to full-scan spectra and a sensitivity comparable to selected-ion recordings. The 3 most abundant fragment ions and the protonated molecular ion for each compound were chosen as suitable for use in confirmation analysis.

Although the technique has been successfully applied only to apples and carrots, the method could readily be adapted to other fruits and vegetables. A minimum detectable level was not investigated in this laboratory; the 0.1 ppm confirmatory level used was sufficient for our purposes because it is 50% of the legal tolerance for these residues in carrots (apples have a higher tolerance). Figure 2 shows a comparison of the full mass spectrum of endosulfan I and a partial mass spectrum of endosulfan I when only the 4 mass ranges are scanned. The full mass spectrum represents endosulfan I standard material, and the partial mass spectrum represents data from a carrot sample to which the pesticide had been added. The GLC-MS data obtained from a confirmation of the 3 compounds added to carrots are presented in Figures 2b, 3, and 4. No measurable response for the 3 endosulfan compounds was obtained from product blanks carried through the entire method.

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Degradation of Methomyl Residues in Frozen Strawberries

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^{14}C-Methomyl (*S*-methyl *N*-[(methylcarbamoyl)-oxy]thioacetimidate) suspended in a commercial formulation was sprayed on strawberries. At 7 and 14 days post-application, mature berries were harvested and chopped. Fresh chopped berries were extracted with methanol using a Polytron homogenizer, and the crop marc was subsequently leached with methanol, freeze-dried, and re-extracted. Portions of the fresh chopped fruit were also frozen at −20°C for 1, 30, and 120 days before extraction with methanol using a Polytron homogenizer, followed by subsequent leaching of the crop marc. For fresh berries, 93.7 and 88.9% of the ^{14}C present at harvest was extractable at 7 and 14 days post-application, respectively. Based on thin layer chromatographic analysis, methomyl represented 83.5 and 68.2% of the ^{14}C extracted from fresh berries at 7 and 14 days, respectively. Freezing of strawberries reduced extraction efficiency and appeared to degrade methomyl. At 7 and 14 days post-application, averages of 80.9 and 77.1%, respectively, of the ^{14}C present were extractable after frozen storage regardless of storage time. Furthermore, only about 10% of the extracted ^{14}C was methomyl; the remainder was of unknown composition.

An important aspect of the analysis of crops for pesticide residues is their stability on or in the harvested crop before analysis. In a review by Kawar et al. (1) several references are given on pesticide degradation in crops and sample extracts during cold storage. Methomyl (*S*-methyl *N* - [(methylcarbamoyl)oxy]thioacetimidate), which is registered for use on a large variety of agricultural crops, is included in the review. Gunther and Westlake (2) reported that methomyl-fortified corn fodder and silage could be stored 4 months at −15°C with no loss of the insecticide. Winterlin (3) stored unchopped samples of corn and tomatoes containing methomyl at 4.5, −17, and −36°C. Corn samples stored at 4.5°C exhibited 18 and 65% loss of methomyl residue after 18 and 35 days of storage, respectively. However, tomatoes stored at 4.5°C did not exhibit any significant losses of methomyl residues. The corn and tomato samples stored at −17 and −36°C were chopped while frozen and then analyzed. These samples showed no loss of methomyl after 111 days of storage.

The stability of field-incurred residues of methomyl as well as other pesticides on chopped samples of mustard greens, radishes, and strawberries is being determined as part of the pesticide extraction efficiency studies being conducted in this laboratory (4).

The purpose of this paper is to present data on the level of methomyl found on chopped strawberries before and after storage at −20°C. In the study reported here, ^{14}C-methomyl was applied to the strawberries in a commercial formulation of the same pesticide to simulate field conditions. The residues were extracted with methanol in a Polytron homogenizer and the extracted ^{14}C was determined.

Experimental

Crop Growth

Florida Bell strawberry plants (acquired from Charles Howard, Agricultural Research Center, Dover, FL) were planted in 19 cm diameter plastic pots containing steam-sterilized soil. The pots were moved into a greenhouse 4 months later. Daily records of water, fertilizer, pest control measures, and temperatures were maintained.

Pesticide Application and Harvest

The ^{14}C-methomyl (*S*-methyl-labeled) was purchased from New England Nuclear Corp., Boston, MA. The identity and purity (99.97%) were verified by thin layer chromatography (TLC), mass spectral analysis, and high performance liquid chromatography. The labeled pesticide was added to formulated material by dissolving the radioactive material (1.0 mCi) in 2-3 mL acetone and adding it to approximately 250 mL water along with Lannate (DuPont) formulation. The mixture was sprayed on the strawberries and surrounding foliage with

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Mention of a pesticide or a commercial product does not constitute a recommendation or endorsement of this product by the U.S. Department of Health and Human Services, Food and Drug Administration (FDA), or the University of Florida. This study was supported in part by FDA Contract 223-76-2220.

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Universal MultiMist aerosol sprayers (No. B-100, Professional Packaging Corp., Chicago, IL) at a rate of 1.30 kg/ha (1.16 lb/acre).

Following pesticide application, the strawberry plants were transferred to growth chambers at 21°C during the 12 h light period and 10°C during the 12 h dark period. The crops were harvested 7 and 14 days post-treatment.

Mature fruit was randomly picked at each harvest and, where necessary, a quantity of untreated strawberries was added to obtain enough crop mass for analysis. The crop was chopped and thoroughly mixed in a Hobart chopper (Model 84141, Hobart Mfg. Co., Troy, OH) and 100 g portions of the chopped sample were weighed into containers. Three portions were immediately extracted and 3 were frozen for later extraction.

Fresh Sample Extraction

The 3 freshly chopped 100 g sample portions were placed in separate 950 mL (32 oz) jars (Tropicana Products, Inc., PO Box 338, Bradenton, FL) for homogenizing with a Model PT-10-35 Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) with a PT 35K generator.

Two hundred mL methanol was added to the jar and the sample was homogenized 30 s at half speed (setting 5) followed by 1 min at full speed (setting 10). The crop and solvent were poured into a 70 mm diameter filter funnel fitted with a 60 mm diameter extra coarse fritted disk covered with a circle of Whatman No. 1 filter paper. The filtrate passed through a vacuum adapter with a variable bore stopcock and was collected in a graduated cylinder, which was also a part of the vacuum system. The homogenizer blades and jar were immediately rinsed with an additional 50 mL methanol, which was added to the solvent in the funnel. The combined filtrates comprised fraction F1. Two hundred mL methanol was then added to the homogenizer jar, and the homogenizer blades were cleaned by blending the solvent 10 s at full speed. This wash was poured over the crop marc and leached under vacuum at ca 5 mL/min. This comprised fraction F2. Three additional 100 mL portions of solvent were added directly to the funnel, leached through the crop marc, and collected as fractions F3, F4, and F5. Two subsequent 25 mL water leaches were combined to form fraction F6. After collection, each fraction (F1–F6) was diluted to a standard volume, and a 20 mL aliquot of each was removed for subsequent quantification. The remaining portions of the fractions were combined in a 2 L round-bottom flask and placed on a rotary evaporator (Rotovapor Model RE, Brinkmann Instruments) to remove the organic solvent from the aqueous component. To assure that radioactivity was not carried over with the solvent distillate, a sample of the distillate was routinely analyzed. The aqueous component of the combined fractions was partitioned three times with 20 mL methylene chloride and the resulting volumes of methylene chloride phase (SP-1) and aqueous phase (AP-1) were recorded. An aliquot of the methylene chloride phase (SP-1) was concentrated and analyzed by TLC.

The crop marc remaining in the filter funnels after the water (F6) fractions were removed was placed in petri dishes and freeze-dried. The residual crop marc was initially frozen (−20°C) and then placed under vacuum on the refrigerated shelves (−40°C) of a freeze-drier (Thermovac Model FDC-8). When the vacuum reached 250 μm, generally after about 4 h, the shelf refrigeration was switched off and the shelf heater was set at 30°C. This caused the chamber pressure to rise, but after 16 h under vacuum, the pressure was again below 300 μm. The residual crop marc was returned to homogenizer jars with 300 mL methanol and homogenized 1 min at full speed in the Polytron. The jar contents were poured into the original filter funnels and the filtrate was collected as fraction FD-1. After filtration, the crop marc was wrapped in filter paper, inserted in a glass extraction thimble fitted with a 45 mm diameter extra coarse fritted disk, and extracted 16 h with 300 mL chloroform–methanol (9 + 1) in a 500 mL Soxhlet extractor. The solvent was collected as fraction S; the residual crop marc was air-dried overnight and then oven-dried at 45°C for 2 days in preweighed beakers to obtain an accurate dry weight. The beaker with dry crop marc was weighed and a small aliquot of extracted tissue was removed for combustion as fraction R.

Frozen Sample Extraction Method

Immediately after chopping, 3 samples were frozen (−20°C) for subsequent extraction with methanol in the Polytron homogenizer after 1 day, ca 30 days, and ca 120 days of storage.

The extraction procedure used for frozen samples was that specified above for fresh samples through the collection of fractions F1–F6 and the partitioning of the combined rotary evaporated extracts resulting in the methylene chloride (SP-1) and aqueous phase fractions (AP-1).

Radioactivity Determination

Liquid samples were prepared for combustion by pipetting 2 mL aliquots into pouches formed with 7.6 cm square pieces of cellophane weighing ca 188 mg, which were cut from rolls of Cello-Flex cellophane (Carolina Biological Supply Co., Burlington, NC). After solvent evaporation, the cellophane was folded and placed in polycarbonate combustion capsules weighing ca 117 mg (Teledyne Intertechnique, No. 59237). The dried crop marc samples (75–300 mg) were weighed directly into combustion capsules. All samples were combusted in duplicate in a IN4101 Oxymat liquid scintillation sample oxidizer (Teledyne Intertechnique); the CO_2 formed was trapped and the $^{14}CO_2$ was quantified using a Packard Model 3375 Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). Note: The ^{14}C recovery of the liquid sample evaporation and combustion procedures averaged 92%. ^{14}C standards of known activity were combusted once every 20 samples and a blank was combusted once every 10 samples to ensure the validity of oxidizer performance.

Thin Layer Chromatography

TLC of extraction and partition fractions was performed on a 250 μm silica gel G layer precoated on 20 × 20 cm glass plates (SIL G-25, Brinkmann Instruments). Samples were applied in 15–17 cm streaks 2.5 cm from the bottom of the plates, using a 250 μL syringe mounted on a TLC streaker (No. 17700, Applied Science Laboratories, Inc., State College, PA). Radiolabeled standards of the parent compound were also spotted at the origin. Plates were developed to 10 cm above the origin in ethyl acetate; after development the plates were air-dried and placed on X-ray films (Kodak No-Screen type NS-ST), generally for 30 days. The developed films were marked and the development heights of the separated labeled components were recorded. The films were aligned with the plates, using radiolabeled ink alignment marks, and 0.5 or 1.0 cm swaths were scraped from the plates where activity was indicated. A 1.0 cm scrape centered 1.5 cm below the origin was always taken to determine radioactivity background levels, and scrapings were always taken from the origin. The scrapings were placed in 22 mL scintillation vials containing 15 mL scintillation cocktail, composed of Aquasol-2 (No. NFF 952, New England Nuclear Corp.) and water (90 + 10).

Results and Discussion

The results show a clear difference between the fresh and frozen strawberries in the amount of extractable methomyl. The major phenomenon observed is a loss of methomyl residue resulting from storage of chopped strawberries under frozen conditions.

The data for extraction of methomyl from fresh strawberries are shown in Table 1. For 7 and 14 day post-application samples, 93.7 and 88.9% of the total ^{14}C was extracted by the blend–wash–leach procedures. The extraction of ^{14}C from strawberries became more difficult with time. This phenomenon was also noted in the extraction of methomyl from radishes and mustard greens (unpublished data) but was distinctly less pronounced.

The extraction data obtained for the chopped strawberries frozen for 1–121 days are shown in Table 2. The extractability of ^{14}C-methomyl residues remained relatively constant whether the sample was frozen for 1 or 2 days or for 4 months. However, the extractability of ^{14}C-methomyl residues from the 7 and 14 day post-application samples decreased from 93.7 and 88.9% for the fresh chopped strawberries to 80.9

Table 1. ^{14}C-Methomyl-treated fresh strawberries extracted 7 and 14 days post-application

Fraction[a]	Radioactivity, cpm[b]	% SD	% ^{14}C[c]	Σ % ^{14}C[d]
7 Days Post-Application				
F1	931 117	7	84.5	84.5
F2	62 398	16	5.7	90.2
F3	15 930	50	1.4	91.6
F4	8 773	25	0.8	92.4
F5	6 947	30	0.6	93.0
F6	7 486	32	0.7	93.7
FD-1	10 697	75	1.0	94.7
S	4 882	14	0.4	95.1
R	53 296	21	4.9	100.0
14 Days Post-Application				
F1	1 280 842	10	77.6	77.6
F2	147 632	24	8.9	86.5
F3	20 334	14	1.2	87.7
F4	9 960	11	0.6	88.3
F5	6 154	11	0.4	88.7
F6	2 930	43	0.2	88.9
FD-1	5 223	18	0.3	89.2
S	4 080	37	0.2	89.4
R	173 471	31	10.6	100.0

[a] Fractions are defined in text.
[b] Mean of 3 replications.
[c] Percent total radioactivity in each fraction.
[d] Cumulative percent radioactivity in each fraction collected at each harvest interval.

Table 2. ^{14}C extracted from methomyl-treated strawberries harvested 7 and 14 days post-application and stored at −20°C

	No. days frozen					
	2		42		121	
Fraction[a]	% ^{14}C[b]	Σ % ^{14}C[c]	% ^{14}C	Σ % ^{14}C	% ^{14}C	Σ % ^{14}C
7 Days Post-Application						
F1	76.5	76.5	64.4	64.4	71.1	71.1
F2	4.2	80.7	12.9	77.2	7.5	78.6
F3	1.1	81.8	1.3	78.6	0.9	79.5
F4	0.4	82.2	0.6	79.1	0.3	79.8
F5	0.3	82.5	0.5	79.6	0.2	80.0
F6	0.2	82.6	0.4	80.0	0.1	80.1
R	17.4	100.0	20.0	100.0	19.9	100.0
	1		35		114	
14 Days Post-Application						
F1	71.3	71.3	67.0	67.0	70.7	70.7
F2	2.0	73.3	6.0	73.0	2.8	73.4
F3	1.3	74.6	1.5	74.4	0.6	74.0
F4	0.5	75.1	0.6	75.0	0.3	74.3
F5	0.6	75.7	0.4	75.4	1.3	75.6
F6	3.5	79.2	0.3	75.7	0.8	76.4
R	20.8	100.0	24.3	100.0	23.6	100.0

[a] Fractions are defined in text.
[b] Percent total radioactivity in each fraction.
[c] Cumulative percent radioactivity in each fraction collected at each harvest interval.

Table 3. Characteristics of ^{14}C extracted from fresh and frozen strawberries

Days post-application	Days frozen	% ^{14}C extracted	% extracted ^{14}C soluble in methylene chloride (SP-1)	% ^{14}C in methylene chloride present as methomyl
Fresh				
7	—	94.5	83.5	98
14	—	98.5	68.2	98
Frozen				
7	2	82.6	26	—[a]
	42	80.0	16	57
	121	80.1	—[a]	63
14	1	79.2	41	—[a]
	35	75.7	26	63
	114	76.7	—[a]	77

[a] Data not available.

and 77.1%, respectively, for the chopped strawberries stored at −20°C after a variable storage period.

Table 3 presents a further comparison of ^{14}C-methomyl extractability data for fresh and frozen strawberries. Of particular importance are the data describing the percentage of ^{14}C which was methylene chloride-soluble and the percentage of that which was intact methomyl. For fresh strawberries 83.5% of the extractable ^{14}C partitioned into methylene chloride (SP-1) and 98% of the ^{14}C in the methylene chloride layer was methomyl at 7 days post-application; at 14 days, 68.2% of the extractable ^{14}C was in the methylene chloride layer and 98% of that was parent insecticide. The data for frozen strawberries reveal that the methomyl residues have undergone substantial changes. As described above, less ^{14}C was extractable after freezing, but the most dramatic change was in the percentage of ^{14}C that was methylene chloride-soluble. For the 7 day post-application samples, 26 and 16% of the extracted ^{14}C partitioned into the methylene chloride layer (SP-1) after being frozen for 2 and 42 days, respectively. For the 14 day post-application samples, 41 and 26% of the extracted ^{14}C partitioned into the methylene chloride when samples were frozen for 1 and 35 days, respectively.

The 7 and 14 days post-application fresh strawberry samples contained 3.0 and 1.9 ppm methomyl, respectively. Freezing of the samples for 5-6 weeks resulted in methomyl residues of 0.1 (7 day samples) and 0.4 ppm (14 day samples). This represents methomyl losses of 97% for the 7 day and 80% for the 14 day frozen samples.

Losses of radioactivity during frozen storage were small. A mean of 91% of the ^{14}C present in fresh strawberries was detected in the frozen samples. This degradation process does not occur during the freezing of radishes (unpublished data) but whether or not this is a widespread occurrence is uncertain. Available data describing storage stability of methomyl on crops indicate great stability of methomyl on frozen crops (3, 4). Further research is needed to determine if the loss of methomyl occurs during freezing and/or thawing of the sample, and if this loss occurs with the whole fruit when frozen.

Unsuccessful efforts were made to identify the methomyl degradation products. TLC showed the presence of only 2 components. One of these exhibited an R_f identical to that of methomyl; the other remained at the origin. The degradation product was not methomyl oxime because standards of this material exhibited a

relative R_f (compared to methomyl) of 1.32–1.35.

The loss of methomyl from frozen chopped strawberries is of analytical significance. Residue analyses are sometimes conducted on harvested crops that have been frozen before analysis, and the level of residue found on the frozen crop is assumed to be that which was on the fresh crop. However, from the findings of this study, the level of methomyl found on frozen strawberries would not reflect the actual level of methomyl present on the fresh fruit. Therefore, to accurately assess the methomyl level present on fresh strawberries, it is essential that samples not be frozen before analysis.

Acknowledgments

The authors thank Linda Cornwell, Laura Kennedy, and Nora Maddox for technical assistance.

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Gas-Liquid Chromatographic Method for Determining Oxamyl in Peppers, Tomatoes, and Cucumbers

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A simple and fast gas-liquid chromatographic method has been developed to determine residues of oxamyl insecticide in peppers, tomatoes, and cucumbers. Following sample extraction with ethyl acetate and cleanup on an alumina column, the compound is determined using a thermionic nitrogen-phosphorus detector. The limit of detection is 0.02 ppm based on a 40 g sample. Average recoveries for oxamyl at 0.025-1.0 ppm for all 3 products were 88-97%.

Oxamyl is the approved common name for methyl *N′,N′*-dimethyl-*N*-[(methylcarbamoyl)-oxy]-1-thiooxamimidate; it is the active ingredient in Du Pont's Vydate insecticide/nematicide. The compound is recommended for use on cucumber, cantaloupe, and honeydew melon for the control of leafminers and nematodes, and on tobacco for control of root knot and lesion nematodes (1).

Several methods for determining oxamyl have been reported. Holt and Pease (2) determined oxamyl as an oximino fragment (methyl *N′,N′*-dimethyl-*N*-hydroxy-1-thiooxamidate) obtained by alkaline hydrolysis. Following several cleanup techniques before and after the hydrolysis step, the compound (oxime) was re-extracted in ethyl acetate and quantitatively determined by gas-liquid chromatography with a flame photometric detector in the sulfur mode. Other techniques using spectrophotometry for oxamyl derivative determination (3) or liquid chromatography (4) for quantitation of oxamyl, both with a limit of detection of 2.0 ppm, have been reported. A liquid chromatographic method for determining oxamyl in citrus leaves was developed by Davis et al. (5). The metabolism of oxamyl in plants, soil, and water was investigated by Harvey et al. and the data obtained were reported in several recent papers (6-8).

In our attempts to determine oxamyl residues by the method described by Holt and Pease (2), major problems were encountered in the final gas-liquid chromatographic (GLC) determination step. We could not detect less than 50-60 ng oximino fragment with the flame photometer detector, and this could not ensure a satisfactory limit of detection.

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The aim of the present study was to develop a rapid and sensitive method for routine analysis, which enables the quantitative determination of oxamyl as such in a more convenient way, and without loss of efficiency and accuracy. In the procedure described, a thermionic nitrogen-phosphorus detector was used for quantitation of oxamyl eluted from the alumina column used to clean up the crude ethyl acetate extract.

METHOD

Apparatus

(a) *Gas chromatograph.*—Tracor Model 560, equipped with Model 702 N-P thermionic detector and 6 ft × 2 mm id coiled glass column packed with 3% Carbowax 20M on 80–100 mesh Chromosorb WHP. Operating conditions: column 170°C, injection port 200°C, detector 250°C, nitrogen 25 mL/min, hydrogen 3.2 mL/min, air 120 mL/min, electrometer setting 8×10^{-11} FSD. Tracor TD-10 recorder; chart speed 0.25 cm/min.

(b) *Rotary evaporator.*—Heidolph.

(c) *Centrifuge.*—BHG-Optima II. Capable of accommodating 250 mL tubes.

(d) *Centrifuge tubes.*—250 mL.

(e) *Chromatographic column.*—Glass 30 × 0.75 cm id with capillary opening and funnel at top.

Reagents

(a) *Solvents.*—Glass distilled ethyl acetate, petroleum ether (bp 40–60°C), and analytical grade acetone, (Chemical Laboratory, Haifa, Israel).

(b) *Aluminum oxide neutral.*—Fluka type 507 C. Heat 4 h at 425°C, cool, and add water (10%), with shaking. Let equilibrate 24 h.

(c) *Oxamyl standard.*—99%. Biochemical Dept, E.I. Du Pont de Nemours and Co., Wilmington, DE.

Stock solution.—1 mg oxamyl/mL ethyl acetate. *Working solutions.*—1, 2, and 5 μg oxamyl/mL ethyl acetate.

Procedure

Extraction for peppers, tomatoes, and cucumber.—Weigh and cut into small pieces 400 g each

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Figure 1. Chromatogram of 25 ng oxamyl standard.

of the 3 products (only edible part for pepper), add 15% ethyl acetate, and macerate 20-30 s in Waring blender to provide representative sample.

Using an aliquot corresponding to 40 g plant material, add 100 mL ethyl acetate and blend 4 min at high speed. Transfer mixture quantitatively to 250 mL centrifuge tube and centrifuge 10 min at 1500 rpm.

Carefully decant ethyl acetate solution, dry through sodium sulfate contained in funnel, and collect in 500 mL round-bottom flask. Repeat extraction using 100 mL ethyl acetate (4 min). Centrifuge, and combine extracts.

Evaporate ethyl acetate solution almost to dryness by rotary evaporator (40°C), add ca 10 mL petroleum ether, and evaporate again (almost to dryness). Add 5 mL petroleum ether for column chromatographic step.

Cleanup for peppers, tomatoes, and cucumber.— Prepare chromatographic tube by adding, in order: small plug of glass wool, 5 g alumina, and 1.5 cm sodium sulfate. Tap tube (with wooden rod) to settle adsorbent. Quantitatively transfer petroleum ether extract to dry column by rinsing flask with five 2 mL portions of 20% acetone in petroleum ether. (If the rate of elution is too slow during washings, increase it by exerting slight pressure by placing glass stirring rod on sodium sulfate layer.) Start eluting column by adding 40 mL acetone-petroleum ether (20 + 80).

Table 1. Recovery of oxamyl added to crops

Crop	Added, ppm	No. of samples	Av. rec., %	SD
Peppers	1.0	3	94	5
	0.5	3	96	5
	0.2	3	93	3
	0.1	3	92	2
	0.05	3	92	6
	0.025	3	90	5
Tomatoes	0.5	3	97	2
	0.2	3	92	2
	0.05	3	92	6
	0.025	3	88	2
Cucumbers	1.0	4	94	6
	0.1	3	93	7
	0.025	3	88	2

Maintain flow rate of 2-3 mL/min. Change receiving flask (100 mL), discard eluate, and add 50 mL acetone-petroleum ether (60 + 40) to elute pesticide. Evaporate solvent of second eluate almost to dryness (rotary evaporator, 40°C), and dissolve residue in ethyl acetate for GLC determination.

Experimental

All samples were analyzed under operating conditions described. Retention time for oxamyl was about 8 min.

Peak height method was used for quantitation. Injections of unknown and fortified samples were bracketed between appropriate standard injections. Peak heights were measured from baseline at rising point to peak top. Range of peak heights was ca 4-25% full scale for 5-30 ng oxamyl standard, and noise level was less than 1%. Peak heights of samples and standards did not vary by more than 5-6%.

A large number of samples cleaned up as described above could be injected consecutively without any decrease in characteristics of sensitivity and peak resolution. If different sample extracts with less efficient cleanup precede injections of oxamyl samples, sensitivity diminishes (peak is decreased and tailing occurs). Changing glass wool in column considerably improves response to oxamyl.

Results and Discussion

The method described requires only 2 steps (extraction and cleanup) in addition to quantitation. Both steps are simple and rapid, and use a minimal volume of organic solvent. Eight samples can be analyzed in one day.

The recovery of oxamyl added to plant material was investigated for tomatoes, peppers, and cucumbers. Oxamyl was added at levels between 0.025 and 1.0 ppm in the first extraction

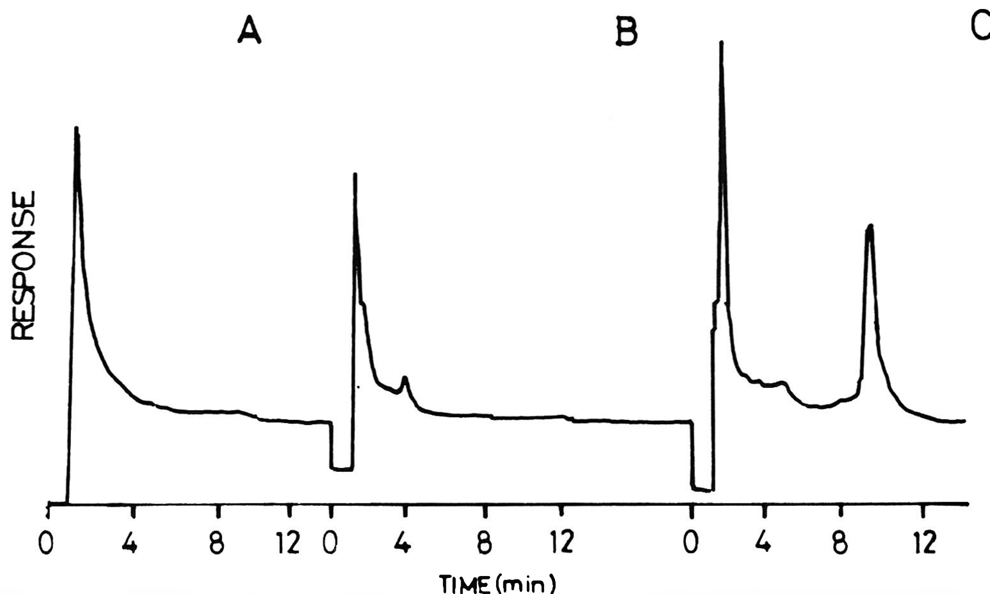

Figure 2. Chromatograms of (A) extract equivalent to 160 mg pepper; (B) extract equivalent to 213 mg pepper; (C) extract equivalent to 213 mg pepper, fortified with 0.1 ppm oxamyl.

step. The average recovery was 90–96% for pepper, 88–97% for tomatoes, and 88–94% for cucumbers (Table 1). Typical chromatograms of standard, untreated products, and samples fortified with oxamyl are shown in Figures 1–4.

The efficiency of the extraction step was checked and it was found that 2 successive extractions with ethyl acetate were sufficient. A third extraction did not change the quantity of oxamyl residue obtained from either spiked or field-treated samples. Figure 5 shows 2 chromatograms of the same homogenate of pepper taken from an agricultural field trial (application: 480 g AI/ha, sampled on the day of the last spraying) and prepared using 2 ethyl acetate extractions for one of the homogenates (Figure 5A) and 2 for the other (Figure 5B). No difference in residue levels was found. The chromatogram obtained for a large injection (8 μL/0.3 mL) of a third, separate extraction of sample shown in Figure 5A did not show any peak at the retention time of oxamyl.

The cleanup method described is efficient enough to allow injection of a large equivalent amount of plant extract. When 8 μL is injected from a final concentrate of 2.0 or 1.5 mL (equivalent to 160 or 210 mg plant material, respectively), none of the control samples (pepper, cucumber, and tomatoes) showed a peak. Their chromatograms (Figures 2A, 3A, 2B, and 4A) are similar to those of the concentrated solvent. The limit of detection is 0.02 ppm in all 3 products.

The first fraction in the cleanup step removes interferences from the plant extracts and separates the oxamyl from other compounds when samples treated with various pesticides are analyzed. Under such circumstances this technique can be used as a multi-detection method for a number of organochlorines and organophosphates extracted with ethyl acetate and eluted in the 20% acetone-in-petroleum ether fraction as described above. The method has so far been tested only with a limited number of representative compounds of these 2 groups. Recovery tests were carried out in tomato and pepper samples for the organochlorines aldrin, dieldrin, endrin, and endosulfan and for the organophosphates dursban, acetellic, diazinon, parathion, ethion, malathion, and Supracide.

For all these compounds that eluted in the first 20% acetone-in-petroleum ether fraction, an additional alumina column with less polar solvents (or other conventional methods) is needed for their gas chromatographic (electron capture or flame photometric detector) determination.

In the method using alumina cleanup, the first eluate extract (free of acetone) was transferred to the column and 2 fractions were collected: aldrin, dieldrin, endrin, and endosulfan I were eluted in the first fraction using 50 mL petroleum ether as eluting solvent, and in the second fraction (3% acetone in petroleum ether), endosulfan II, diazinon, parathion, acetellic, malathion, Supracide, and ethion were eluted. The recovery

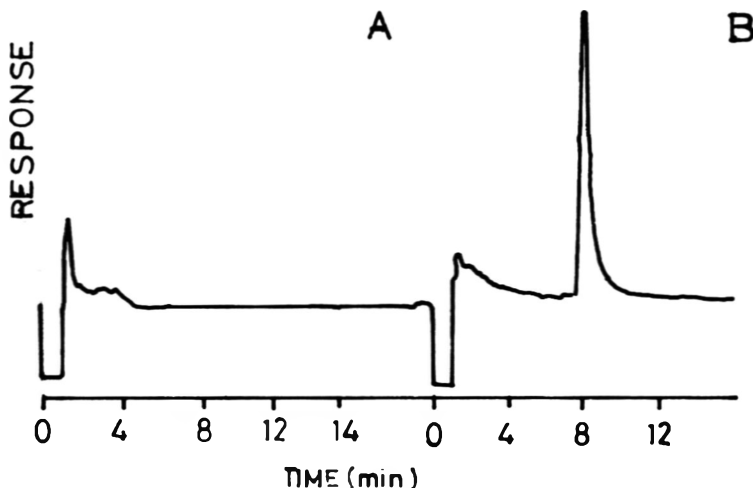

Figure 3. Chromatograms of (A) extract equivalent to 160 mg tomatoes; (B) extract equivalent to 50 mg tomatoes, fortified with 0.5 ppm oxamyl.

values obtained for all these compounds exceeded 90% at the 0.025–0.05 ppm level for organochlorines and at the 0.05 ppm level for organophosphates.

A limit of detection of 0.01 ppm for organochlorines and 0.02–0.03 ppm (depending on the compound) for organophosphates can be expected. (A glass column packed with 2% XE-60 on 60–80 mesh Supelcoport was used for GLC-EC determination and a column packed with 5% SE-30 on 60–80 mesh Gas-Chrom Q was used for FPD determination.)

Other cleanup methods such as that using a Florisil column (4 g) with the same eluting solvents or a mixture of charcoal and Celite (2 g; 4 g) with ethyl acetate were investigated for determining oxamyl in peppers and tomatoes. The pesticide was eluted quantitatively, but an un-

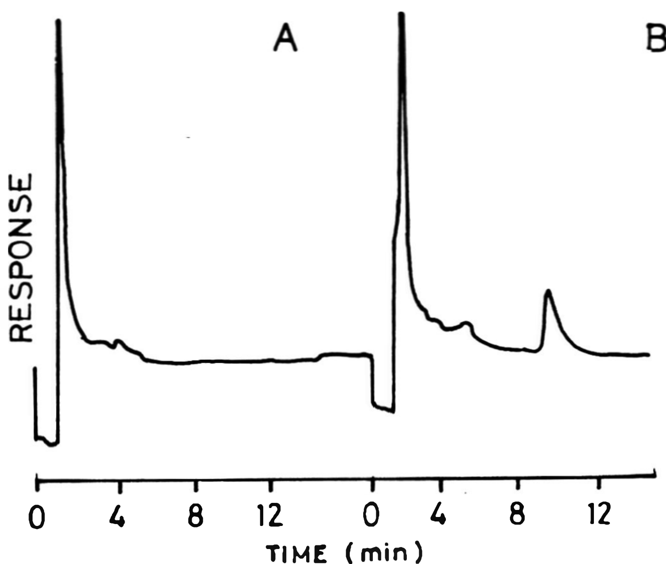

Figure 4. Chromatograms of (A) extract equivalent to 213 mg cucumber; (B) extract equivalent to 213 mg cucumber, fortified with 0.025 ppm oxamyl.

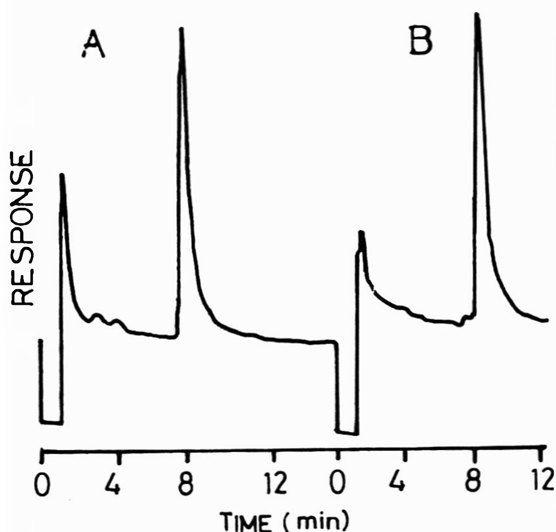

Figure 5. Chromatograms of (A) sample prepared using 2 ethyl acetate extractions, extract equivalent to 213 mg pepper (treatment: 480 g AI/ha, sampled on the day after the last application); (B) duplicate of sample 5A prepared using 3 ethyl acetate extractions, extract equivalent to 213 mg pepper.

known compound in the chromatograms of control samples, with a retention time close to that of oxamyl, was difficult to separate.

A modified procedure for residue analysis of one of the oxamyl metabolites, the oximino fragment (see introduction), in samples of peppers is in progress.

The method described above for oxamyl residues is characterized by its simplicity. This is due to the direct determination of oxamyl itself, to the efficiency of the alumina column which ensures clean samples and high recovery, and to the relatively good response of the nitrogen-phosphorus detector to the oxamyl compound.

Acknowledgment

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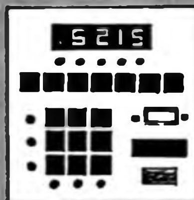

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High Performance Liquid Chromatographic Determination of Cyanuric Acid in Human Urine and Pool Water

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A reverse phase high performance liquid chromatographic (HPLC) assay for the quantitative determination of cyanuric acid (CA) in urine and water is described. For purification, samples are passed through a pre-activated reverse phase C_{18} column. The effluent is dried by lyophilization, and the residue is reconstituted in hexane-washed water and then passed through a prewashed Dowex-1 column. The effluent is again dried by lyophilization, and the dry residue is extracted with hot dioxane. The solution is cooled to ambient temperature and centrifuged. The supernatant liquid is removed, dried under a nitrogen steam, and dissolved in water for final extraction by reverse phase chromatography. This effluent is dried, dissolved in the sodium phosphate monohydrate in methanol (pH 7.0) mobile phase, and injected into a pre-equilibrated chromatographic system. An external standard is used for quantification by peak height comparison. A sample of HPLC column effluent is collected, dried, dissolved in methanol, and used for mass spectrometric confirmation by a solid probe insert procedure. Average combined recovery determined at 1.0, 5.0 and 10.0 μg CA/mL is 103 ± 3% with an average coefficient of variation of 8.6%. Standard deviations for the 3 concentration levels are 0.04, 0.58, and 0.76, respectively, with average precisions of 4.28, 10.92, and 7.61%. The limits of detection are approximately 0.05 μg/mL for urine and 0.1 μg/mL for swimming pool water. Recorder response to CA is linear over the concentration range 1-10 μg/mL.

Cyanuric acid was first synthesized by Scheale in 1776, but did not come into general use until the late 1950s as a swimming pool chlorinator, stabilizer, and bleaching agent (1). The effective concentration range for cyanuric acid was eventually established as 25–100 ppm (2, 3), and its use as a pesticide began to be monitored in 1959. Possible health effects resulting from exposure to cyanuric acid prompted this study. Several investigators have become concerned about the potential toxicity and carcinogenicity of the compound, particularly because of its widespread use as a pool water bactericide. Over the past few years, several methods have been published for the separation, and in some cases quantification, of s-triazine pesticides in reference standards and in various biological materials. The methods have included paper (4, 5), thin layer (6, 7), gas-liquid (8, 9), and high performance liquid (HPLC) chromatography (10–14). None of the reported procedures has been directed towards extracting and quantifying CA in swimming pool water or in the urine of exposed swimmers. Only two have dealt with CA directly; most have been concerned with either s-triazine or derivatized s-triazine pesticides. The procedure reported here allows the quantitative extraction and determination of cyanuric acid from human urine by reverse phase HPLC. No extraction step is required to analyze swimming pool water by this procedure.

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METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—With Model U6K universal injector and Model 6000A solvent delivery system attached to Model 450 variable wavelength detector (Waters Associates, Milford, MA) and Model LD11B strip chart recorder (Westronics Inc., Fort Worth, TX).

(b) *HPLC column.*—μBondapak C_{18} (Waters Associates).

(c) *Syringe.*—10 μL, Model 820 (Hamilton Corp., Reno, NV).

(d) *Elution solvent.*—0.005M dibasic sodium phosphate monohydrate (Mallinckrodt Chemical Co., St. Louis, MO) in 5% methanol (95% water; adjusted to pH 7.0 with 1N HCl) filtered through 0.2 μm Millipore filter under reduced pressure. Buffer solution was always degassed before use and all water was deionized. Nanograde® methanol was obtained from Mallinckrodt Chemical Co.

(e) *Gas chromatograph/mass spectrometer.*—Finnigan Model 4000 GC/MS unit interfaced with Incos data system (Finnigan Corp., Sunnyvale, CA).

(f) *Lyophilizer.*—Virtis Model 10-100 Uni-Trap (Virtis Co., Gardner, NY).

(g) *Extraction components.*—Sep-Pak® C_{18} cartridge (Waters Associates) for reverse phase extraction step. 5 × 25 mm disposable glass chro-

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matographic columns packed with Dowex®-1 (Chloride form), 50–100 dry mesh (Sigma Chemical Co., St. Louis, MO), previously washed and regenerated into formate form with 0.010M formic acid. Pesticide grade dioxane (Burdick and Jackson Laboratories, Inc., Muskegon, MI).

(h) *Stock solution.*—300 μg cyanuric acid (Eastman Kodak Co., Rochester, NY)/mL elution solvent.

(i) *Reference solutions.*—50.0, 10.0, 5.0, 2.0, and 1.0 μg CA/mL. Serially dilute CA stock solution with elution solvent to obtain concentrations shown.

(j) *Wash solvent.*—Wash test tubes with Nanograde hexane (Mallinckrodt).

Urine Extraction

Successively wash Sep-Pak C_{18} cartridge with 5 mL methanol, 10 mL freshly deionized water, and 5 mL methanol to ensure cleaning and activation of cartridge. Use 1 mL disposable syringe to transfer 1 mL undiluted urine to washed cartridge, followed by 0.5 mL deionized water. Collect both fractions in clean, dry, hexane-washed test tube. Dry effluent by rotary evaporation at 80°C under 5 μm vacuum (or by lyophilization). Reconstitute dry sample in 1 mL deionized water and pass it through 5 × 25 mm column containing Dowex-1 resin which has been prewashed with 10 mL 0.010M formic acid. To ensure quantitative elution, pass 0.5 mL deionized water through column. Collect the 2 effluents in dry, hexane-washed 13 × 100 mm test tube and dry by lyophilization. Add 3 mL dioxane (80°C) to the dry residue, and maintain mixture 1 h at 80°C, vigorously mixing (vortex) solution 3–5 times during this period. Then centrifuge sample 5 min at 10 000 × g at ambient temperature to remove particulate matter. Transfer supernatant solution to clean 13 × 100 mm test tube, dry at 37°C with aid of gentle nitrogen stream, and re-extract with 3 mL hot (80°C) dioxane. Caution: Dioxane is suspect carcinogen; carry out drying in well ventilated fume hood. Prepare a second Sep-Pak cartridge as described above. Reconstitute sample in 1.0 mL deionized water, and pass solution through the pre-washed cartridge. Dry eluate by rotary film evaporation or under nitrogen stream at 37°C. Dissolve dry residue in 200 μL elution solvent and inject 5 μL into pre-equilibrated HPLC system and analyze as described.

Mass Spectrometry

Collect a 0.1 mL aliquot of the HPLC column effluent and dry it at 37°C under a nitrogen stream. Reconstitute this sample in 50 μL methanol, and transfer 5 μL to clean solid-probe sample tube for MS confirmation of CA. After solvent has been completely evaporated by gently warming solid probe tip (25–45°C), insert probe into mass spectrometer at ambient temperature, then program to 250°C at 10°/min. From start of temperature program, data system scans for spectra every 2 s, resulting in collection of 200 scans. Cyanuric acid is identified as that component with maximum response at scan 133. Standard MS operating parameters include: 70 eV electron energy, 9.5×10^{-8} torr vacuum, and 1500 mV emission voltage.

Column and Buffer Test

Test elution pattern of a CA reference solution daily and after each new buffer preparation to ascertain proper column performance and assure integrity of buffer solution.

Chromatography of Cyanuric Acid

When 10 μL reference solution (1 μg/mL) is injected into pre-equilibrated HPLC system, relative retention time of 9.75 ± 0.33 min is obtained. Adjust sensitivity of recorder to obtain response of 50% full scale. Conditions: flow rate 0.3 mL/min at 300 psi; chart speed 4 mm/min; AUFS 0.04 (0.004 absorbance unit full scale).

Results and Discussion

Reproducibility

The reproducibility of the HPLC analysis was determined by injecting 5.0 μL aliquots of a standard solution of CA (2 μg/mL) 10 times. These injections resulted in a 50% recorder response. The resulting peak heights varied from 136.6 to 138.2 mm with a variance of 0.3 mm and a standard deviation of 0.6 mm.

Extraction Efficiency

Urine from 10 volunteers having no known exposure to cyanuric acid (chromatograms were negative) were spiked with a known amount of reference CA. The samples were extracted as outlined above. Recovery data obtained from analyzing ten 1 mL urine samples containing 10 μg cyanuric acid included an average recovery of 9.98 μg, indicating an average recovery of 99.8 ± 0.5% and a coefficient of variation (CV) of 7.6%.

Figure 1 is a representative pair of chromatograms of urine samples carried through the entire extraction procedure and analyzed as de-

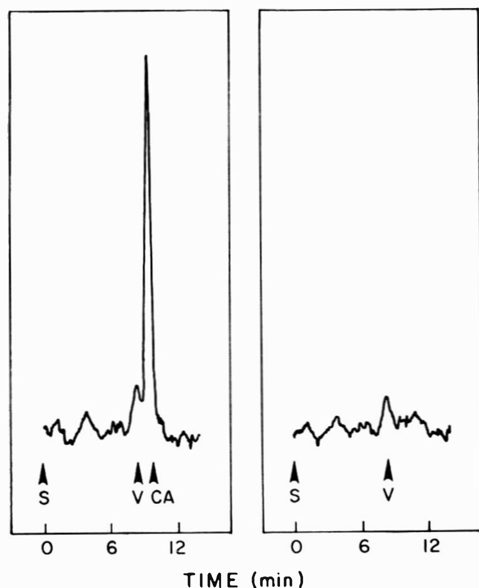

Figure 1. Representative chromatograms obtained for 2 aliquots of urine carried through the extraction procedure and HPLC analysis described. No cyanuric acid was added to the sample on the right; 10 μg CA had been added to the other aliquot. Start (S) of the chromatograms, void volume (V), and cyanuric acid component (CA) are indicated.

scribed. Two samples of the same urine were used; the sample on the left contained 10 μg CA. The start (S) of the chromatograms, void volumes (V), and cyanuric acid component (CA) are indicated on the charts.

Reference Standard and Instrument Calibration

Occasionally the resolution of CA from other urinary components was obscured. In these instances, between 1 and 10 μg of CA was added to the sample and a second analysis was performed. This procedure facilitated measurement of the recorder response obtained for the CA component and allowed differential quantitation of the material.

Calculations

Quantification of CA levels was accomplished by measuring recorder responses and following the peak comparison, external standard method discussed by Cieri (11).

Analysis at Other Concentrations

After establishing the reproducibility of the method using 10 different urine samples spiked with 10 μg CA, 5 series of ten 1 mL aliquots, all

Table 1. Recovery of cyanuric acid added to urine [a]

Added, μg	Rec.,[b] μg	SD	CV, %	Range, μg
1.00	1.02	0.04	4.28	0.10
2.00	2.20	0.20	10.00	0.11
5.00	5.31	0.58	10.92	0.45
7.00	6.90	0.69	10.00	0.78
10.00	9.98	0.76	7.61	0.94

[a] CA added to ten 1-mL aliquots of urine at each level. Urine for this recovery study was from one urinary collection.

[b] Average of 10 determinations.

from one urinary collection, were fortified with CA as shown in Table 1 and the reported data were obtained. The correlation coefficient between the known and observed value was 0.999 with a slope of 0.982 ± 0.024 and an intercept of 0.173 ± 0.45. The same curve, when forced through zero, gave a correlation coefficient of 0.999 and a slope of 1.001 ± 0.014.

The overall cyanuric acid recovery for the entire series was 103 ± 3% (SD = 0.45). Over the 1–10 μg range studied, the standard deviation was 0.45 μg, and the average precision was 8.56%. Linear regression analysis over this range yielded a slope of 1.06.

Special Considerations

Although maximum absorbance for CA occurs at 213 nm (pH > 7.0), acceptable absorption (60%) occurs as high as 225 nm. Analysis at 225 nm often circumvents a number of otherwise interfering components. Use of an increased wavelength also increases baseline stability and component resolution which are, at times, nonideal.

No correlation was apparent between such urinary parameters as hematuria, ketonuria, and glucosuria and the atypical chromatograms obtained when analyzing urinary collections from very young children, 1–3 years of age. In these cases, lowering the column flow rate did not improve resolution. Addition of a second column in series with the first did increase both resolution and analysis time. Addition of a third column was of little benefit.

Mass Spectral Analysis

Solid probe insert analysis was used to confirm the identity of the component labeled CA in Figure 1. Figure 2 is a representative mass spectrum of cyanuric acid collected from a spiked urine sample. The large molecular ion occurring at m/z = 129 represents the intact compound. The fragment at m/z = 86 probably represents

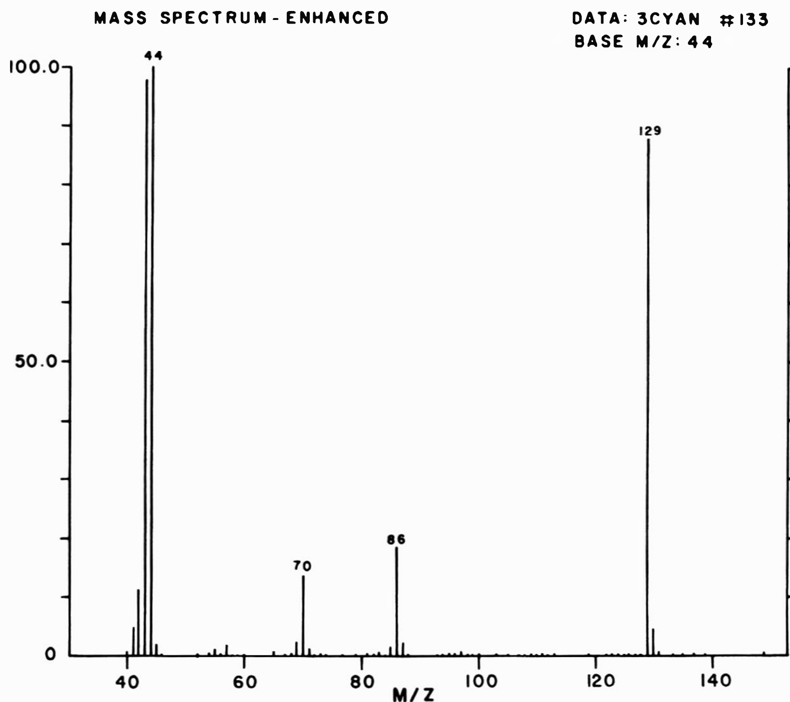

Figure 2. The component labeled CA in Figure 1 was collected and analyzed by solid probe insert mass spectrometry to yield this normalized fragmentation pattern. See text for discussion.

the $C_2H_2O_2N_2^+$ ion which can expel oxygen to yield the fragment appearing at m/z = 70. The base ion occurring at m/z = 44 is most likely the protonated form of the fragment occurring at m/z = 43, $CHON^+$.

Future Studies

This method was specifically developed to study both the concentration of CA in the urine of swimmers and in water from a group of privately treated swimming pools located in Dade County, FL. Figure 3 is a chromatogram of a urine sample of a long-distance swimmer, showing a urinary CA level of 8.8 μg/mL. This level was detected in the first void (21 mL) collected after swimming 2 h in a pool containing 29.9 μg CA/mL. No CA was detected in urine collected just before the swim.

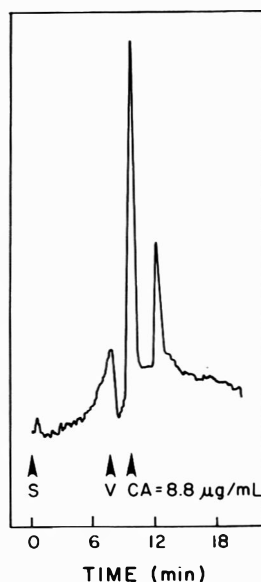

Figure 3. Chromatogram of urine from a long-distance swimmer, containing 8.8 μg/mL free cyanuric acid. No CA was detected in urine collected just before the swim.

Acknowledgments

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Rapid Cleanup Procedure for Gas-Liquid Chromatographic Determination of Chlorpyrifos-Methyl Residues in Cat Food

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A simple and rapid gas-liquid chromatographic (GLC) method was developed for the determination of residues of chlorpyrifos-methyl in dry cat food. Cat food fortified with chlorpyrifos-methyl was extracted with acetone and cleaned up on a modified acetonitrile-on-Florisil partitioning column. The chlorpyrifos-methyl was determined by GLC with a flame photometric detector. Results were compared with those for known standards that had undergone the same cleanup procedure. The quantitative electronic integration limit of determination was 0.03 ppm. Average recoveries for 5 analyses at fortification levels from 0.052 to 51 ppm ranged from 90 to 102% and averaged 97%.

Chlorpyrifos-methyl (*O,O*-dimethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate), also known as Dowco 214, Reldan, OMS 1155, and USDA ENT 27520, is an organophosphate compound with a broad spectrum of insecticide activity. The compound kills insects on contact or by ingestion, but is low in mammalian toxicity. It is relatively stable to hydrolysis under neutral conditions at room temperatures. The promising qualities of chlorpyrifos-methyl have stimulated interest in its use as a protectant against insect damage to packaged dry cat food. However, before this apparent potential could be fully evaluated, a method was needed for the quantitative determination of residues of chlorpyrifos-methyl in dry cat food, a commodity with moderate to high levels of fat. This paper describes a rapid and efficient cleanup procedure for a gas-liquid chromatographic (GLC) determination of chlorpyrifos-methyl residues in dry cat food.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard Model 5750 (Hewlett-Packard Co., Avondale, PA 19311) equipped with Model 100AT flame photometric detector (Meloy Laboratories, Inc., Springfield, VA 22151), Model 7670A automatic sampler, Model 3370B digital electronic integrator (Hewlett-Packard Co.), and 1 mV recorder. 122 cm × 4 mm id glass column packed with mixed liquid phase of 2% HI-EFF-8AP (cyclohexanedimethanol adipate) plus 8% OV-101 (methyl silicone) on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA 16801). Column was conditioned overnight at 250°C with nitrogen purging. Operating conditions: flow rates (mL/min)—nitrogen (carrier), 225; hydrogen, 200; oxygen, 25; temperatures (°C)—injection port, 310; detector, 220; column oven, 225.

(b) *Solvents and solutions.*—Acetonitrile, acetone, and pentane (all pesticide grade); diethylene glycol and 0.00025% tributyl phosphate (Fisher Scientific Co., Pittsburgh, PA 15219) (w/v)-in-acetone solution.

(c) *Florisil-PR.*—60–100 mesh (Floridin Co., Pittsburgh, PA 15235).

(d) *Analytical pesticide standards.*—Chlorpyrifos-methyl (99.2%) analytical grade (Dow Chemical Co., Midland, MI 48640).

(e) *Chromatographic column.*—Chromaflex, 30 cm × 10 mm with 8 cm × 3 mm bore capillary tip, 50 mL reservoir (Kontes Glass Co., Vineland, NJ 08360).

Preparation and Extraction of Sample

Grind cat food to fine consistency in blender and transfer 50 g thoroughly mixed sample to 300 mL Erlenmeyer flask fitted with ground glass stopper. To test efficiency of cleanup procedure, fortify with known concentrations of chlorpyrifos-methyl solutions. Add 150 mL acetone and stopper, and then shake on wrist-action mechanical shaker for 3 h. Filter extract through Whatman 2V paper into 4 oz bottles and stopper. Store extracts in freezer at −5°C until ready for further analysis or proceed by transferring 30 mL aliquot (equivalent to extract from 10 g cat food) to 50 mL Erlenmeyer flask. Place flask on 60°C water bath under gentle stream of dry nitrogen and evaporate all traces of acetone extraction solvent. Oily residue that remains is now ready for chromatographic cleanup.

This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the U.S. Department of Agriculture nor does it imply registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) as amended. Mention of a commercial or proprietary product does not constitute an endorsement of this product by the USDA.

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Table 1. Average recoveries [a] of chlorpyrifos-methyl from dry cat food fortified before extraction

Added, ppm	Found, ppm	Rec., %	Std dev.
0.0	<0.03	—	—
0.052	0.047	90	0.0098
0.10	0.10	100	0.013
0.52	0.52	100	0.029
1.0	1.0	100	0.10
1.5	1.4	93	0.12
5.1	5.0	98	0.36
10	9.4	94	0.79
51	52	102	0.81

[a] Each value is the average of 5 analyses. Minimum amount detected, based on a peak twice the noise level, was <0.03 ppm.

Chromatographic Cleanup

Pack chromatographic column with glass wool plug and 15 cm Florisil-PR (ca 7.5 g Florisil). Tap sides of column to produce even packing of adsorbent. Secure packing by placing small plug of glass wool on top. Add 15 mL acetonitrile saturated with pentane, followed by 15 mL pentane saturated with acetonitrile. Let eluate flow into 150 mL beaker at ca 50–60 drops/min. When level of solvent reaches ca 1–3 mm above packing, dissolve oily sample residue contained in 50 mL Erlenmeyer flask in 2 mL pentane saturated with acetonitrile. Quantitatively transfer to column and rinse with 3 successive 2 mL portions of pentane saturated with acetonitrile. After sample and rinses have been added to column, elute column with 12 mL pentane saturated with acetonitrile. Collect eluate in same 150 mL beaker and discard. Place 125 mL Erlenmeyer flask under column, elute column with 35 mL 5% acetone-in-pentane, and collect eluate. Add 1 drop diethylene glycol (keeper) to eluate and evaporate all traces of solvent on 60°C water bath under gentle stream of nitrogen. Dissolve residue in ca 2 mL acetone solution containing 0.00025% tributyl phosphate and transfer to calibrated centrifuge tube or volumetric flask of appropriate size for diluting residue to desired concentration of 5–20 μg/mL. Dilute to volume with acetone solution containing 0.00025% tributyl phosphate. Fill 2 mL vial ca ⅔ full with portion of sample. Cap vial with aluminum septum cap and store in freezer at −5°C until ready for GLC analysis.

Gas-Liquid Chromatographic Analysis

Bring all solutions to room temperature. Adjust GLC operating conditions as described under *Apparatus*. With automatic sampler, inject 3 μL aliquots of analytical standard solution until integrator counts vary ≤10%. Place vials containing analytical standard before and after vials containing sample solutions. Calculate concentration of chlorpyrifos-methyl by comparing

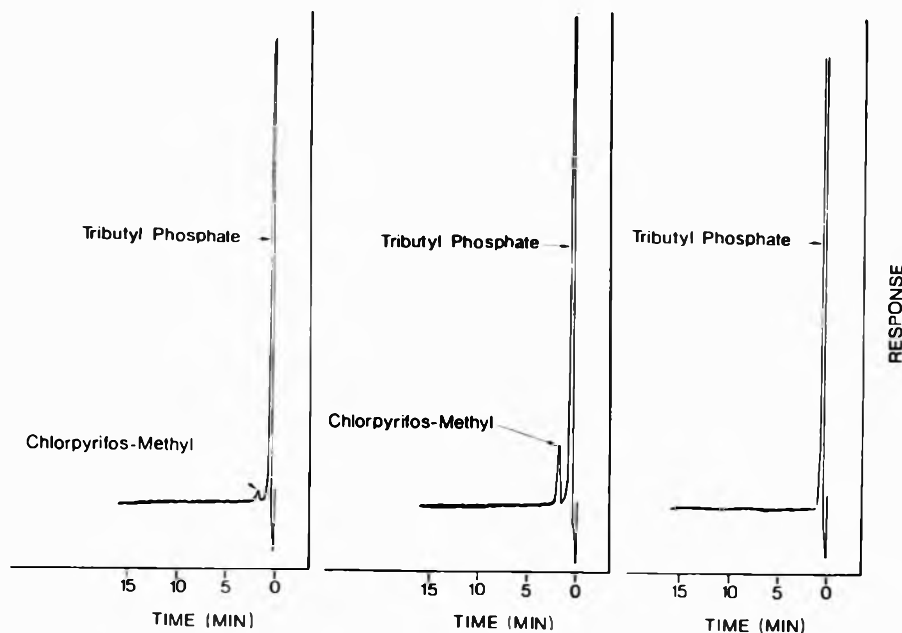

Figure 1. Chromatograms of extract from cat food fortified with 0.052 ppm chlorpyrifos-methyl; extract from cat food fortified with 0.52 ppm chlorpyrifos-methyl; extract from unfortified cat food.

integration counts obtained for sample with those obtained for standard.

Results and Discussion

The cleanup procedure described in this paper is a modification of the procedure developed by Dale and Miles (1) for separating pesticides from fats. The efficiency of the cleanup column of the Dale and Miles procedure was between 97 and 100% for pesticides with partition coefficients (*P*-values) between *n*-hexane and acetonitrile of ≤0.044. However, when the *P*-values were >0.044, the recovery drastically declined, since most or all of the pesticides were eluted with the fats in the *n*-hexane elutions. Consequently, chlorpyrifos-methyl, with a reported *P*-value of 0.17, did not partition satisfactorily with either of the 2 column lengths examined by Dale and Miles. In the modified cleanup procedure described in this paper, the Florisil adsorbent was extended to a depth of 15 cm, pentane was substituted for *n*-hexane, and the total pentane volume was reduced to 20 mL. (By collection and analysis of multiple 5-mL fraction pentane elutions of a chromatographic column to which a known amount of chlorpyrifos-methyl had been added, we established that no trace of chlorpyrifos-methyl was found until the 5th elution.) The final modification was use of 35 mL of 5% acetone-in-pentane solution instead of 30 mL acetone alone to elute and collect the chlorpyrifos-methyl. The 5% acetone-in-pentane solution was preferred because it eluted fewer of the retained impurities when the pesticide was collected. The efficiency of the modified cleanup procedure was determined by evaporating the solvent from triplicate 10 g samples of cat food extract. The weights of the remaining fats and other nonvolatile material were 0.54, 0.62, and 0.63 g. The residues were dissolved and carried through the modified cleanup procedure. The 5% acetone eluates were collected, and the solvent was evaporated. The remaining residue weights of 0.061, 0.049, and 0.070 g, respectively, showed that an average of 90% of fats and other nonvolatile material had been removed from the extracted 10 g samples of cat food. Recovery data for chlorpyrifos-methyl in dry cat food are presented in Table 1. Five independent samples were fortified at 8 treatment levels ranging from 0.052 to 51 ppm. Recoveries averaged 97.1%. Figure 1 shows typical chromatograms of cat food samples unfortified and fortified with 0.052 and 0.5 ppm chlorpyrifos-methyl. No interfering peaks were observed in any of the samples analyzed. Inaccurate integration of the chlorpyrifos-methyl peak because of a negative peak that appears immediately after injection was avoided by making all final sample dilutions in acetone solution containing 0.00025% tributyl phosphate (2). Tributyl phosphate was selected because it elutes just before the chlorpyrifos-methyl and thus acts as a trigger to ensure correct integration. This procedure is particularly beneficial for standards that contain no sample residue. Also, the addition of a drop of diethylene glycol helped to prevent loss of pesticides when the eluates were evaporated on the water bath. The chromatogram of the reagent blank in Figure 2 shows no interfering peaks; the only peak present was tributyl phosphate. The linearity of the detector response was determined by plotting the integrator counts obtained vs the concentration of chlorpyrifos-methyl injected. The volume injected was kept constant. The response of the flame photometric detector to chlorpyrifos-methyl was linear over the range examined, from 0.52 to 510 ng/3 μL injection. The retention times for chlorpyrifos-methyl and tributyl phosphate under the conditions described were 1.98 and 0.69 min, respectively.

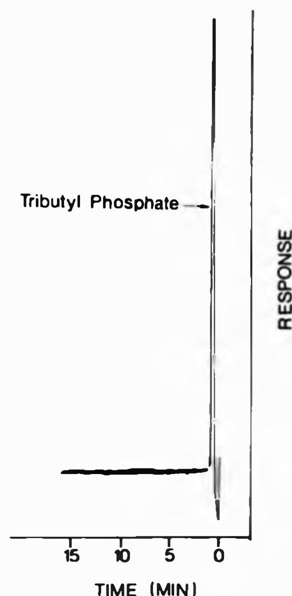

Figure 2. Chromatogram of reagent blank.

Various other procedures were studied in the attempt to find an effective cleanup for chlorpyrifos-methyl in dry cat food. Table 2 gives details on the other procedures investigated, as well as the method described in this paper, and the results obtained. Only one other procedure, a solvent partition between pentane and aceto-

Table 2. Recovery of chlorpyrifos-methyl added to dry cat food, by using various cleanup procedures

Exp. No.[a]	Type of cleanup	Packing or solvents	Eluant	Cleanup procedure	Chlorpyrifos-methyl			Nonvolatile material		
					Added, μg	Found, μg	Rec., %	Before cleanup, g	After cleanup, g	% removed by cleanup
1	column, 40 cm × 20 mm id	10 cm Florisil, 0.5% moisture	1% ethyl acetate in pentane	Sample transferred to column with pentane, and eluted with 60 mL eluant. Eluate discarded. Eluted with 90 mL eluant and collected.	25.3	6.11	24.1	1.413	0.3545	74.9
2	column, 40 cm × 20 mm id	10 cm Florisil 0.5% moisture	3% ethyl acetate in pentane	Sample transferred to column with pentane and eluted with 30 mL eluant. Eluate discarded. Eluted with 60 mL eluant and collected.	25.3	23.6	93.3	1.281	0.9445	26.3
3	column, 40 cm × 20 mm id	10 cm Florex, 0.5% moisture	3% ethyl acetate in pentane	Sample transferred to column with pentane and eluted with 20 mL eluant. Eluate discarded. Eluted with 60 mL eluant and collected.	25.3	15.8	62.5	1.314	0.8892	32.3
4	column, 40 cm × 20 mm id	10 cm Florex, 0.5% moisture	5% ethyl acetate in pentane	Sample transferred to column with pentane and eluted with 20 mL eluant. Eluate discarded. Eluted with 70 mL eluant and collected.	25.3	18.7	73.9	1.402	1.018	27.4
5	column, Chromaflex, 23 cm × 13 mm with 8 cm × 2 mm bore	11 cm alumina, acid, 80–200 mesh	3% ethyl acetate in pentane	Note: 62% of pesticide eluted with pentane transfer solvent only. Column was eluted with additional 30 mL eluant. Both volumes collected.	25.3	22.9	90.5	1.314	0.9896	24.7
6	column, Chromaflex, 23 cm × 13 mm with 8 cm × 2 mm bore	11 cm silica gel, 60–200 mesh	3% ethyl acetate in pentane	Note: 97% of pesticide eluted from column with pentane transfer solvent only. Column was eluted with 20 mL additional eluant. Both volumes collected.	25.3	22.9	90.5	1.312	1.080	17.7
7	solvent partition	pentane extracted with acetonitrile		In separatory funnels, sample in 100 mL pentane was extracted 5 times with 25 mL volumes of saturated acetonitrile. Sample in acetonitrile diluted 1:1 with salt water and extracted with 50 mL pentane.	25.3	21.9	86.6	1.384	0.119	91.4
8	column, Chromaflex, 30 cm × 10 mm with 8 cm × 2 mm bore	15 cm Florisil-PR, 60–100 mesh	5% acetone in pentane	Column was washed with 15 mL volumes of mutually saturated acetonitrile and pentane. Sample was transferred to columns washed with 20 mL saturated pentane and eluted with 35 mL eluant.	25.5	24.3	95.3	0.536	0.0607	88.7

[a] Extraction: 25 g samples of dry cat food were ground to a fine mesh in a Waring blender and extracted by shaking 3 h (1 g cat food to 3 mL solvent). The extract was filtered, aliquots were treated with chlorpyrifos-methyl, and then evaporated to dryness under nitrogen on a 60°C water bath.

nitrile, provided acceptable results; however, it was time consuming. No method investigated was sufficiently rapid and efficient except experiment 8, which is the procedure presented in this paper. Although the present procedure was developed primarily for determining chlorpyrifos-methyl in dry cat food, the method should require little or no modification for use with other commodities containing moderate to high levels of fats.

Acknowledgment

The authors are grateful for the advice and suggestions of W. E. Dale in the preparation of this paper.

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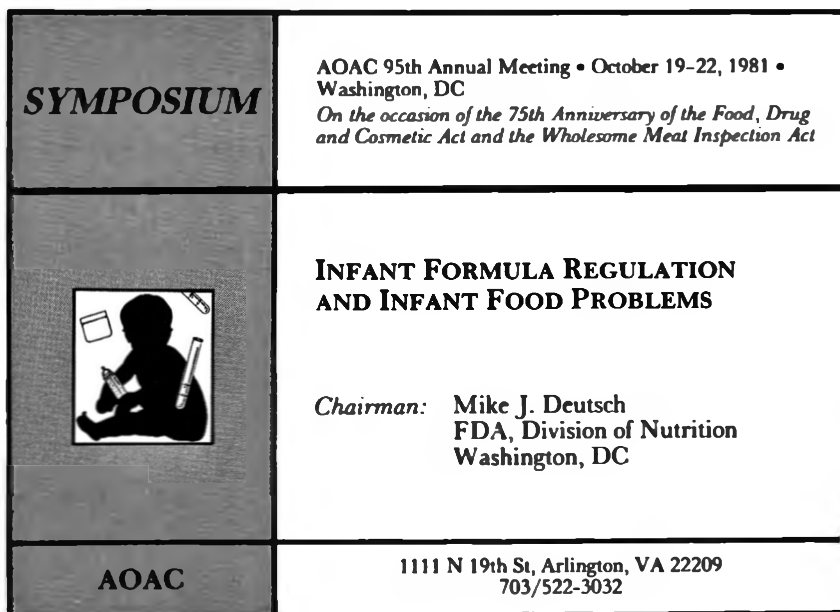

Gas Chromatographic-Mass Spectrometric Characterization of an Alteration Product of Malathion Detected in Stored Rice

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Four alteration products of malathion have been observed in crop extracts of rice, which may not be readily identifiable in a typical gas chromatographic-mass spectrometric (GC-MS) analysis of the residues. Extracts of rice containing malathion and several related compounds were analyzed on gas-liquid chromatographic (GLC) columns of different polarities. The methyl ethyl succinate ester of malathion co-elutes with malaoxon from a nonpolar GLC column. Retention data and GC-MS analysis indicate the presence of 3 previously reported compounds. Evidence was obtained for a fourth compound, *O*-methyl *O*-ethyl *S*-(1,2-bis-carbethoxy) ethyl phosphorodithioate, apparently resulting from the environmental alteration of malathion.

Malathion (*O,O*-dimethyl *S*-(1,2-bis-carbethoxy) ethyl phosphorodithioate) is used to control insects in stored grain. Malathion can be contaminated by impurities in manufacturing and is altered by the environment into a variety of compounds which differ in their persistence and toxicity (1, 2). This presents problems in residue analysis and interpretation of the significance of the results.

Preliminary residue analysis of several samples of rice held in storage indicated the presence of malathion and 3 related compounds which have been reported previously (1, 2). By using gas-liquid chromatographic (GLC) columns with different polarities and gas chromatographic-mass spectrometric (GC-MS) analysis, evidence was obtained for a fourth compound which appears to result from environmental alteration of malathion. The gas chromatographic data and mass spectra of malathion and 3 alteration products are presented as an aid in characterizing the fourth product.

Experimental

Extraction and Cleanup

The rice and rice hull samples were extracted with 35% H_2O-acetonitrile, partitioned between petroleum ether and acetonitrile, and fractionated with 6, 15, and 50% ethyl ether-petroleum ether on a Florisil column (3).

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GLC Determination

All 3 residue fractions were analyzed on both polar (5% OV-101 + 7.5% OV-210) and nonpolar (5% OV-101) columns operated at 200°C. All columns were 2 m × 4 mm glass; liquid phases were coated on 80–100 mesh Gas-Chrom Q (Analabs). The elution time for malathion was 4.2 min on the nonpolar column and 7.5 min on the polar column. The flame photometric detectors were equipped with 526 nm filters (phosphorus mode) and were adjusted for half scale deflection for 2 ng parathion. Sample residue volumes were adjusted on a 5 μL injection representing 20 mg of product.

Mass Spectrometry

A Hewlett-Packard 5982A gas chromatograph-mass spectrometer-data system was used in the EI mode (70 eV). A 2 m × 2 mm coiled glass column packed with 3% OV-101 on 100–120 mesh Gas-Chrom Q was temperature-programmed from 150 to 240°C at a rate of 8°/min. The 15 and 50% Florisil residues were concentrated to 0.5 mL for MS analysis.

Results

Gas Chromatography

A chromatogram from a nonpolar column of the 50% ethyl ether-petroleum ether elution fraction is shown in Figure 1. A chromatogram of the same residue obtained from a polar column had similar peaks; however, about half of Peak I appeared to have moved to Peak III in the second chromatogram. This indicates that a highly polar compound is present along with several nonpolar compounds. Published retention data for the columns used (see Table 1) indicate that malaoxon (*O,O*-dimethyl *S*-(1,2-bis-carbethoxy) ethyl phosphorothiolate) is the polar material moving from Peak I to Peak III in the 2 chromatograms. The remaining component of Peak I could be the methyl ethyl succinate ester of malathion (*O,O*-dimethyl *S*-(1-carbomethoxy-2-carbethoxy) ethyl phosphorodithioate or *O,O*-dimethyl *S*-(1-carbethoxy-2-carbomethoxy) ethyl phosphorodithioate) (2, 4). Peaks II and III appear to be malathion and parathion, re-

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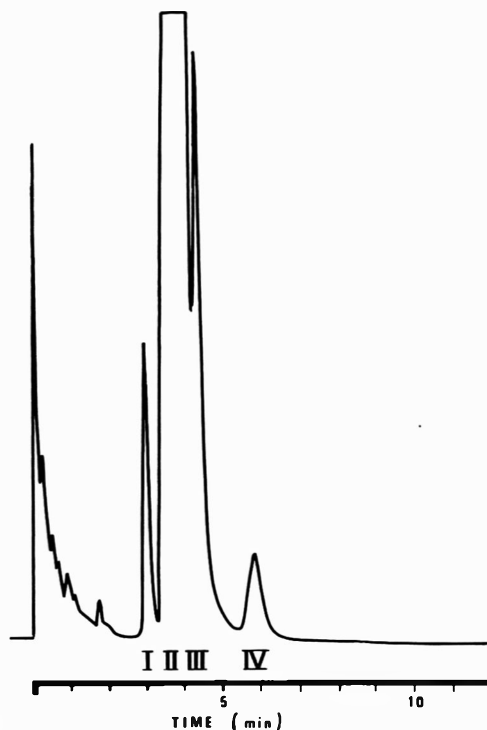


Figure 1. Chromatogram of 50% rice extract obtained with flame photometric detector (phosphorus mode) from OV-101 column at 200°C. See text and Table 1 for peak identification.

spectively. Peak IV did not fit any reported retention data.

Mass Spectrometry

Two sets of fragmentation in the mass spectra provide structural information about malathion and its analogs (5, 6). The major fragments for malathion and its analogs are shown in Figures 2 and 3. The phosphate (P) moiety of malathion produces several prominent fragments (B series, Figure 2). The succinate ester (Z) portion also yields several prominent fragments (F series, Figure 3). Note that a common ion (m/z 99) is possible in malathion and any analogs.

The residue from the 50% ethyl ether–petroleum ether eluate was analyzed using a temperature-programmed 3% OV-101 column. Figure 4 shows a total ion (TI) and a single ion (SI) (m/z 99) mass chromatogram reconstructed from the MS analysis. Peak I appears as a doublet in the SI mass chromatogram. The peak locations were established on the temperature-programmed column with standard pesticide solutions of malathion and malaoxon and their known impurities. Peak IV was located in the temperature-programmed chromatogram by using the elution time of merphos (tributyl phosphorotrithioite), which has a similar retention time on an isothermal OV-101 column.

Four sets of fragments observed in the mass spectrum (Figure 5) of Peak II from the rice residue confirm malathion as the major component of the residue. The expected set of P fragments with m/z 158, 125, and 93 are found. A less prominent set of fragments is found at m/z 143 and 79. One set of Z fragmentation consists of m/z 173, 127, and 99. A set of higher m/z fragments appears at m/z 285, 256, and 211, representing ester fragmentation (5).

The mass spectrum (Figure 6) of Peak IV indicates the presence of the ethylbutyl succinate of malathion (*O,O*-dimethyl *S*-(1-carbethoxy-2-carbobutoxy) ethyl phosphorodithioate or *O,O*-dimethyl *S*-(1-carbobutoxy-2-carbethoxy) ethyl phosphorodithioate) (1, 7). Fragments with m/z 158, 125, and 93 indicate that the P moiety is B in Figure 2. The prominent fragment with m/z 201 indicates that G in Figure 3 is the Z moiety of the analog. The preferred pathway of the butyl ester fragmentation is demonstrated by the almost complete absence of m/z 155 in the mass spec-

Table 1. Retention times (relative to parathion) of malathion and several of its analogs

Peak[a]	Compound	MW	Retention time: Nonpolar[b]	Retention time: Polar[c]
I-A	*O,O*-Dimethyl *S*-(carbomethoxy-carbethoxy) ethyl phosphorodithioate	316	0.68	0.68
I-B	*O,O*-Dimethyl *S*-(1,2-bis-carbethoxy) ethyl phosphorothiolate (malaoxon)	314	0.70	0.93
II	*O,O*-Dimethyl *S*-(1,2-bis-carbethoxy) ethyl phosphorodithioate (malathion)	330	0.80	0.83
III	*O*-Methyl *O*-ethyl *S*-(1,2-bis-carbethoxy) ethyl phosphorodithioate	344	1.00	0.98
IV	*O,O*-Dimethyl *S*-(1-carbethoxy-2-carbobutoxy) ethyl phosphorodithioate or *O,O*-dimethyl *S*-(1-carbobutoxy-2-carbethoxy) ethyl phosphorodithioate	358	1.25	1.31

[a] Peak location in nonpolar chromatogram (OV-101) (Figure 1).
[b] 6 in. × 4 mm, 5% OV-101 on 80–100 mesh Gas-Chrom Q.
[c] 6 in. × 4 mm, 5% OV-101 and 7.5% OV-210 on 80–100 mesh Gas-Chrom Q.

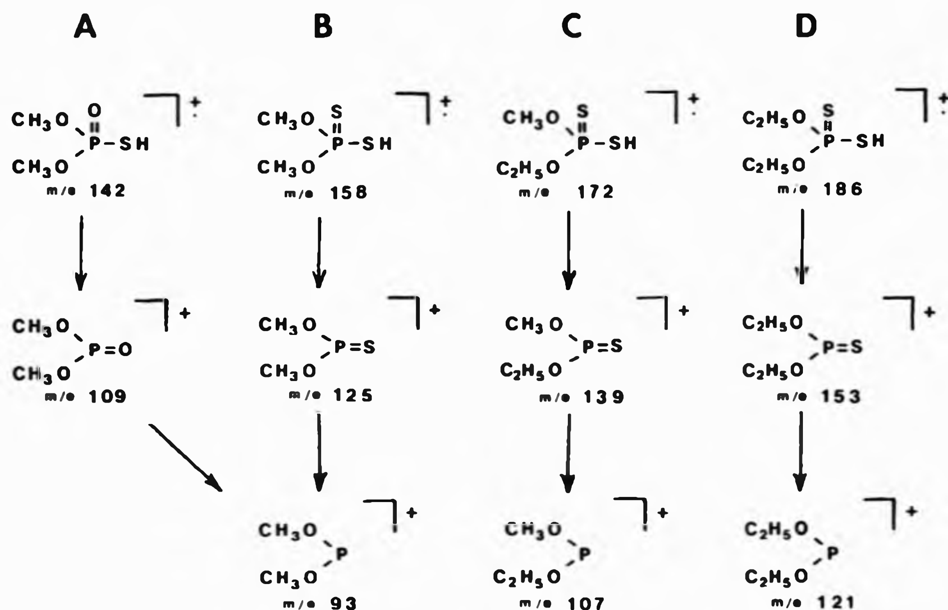


Figure 2. Fragmentation series for the phosphate (P) portion of malathion (B) and its analogs.

trum. (At this trace level the relatively high background obscures the ester mass fragmentation at higher m/z.)

Figure 7A shows the mass spectrum of Peak I of the rice residue. The peak pattern in the m/z 159 region is characteristic of the m/z 173 (succinate) region of malathion. The remaining peaks typical of malathion fragments are also present. Standard solutions of malathion are known to contain a methyl ethyl succinate ester of malathion as a low level contaminant (4). A standard solution of malathion containing this peak was analyzed by GC-MS in the same manner as the above sample. The spectrum of this

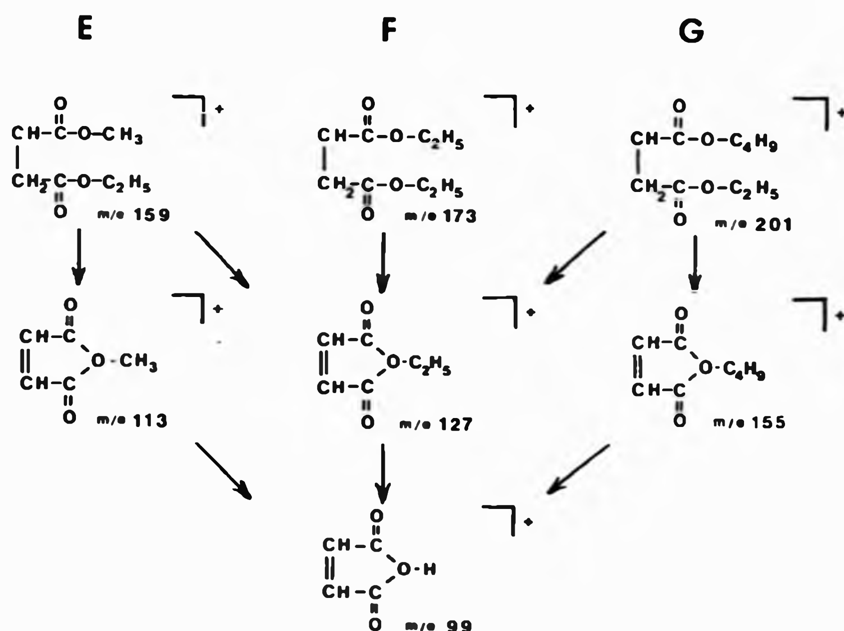


Figure 3. Fragmentation series for the thiosuccinate ester (Z) portion of malathion (F) and its analogs.

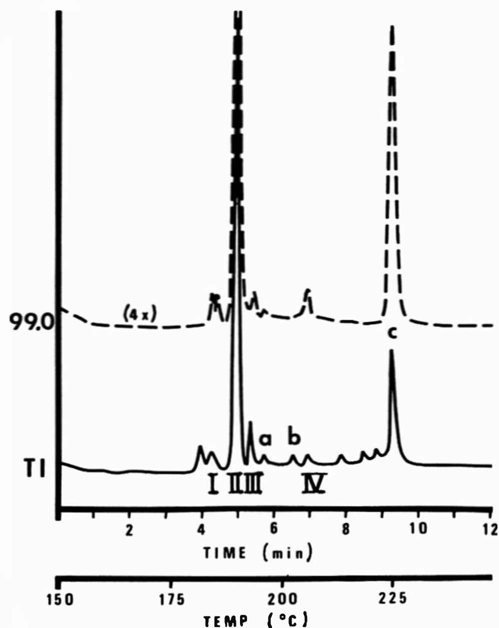
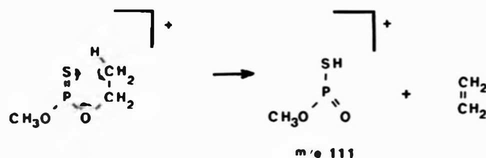


Figure 4. Total ion (TI) and single mass (m/z 99) chromatogram of temperature-programmed GC-MS analysis of 50% rice residue on OV-101 column. See Table I for peak identification.

peak (Figure 7B) reveals a base peak at m/z 159 and an m/z 113 peak, most probably resulting from fragmentation of a methyl ethyl succinate moiety (E in Figure 3). The m/z 159, 125, and 93 fragments are in agreement with assignment of the structure as the methyl ethyl succinate ester analog of malathion.

The spectrum shown in Figure 7C is that of malaoxon standard. The m/z 127 fragment predominates over the other typical phosphate moiety fragments observed in the malathion spectrum. The substitution of oxygen for sulfur produces different fragmentation characteristics (5). The mass spectrum from Peak I (Figure 7A) indicates the presence of both compounds in the rice residue. Although the reconstructed single ion chromatogram (m/z 99, Figure 4) hints at 2 components at location I, distinct front edge and back edge spectra could not be obtained from the analysis.

Peak III is difficult to analyze because it occurs on the overload tail of the malathion peak. The spectrum obtained from Peak III (Figure 8) appears to be a typical malathion spectrum with the addition of fragment peaks at m/z 107, 111, and 139. Several cluster sets are present at 14 amu above the heavier fragments of malathion (m/z 225, 270, and 299). The fragment series C in Figure 2 (the *O*-methyl *O*-ethyl phosphate) can be expected to show the observed fragments. The prominent fragment with m/z 111 offers additional structural evidence. Provided an ethyl (or larger) group is present (8), cleavage with hydrogen rearrangement or migration can produce this fragment:



This is the only malathion analog capable of this rearrangement; a mass spectrum of this analog was not possible since it could not be chromatographically separated from malathion. The chromatographic location of this compound on both polar and nonpolar columns, eluting slightly after the *O,O*-dimethyl compound (malathion), also is in agreement with the *O*-methyl *O*-ethyl phosphate structure.

The observed relative retention times of the 4 peaks are given in Table 1 for several columns. Malaoxon is the only compound whose relative

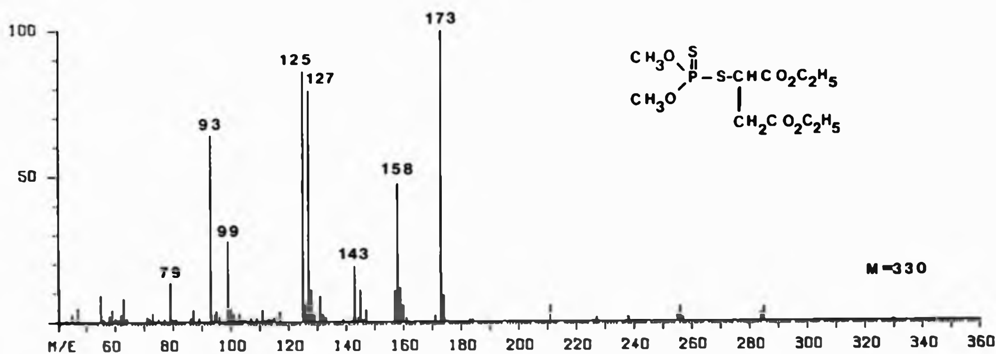


Figure 5. Mass spectrum of malathion.

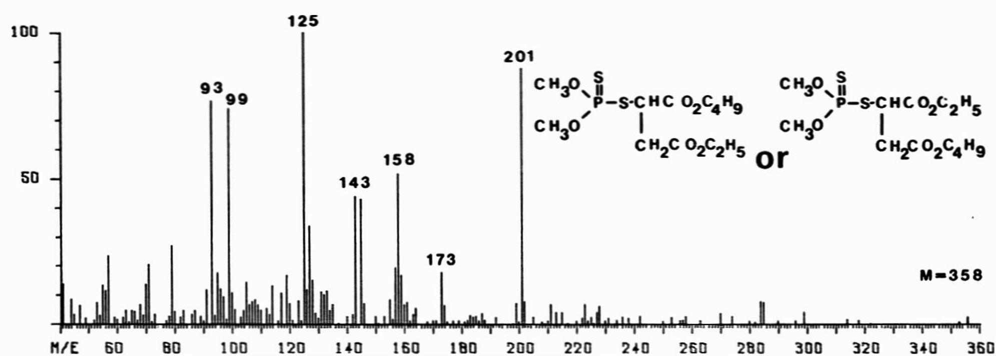


Figure 6. Mass spectrum of Peak IV in residue, ethyl butyl mercaptosuccinate ester analog of malathion.

Figure 7. Mass spectra of A, Peak I in residue; B, methyl ethyl succinate ester analog of malathion; C, malaoxon, the oxygen analog of malathion.

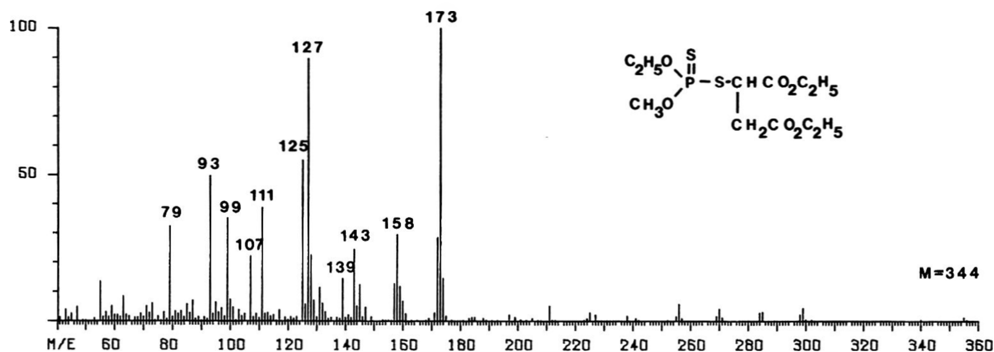


Figure 8. Mixed spectrum of Peak III in residue; malathion and *O*-ethyl *O*-methyl phosphate analog of malathion.

order of elution varies with column polarity. The insensitivity of Peaks I-A, II, III, and IV to changes in column polarity is indicative of nonpolar differences in these analogs.

Several peaks in the total ion and the m/z 99 single ion chromatograms (Figure 4) indicate the possible presence of additional phosphates in the residue. The mass spectra of peaks a and c consist almost entirely of m/z 99 with only small clusters 14 amu apart, which is typical of aliphatic phosphates. A re-examination of the crop residue using an FPD detector and a temperature-programmed GLC column showed no phosphate sensitivity at these corresponding locations. Thus, although these compounds are not identified they are not related to malathion. Because malaoxon presents a greater physiological hazard than does the methyl ethyl succinate ester of malathion (2), the public health hazard of a crop or food product could be overstated if the GLC peak is assumed to be only malaoxon.

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FOOD ADDITIVES

Volatile *N*-Nitrosamines in Dried Foods

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An investigation was carried out to determine the levels of volatile *N*-nitrosamines in several dried food commodities such as malt, instant skim milk powder, infant formula containing milk powder, instant coffee, soup and soup bases, and baby cereals. The method involves vacuum distillation of the sample from 3N KOH or 1% sulfamic acid, followed by extraction of the aqueous distillate with dichloromethane. The dichloromethane extract is then washed with an acidic buffer (to remove amines), dried over anhydrous sodium sulfate, concentrated in a Kuderna-Danish concentrator, and analyzed by gas-liquid chromatography, using a thermal energy analyzer. Detection limit is 0.1-0.5 ppb. Of the samples analyzed thus far, all malts (22 samples) and instant skim milk powders (11 samples) were positive for *N*-nitrosodimethylamine (NDMA); average levels were 7.4 ppb (1.3-67.0 ppb) and 0.4 ppb (0.3-0.7 ppb), respectively. Traces of NDMA and/or *N*-nitrosopyrrolidine were also detected in 3 of 20 dried soups and 5 of 10 instant coffees analyzed. Traces of NDMA and/or *N*-nitrosopiperidine (NPIP) were detected in 3 of 8 powdered infant formulas. All 4 baby cereal samples were negative. The identity of NDMA in 14 samples of malt and that of NDMA and NPIP in an infant formula was confirmed by gas-liquid chromatography-mass spectrometry; identity of *N*-nitrosamines in 3 instant coffees and 1 infant formula was independently verified by high pressure liquid chromatographic analysis.

During the last 8–10 years, most of the work on the occurrence and determination of volatile *N*-nitrosamines in foods has been concentrated on food products that have been preserved or processed with the addition of nitrate and nitrite. Thus, the literature is full of references (1, 2) relating to the presence of volatile *N*-nitrosamines in various cured meats such as sausages, salami, wieners, fried bacon, and nitrite-treated fish. The recent discovery (3) of the occurrence of fairly high levels of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) in malt suggested that the formation of *N*-nitrosamines can take place even in foods that have not been preserved with added nitrite. The formation of *N*-nitrosamines in malt is believed to be caused by the interaction of amines in the malt and gaseous nitrogen oxides (NO_x) present in the hot flue gas used for drying the malt (3). A similar mechanism for the formation of NDMA in fish meal that has not been treated with nitrite has also been suggested (4).

Although the formation of NDMA is more of a problem with malts dried by the direct heating technique, traces of NDMA have also been detected in malts dried by electric heat (3) or by indirect heating (e.g., by circulating hot air instead of hot flue gas). In such cases, traces of NO_x, which are present in the ambient air, are believed to be responsible for the formation of NDMA.

The fact that both the direct and indirect drying methods can result in the formation of detectable levels of NDMA in malt, and also the fact that there are many other foods which are dried by direct heating (where hot flue gas comes in direct contact with the food), prompted us to carry out a study on the volatile *N*-nitrosamine contents of various dried foods. Our findings are presented in this report.

Experimental

Precaution. Because *N*-nitrosamines are potent carcinogens, take adequate precautions while handling the chemicals or working with food extracts containing *N*-nitrosamines. Follow guidelines outlined previously (5).

Samples

Most samples, except malts, were purchased from local retail outlets in Ottawa. Malt samples were obtained from various breweries across Canada.

Homogenization of Samples

A 125 g aliquot of each malt sample was finely ground in a small blender, and the ground malt was mixed well with a spatula just before an aliquot was taken for analysis. All other samples

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were already ground or well mixed, so they were analyzed without any further grinding or homogenization.

Analysis for Volatile N-Nitrosamines

The method used was essentially the same as reported in detail previously (6). It can be described briefly as follows:

A 20 g aliquot of each sample was distilled from 200 mL 3N KOH or 1% sulfamic acid, using a flash evaporator (water bath, 45–50°C). The aqueous distillate, which contained the volatile *N*-nitrosamines (if present), was made alkaline and extracted with dichloromethane. The dichloromethane extract was further washed with acidic aqueous buffer (to remove amines) and dilute KOH (6), and dried over anhydrous sodium sulfate. The dried dichloromethane extract was then concentrated to 1.0 mL, using a Kuderna–Danish-type concentrator, and a 5–10 μL aliquot of the concentrated extract was analyzed by gas–liquid chromatography (GLC) using a thermal energy analyzer (TEA) detector which is highly specific for *N*-nitroso compounds. For low level detection of *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosopiperidine (NPIP), NPYR, and *N*-nitrosomorpholine (NMOR), the TEA detector was operated at attenuation 2. The detection limit of the method is 0.1–0.5 ppb. The same concentrated extract was used for high pressure liquid chromatographic (HPLC)–TEA analysis described later.

Some samples (e. g., skim milk powder, powdered infant formulas, baby cereals) caused excessive foaming during vacuum distillation from 3N KOH but could be easily distilled from 1% sulfamic acid without addition of antifoam agent. In general, malt and coffee samples were distilled from 3N KOH solution, and the rest of the commodities were distilled from 1% sulfamic acid (mainly because of the foaming problem). To check against artifactual formation of *N*-nitrosamines, some samples (as described later) were analyzed by both distillation methods.

HPLC-TEA Confirmation

In a few cases, the identity of the detected volatile *N*-nitrosamines was independently verified by HPLC–TEA analysis. HPLC conditions were as follows: 250 mm × 2.1 mm column of LiChrosorb Si60 (5 μm); 5% acetone in *n*-hexane at 0.5 mL/min; acetone–dry ice (−80°C) TEA slush bath; 150 μL sample; and attenuation 8 or 16. The instrument was operated as described in the manual for Model TEA 502.

GLC-Mass Spectrometry (GLC-MS)

Before GLC-MS analysis, extracts were further concentrated to 0.5 mL by bubbling a gentle stream (through a hypodermic needle) of nitrogen through the extract. GLC-MS confirmation was carried out by the specific ion current monitoring technique for the molecular ions of NDMA at a resolution of 5000, and at a resolution of 10 000 for NPIP (6).

Results and Discussion

Table 1 summarizes the type of dried foods analyzed and their *N*-nitrosamine contents. Of the samples analyzed, the malts and the instant skim milk powders consistently contained traces of NDMA, although the latter contained much lower levels of NDMA than the former. All malts analyzed during the first half of 1980 contained very low levels of NDMA; the highest level detected was 6.6 ppb which is much lower than the highest level (67 ppb) detected in the 4 samples analyzed during 1979. This general decrease in the NDMA level of malt is believed to be due to a prolongation of sulfur burning carried out during kilning of malt.

The detection of extremely low levels (average 0.4 ppb) of NDMA in instant skim milk powder is of considerable interest. We gave a preliminary report on this finding earlier (7). To our knowledge, this was the first reported finding of NDMA in these products. In the United States, Hotchkiss et al. (8) reported similar findings and observed a much higher average level of NDMA in nonfat dried milk powders. Since most instant skim milk powders are prepared by some kind of heating techniques, it is conceivable that the NDMA is formed by the interaction of NO_x in the hot air used to dry the milk and the amines naturally occurring in the milk. Because NDMA is steam volatile, the NDMA present (if any) in the raw milk would be expected to be lost during the spray-drying process. Further work is needed, however, to fully understand the mechanism of NDMA formation in instant skim milk powder.

Most of the dried soups and soup bases were negative; only 3 contained extremely low levels of either NDMA or NPYR (Table 1). The finding of traces of NPYR in 5 of 10 instant coffees is noteworthy. The source of the contamination or its mechanism of formation is, however, not clear. Of the few powdered infant formulas analyzed, only 3 contained detectable levels of NDMA and/or NPIP. It should be mentioned that 2 other samples (different batch number) of the same brand of infant formula (1st sample of

Table 1. *N*-Nitrosamine contents of various foods analyzed in this survey

Commodity	No. samples positive/No. analyzed	*N*-Nitrosamine levels, ppb[a]		
		NDMA	NPIP	NPYR
Malt				
Samples analyzed in 1979	4/4	30.3		
		67.0		
		4.2		
		3.1		
Samples analyzed in 1980[b]	18/18	4.5		
		3.9		
		5.0		
		4.4		
		4.9		
		3.1		
		1.4		
		1.3		
		2.6		
		2.5		
		2.9		
		4.0		
		3.4		
		1.8		
		1.3		
		1.9		
		3.6		
		6.6		
Dried soups and soup bases (chicken noodle, tomato, beef or chicken cubes, mushroom vegetable noodle, onion, etc.)	3/20	0.25		
		0.20		
		NEG.		0.6
Instant skim milk powder	11/11	0.4		
		0.3		
		0.3		
		0.3		
		0.4		
		0.7		
		0.6		
		0.4		
		0.5		
		0.6		
		0.4		
Instant coffee	5/10			0.3
				0.8[c]
				0.6[c]
				0.4
		0.3		1.4[c]
Powdered infant formulas containing skim milk powder	3/8	1.0[b,c]	0.8[b,c]	
		trace (<0.2)		
		0.2		
Baby cereals (oatmeal, rice, and mixed cereals)	0/4			

[a] Only positive results of individual samples are indicated. Samples were negative for other volatile nitrosamines (e.g., *N*-nitrosodiethylamine, NDBA, and NMOR). All results are uncorrected for percentage recoveries and represent single analysis. Most results, except those noted below, are based on GLC-TEA analysis.
[b] Confirmed by GLC-MS (see Table 2 for details of malt data).
[c] Confirmed by HPLC-TEA analysis.

infant formula in Table 1) were negative. Therefore, the occurrence of NDMA and NPIP in this infant formula may be just an isolated instance. All 4 baby cereals analyzed were negative.

The identity of the NDMA in most of the malt samples and that of the *N*-nitrosamines in an infant formula (Table 1) was confirmed by GLC-MS analysis. As can be seen from the data (Table 2) the correlation between the 2 sets of results in most of the samples, except the last 4, was very good. The levels of *N*-nitrosamines in the other food commodities were too low to be confirmed by GLC-MS. The results in these cases, therefore, should not be considered as unequivocal proof of the presence of *N*-nitrosamines. The identity of both NDMA and NPIP in the above-mentioned infant formula and that

Table 2. Correlation of GLC–TEA results for malts with those obtained by GLC–MS analysis

NDMA levels, ppb	
GLC-TEA	GLC-MS[a]
6.6	7.5
3.6	3.8
1.9	1.5
1.3	1.2
1.8	1.5
3.4	3.2
4.0	4.4
2.6	2.0
1.3	1.4
1.4	1.1
2.5	5.2
3.1	1.9
4.9	2.9
4.4	2.8

[a] Specific ion monitoring for molecular ion of NDMA at resolution of 5000 (6).

of NPYR in 3 coffee samples was independently verified by HPLC-TEA analysis (Table 1).

Each sample was analyzed with the addition of either 10 or 2.5 ppb *N*-nitrosodi-*n*-propylamine (NDPA) as an internal standard. The percentage recoveries of the internal standard usually varied between 80 and 100, but all the results reported in Table 1 are uncorrected values.

In trace analysis there is always a possibility of contamination and artifact formation. To avoid the possibility of contamination, each batch of dichloromethane, water, anhydrous sodium sulfate, etc., was pretested for *N*-nitrosamine contamination. Furthermore, a complete reagent blank (taken through all the steps) was carried out with each batch of 3N KOH, 1% sulfamic acid, or the acidic buffer. To guard against artifactual formation of *N*-nitrosamines, 4 samples of instant skim milk powder, 3 of the positive coffees, and 1 of the positive powdered infant formulas were analyzed by the regular sulfamic acid distillation method in the presence of 5000 ppb added di-*n*-butylamine free base. No evidence of formation of NDBA was noted in any sample but they all contained the respective nitrosamines as originally detected. In addition, the 3 coffee samples mentioned above were reanalyzed by a modified KOH distillation method in which the samples were pre-incubated with 1 mL 1N HCl and 25 mL 1% sulfamic acid for 10 min before adding 175 mL 3N KOH and distilling the sample. Results were similar to those obtained earlier. The purpose of the pre-incubation step was to destroy any nitrosating agent that may have been present. The infant formula (Table 1, 1st sample of infant formula) was also

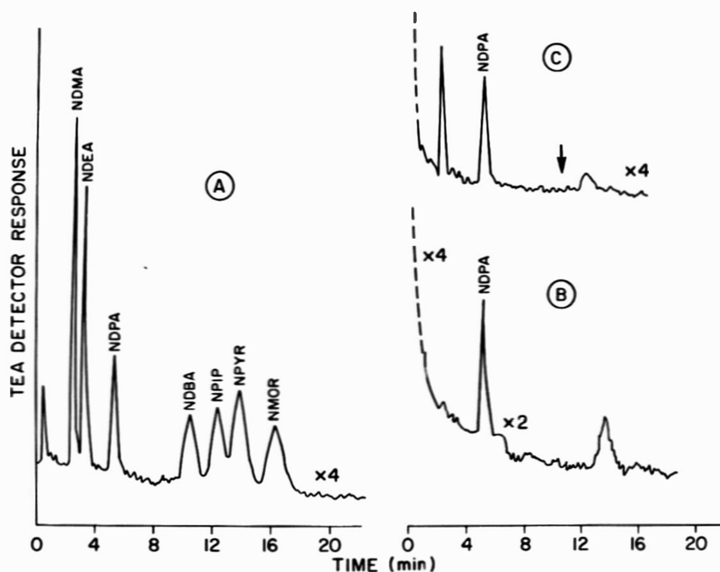

Figure 1. GLC-TEA chromatograms: A, 500 pg of each standard; NDEA = *N*-nitrosodiethylamine. B, 10 µL/1.0 mL extract of instant coffee (containing 0.6 ppb NPYR). Sample was first treated with 1 mL 1N HCl and 25 mL 1% sulfamic acid for 10 min, made alkaline with 175 mL 3N KOH, and distilled under vacuum. C, 10 µL/1.0 mL extract of infant formula (Table 1, 1st sample of infant formula) analyzed by acidic sulfamic acid distillation method with 10 000 ppb added di-n-butylamine hydrochloride. Note absence of NDBA formation at point marked with arrow. Attenuation settings are shown on diagrams. NDPA peaks are due to internal standard added at 2.5 ppb in each case.

analyzed from acidic sulfamic acid (2 mL 3N H_2SO_4 + 198 mL 1% sulfamic acid) in the presence of 10 000 ppb added di-*n*-butylamine hydrochloride. There was no evidence of NDBA formation (Fig. 1) but both NDMA and NPIP were detected. Because there was no evidence of formation of NDBA when the analysis was carried out with added dibutylamine, it is highly unlikely that the *N*-nitrosamines detected in the samples were formed as artifacts. Under the conditions used, the addition of sulfamic acid and 2 mL 3N H_2SO_4 to the sample did not cause any detectable loss of the 2 nitrosamines.

The present study suggests that besides malt, other dried foods may contain traces of volatile *N*-nitrosamines. Because the number of samples of various commodities analyzed in this survey is extremely small, one should be careful in extrapolating the results to entire samples sold in retail outlets in Canada. A much more extensive survey must be carried out before one could get a clearer idea as to the extent and nature of the contamination level in the various products. Additional work is also needed to understand the mechanism of formation of these *N*-nitrosamines in these foods.

Acknowledgment

We thank P.-Y. Lau for carrying out the GLC-MS analysis, and the Field Operations Directorate, Health Protection Branch, for its assistance in procuring the malt samples. The technical assistance of M. McPherson and H. Jung is also gratefully acknowledged.

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CEREAL PRODUCTS

Colorimetric Alpha-Amylase, Falling Number, and Amylograph Assays of Sprouted Wheat: Collaborative Study

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Results are reported of a collaborative study on the determination of sprout damage in wheat. Methods of analysis included falling number, amylograph, and a colorimetric α-amylase assay. Data for the 3 methods were linearly interrelated. Primary source of error for each method was lack of agreement among collaborators. The 3 tests adequately differentiated among sprout damage levels within a single laboratory. The colorimetric test was the most sensitive to change in α-amylase content and appeared to have greater potential for standardization than the other 2 methods.

The accurate determination of quality in cereal grains is of interest in all countries involved in marketing grain. The quality evaluation is, however, often complicated by different requirements among users and by the use of imprecise subjective methods of evaluation. The current visual inspection method (1, 2) is inadequate as an index of sprout damage. Although visual inspection can estimate the percentage of sprouted kernels, it does not quantitate the extent of sprout damage, which is the more important of the 2 characteristics.

The amylograph and falling number methods provide an indication of wheat quality by measuring the change in viscosity of a suspension of ground wheat or flour as it is heated through its gelatinization temperature. These tests primarily measure the effect of hydrolysis of the starch component of wheat or flour. Both tests are objective and, consequently, are more precise than visual inspection. Although the falling number method is widely used in Europe, neither the amylograph nor falling number method is widely used in U.S. grain marketing channels. The falling number method is used at some large export facilities in the United States, but at smaller elevators, visual inspection is the primary means of evaluating sprout damage. Because of the inherent imprecision of visual inspection, sprouted wheat may pass local inspectors and enter marketing channels. Repeated blending of these wheats does tend to eliminate extremes of sprout damage, but the dilution makes detection of sprouted wheat more difficult. The inability of local elevator personnel to accurately identify sprout-damaged wheat can thus result in significant problems at the export terminal.

Mathewson and Pomeranz (3) developed a simple rapid colorimetric method for assay of α-amylase (using Cibacron blue-amylose (CBA) as substrate) as an index of sprout damage in cereal grains. The results, expressed as absorbance, are linear over a wide range of α-amylase activity.

The objective of our collaborative study was to measure sprout damage in wheat by the colorimetric, amylograph, and falling number methods, using wheat samples containing 0–5% sprout damage. We were particularly interested in intra- and interlaboratory variation, determining how the data obtained by the colorimetric method related to those data obtained from the amylograph and falling number methods, and

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how well the colorimetric test would predict the α-amylase content of the sprout-damaged samples.

METHODS

Materials

Two hundred pounds of untreated sound hard red winter wheat flour was obtained from the Department of Grain Science and Industry, Kansas State University, Manhattan, KS. One hundred pounds of sound western white wheat and 2 samples of sprout-damaged western white wheat containing 6 and 35% sprout damage (as determined by federal inspectors) were obtained from North Pacific Grain Growers, Inc., Portland, OR. Check samples of malted barley were obtained from the Malt Analysis Check Service, American Society of Brewing Chemists (ASBC), Madison, WI.

Preparation of Samples

Samples differing in level of sprout damage consisted of sound and sprouted wheats blended in weight ratios of 10:1, 6:1, 3:1, and 2:1. The sprouted wheat was a composite of 6 lb of 35% sprout-damaged wheat and 12 lb of 6% sprout-damaged wheat. The composite sprout-damaged sample, therefore, contained about 15% sprout-damaged grain, and the blends contained about 1.4, 2.1, 3.8, and 5.0% sprout-damaged grain.

Individual samples for collaborative study were prepared by repeatedly passing sound wheat and each blended mixture of sound and sprouted wheat through a Boerner divider. For each level of sprout damage, approximately 90 plastic vials were filled with 75 g grain. Each collaborator received 3 sets of 8 vials of whole grain, one set to be used for each test method. The 8 vials in each set contained duplicate samples of sound wheat, 10:1 mix, and 3:1 mix, and one sample each of 6:1 and 2:1 mixes. The collaborators were not aware of the internal duplicates. The 8 vials in each set were randomly numbered from 1 to 8.

Instructions to Collaborators

Twelve collaborators were located in the United States and 11 were located in Canada and overseas countries. Each collaborator was sent 24 vials of whole grain, 1600 g sound wheat flour (to be used for the amylograph test), a vial of CBA tablets, 2 tubes containing colorimetric standard solutions, and a set of detailed instructions. Each collaborator was asked to perform falling number tests according to AACC method 56-81B, amylograph tests according to AACC Method 22-10 (4) with some modifications, and the colorimetric test for α-amylase. Modifications to the amylograph test included use of distilled water in place of McIlvaine buffer. We suggested use of an egg beater or a blender to mix the flour-water slurry to ensure uniform mixing with no lumps. We further suggested that 82.00 g sound untreated flour (12.8% moisture, dry basis) in 460 mL deionized water should give a maximum amylograph viscosity value (BU) not to exceed 950. All collaborators were asked to use this flour to establish a standard amylograph viscosity curve for flour with no α-amylase activity. To test the sprout-damaged samples, workers ground the samples, and mixed 15.0 g ground wheat with 67.00 g sound wheat flour (12.8% moisture, dry basis).

The colorimetric α-amylase test was described by Mathewson and Pomeranz (3). A standard curve for the colorimetric α-amylase assay was prepared at the U.S. Grain Marketing Research Laboratory (USGMRL), using malted barley check samples. Sprout-damaged samples were also assayed at the USGMRL for α-amylase activity according to the micro-SKB method described by Perten (5).

Results and Discussion

Transformation of Data

Table 1 shows the averaged analytical data, as reported, from all collaborators. These data were transformed to units which would be linear with α-amylase units. Falling number values were converted to liquefaction number (LN) (6) and peak amylograph viscosity values were converted to mobility number (7). The results of the colorimetric test (CBA), reported as absorbance at 620 nm, were converted to dextrinizing units/100 g by use of the regression equation for the standard curve. A dextrinizing unit (DU) is defined as "the quantity of α-amylase which will dextrinize soluble starch in the presence of an excess of β-amylase at the rate of 1 g/h at 20°C" (8).

Statistical Analyses

Data were analyzed graphically and on the basis of the different sources of variation. Table 2 shows the assay means and variance components for each of the methods used to measure the extent of sprout damage. The among-collaborator variance is a measure of that variation caused by differences from one collaborator to

Table 1. Summarized data for sprout-damaged blends

Parameter	Sound	Ratio of sound:sprouted wheat			
		10:1	6:1	3:1	2:1
Sprout damage, %	0	1.4	2.1	3.8	5.0
Colorimetric test, absorbance 620 nm	0.07	0.21	0.29	0.43	0.53
Falling number, s	363	278	249	213	193
Amylograph Brabender units	719	615	555	459	418

another. The between-duplicate variance is primarily a measure of the heterogeneity of samples with the same level of sprout damage. The within-sample variance is a measure of the reproducibility of a particular method on the same sample.

Sources of Variation

Several points need to be made concerning the sources of error in this study. There were 23 participants representing 10 countries, and, as expected, all used different types of equipment to perform the tests. The falling number tests were done with 3 models of the device at widely different elevations. Amylographs were obtained from 2 manufacturers and represented an undetermined number of models. Many types of colorimeters and spectrophotometers (with no common cell size) were used for the colorimetric test. While we were aware that such differences in equipment would cause differences among collaborators, we proceeded with the study for 2 reasons: First, it would have been impractical to require all participants to use the same equipment. Second, we believed that use of the available equipment would give a more realistic comparison of how these tests truly perform around the world, especially the falling number and amylograph tests, which have been used for many years. The procedures for these 2 tests are well known to those using the instruments.

Another source of error resulted from sampling. The samples were prepared by blending sound and sprouted wheat for 20 min in a McLellan blender. Still, obtaining a truly homogeneous blend of sound and sprouted wheat was virtually impossible. Sprouted wheat itself is not homogeneous, because the kernels are usually sprouted to different extents and thus differ in degree of physiological change. This nonhomogeneity, combined with differences in density between sound and sprouted wheats,

Table 2. Results of statistical analysis of data from falling number, amylograph, and colorimetric tests

Statistic	Sound	Ratio of sound:sprouted wheat			
		10:1	6:1	3:1	2:1
Falling Number Results (Liquefaction No.)					
Mean	19.19	26.29	30.01	36.74	41.80
n	90	89	44	89	45
Variance components					
Among-collaborators	3.26	9.79	14.57	24.12	36.51
Between-duplicates	2.72	3.48		8.69	
Within-samples	1.24	1.03	0.87	1.27	1.00
Amylograph Results (Mobility No. × 10^4)					
Mean	13.90	16.26	18.01	21.79	23.89
n	67	67	33	67	34
Variance components:					
Among-collaborators	4.20	6.29	7.37	37.92	28.53
Between-duplicates	0.10	0.32		7.24	
Within-samples	0.05	0.06	0.06	0.24	0.48
Colorimetric results (DU/100 g)					
Mean	2.15	5.48	7.32	10.70	13.06
n	84	84	41	84	41
Variance components:					
Among-collaborators	0.28	1.36	2.97	3.13	11.18
Between-duplicates	0.31	1.17		2.12	
Within-samples	0.05	0.24	0.38	0.32	0.30

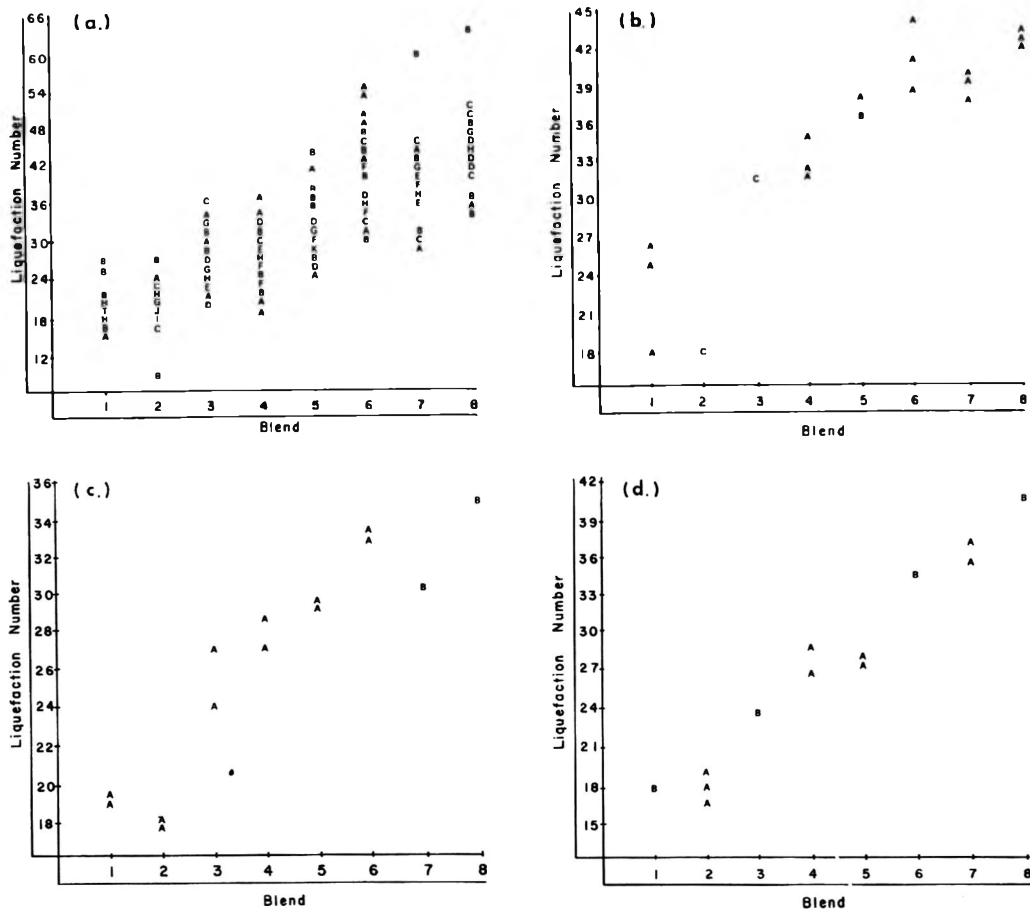

Figure 1. Reported results of falling number tests (expressed as liquefaction numbers).

(a) Results from all collaborators; (b–d) results from 3 randomly selected laboratories. Sample denotations 1 and 2 represent duplicates of sound wheat; 3 and 4, duplicates of 10:1 blend; 5, the 6:1 blend; 6 and 7, duplicates of 3:1 blend; and 8, the 2:1 blend, prepared as described in text. A, B, and C denote single, double, and triple observations, respectively.

make the sampling of a blend of those wheats an imprecise process.

Falling Number Results

Falling number data are expressed as liquefaction number (LN). The means (Table 2) show that there was a 2-fold increase in LN from the sound wheat to the most sprout-damaged sample. Variance analysis indicates that though both among-collaborator and between-duplicate components increased as the amount of sprout damage increased, the within-sample variance did not. The ability of the instrument to provide reproducible data on replicates of the same sample was approximately equal over the range of sprout damage tested.

Figure 1a shows falling number results from all collaborators for each of the 8 samples. There was considerable spread in the range of values reported for each level of sprout damage.

The highest LN values for the 6:1, 3:1 duplicates, and 2:1 mixtures were all obtained from one collaborator. Those outlying values probably contributed appreciably to the magnitude of the among-collaborator variance component. Even though the apparent increase in the among-collaborator variance appears to have been due largely to one collaborator, overlap in LN values for the different levels of sprout damage was considerable; hence Figure 1a indicates that adjacent levels of sprout damage could not be reliably distinguished. On the other

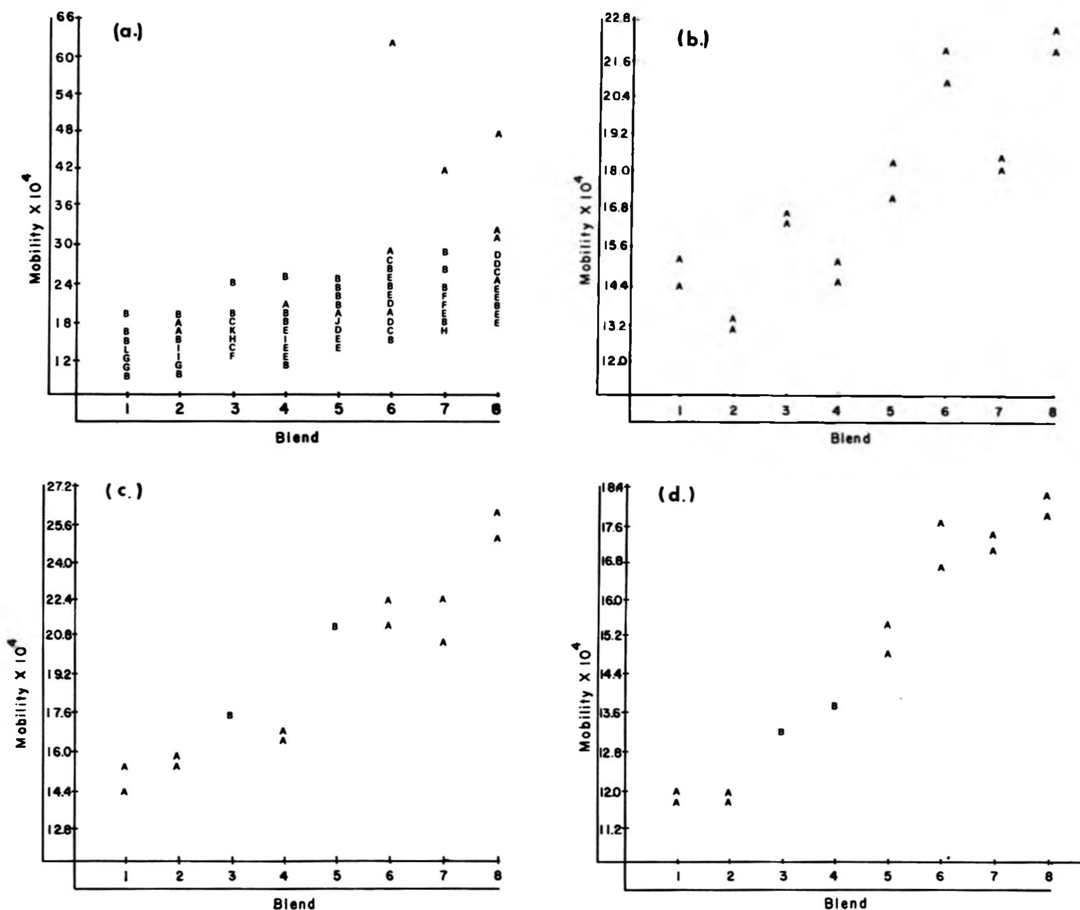

Figure 2. Reported results of amylograph tests (expressed as mobility numbers). Designations same as in Figure 1.

hand, the decrease in falling number values (Table 1) with increasing levels of sprout damage averaged 14.5%. While this is not a large change, it would be expected to be adequate for distinguishing adjacent levels of sprout damage. Data for 3 randomly chosen individual collaborators (Figure 1 b–d) illustrates that this was true. In many cases, reproducibility in a single laboratory was adequate enough that most levels could be distinguished.

Amylograph Results

As observed with falling number data, both the among-collaborator and between-duplicate variance components of the amylograph data increased as sprout damage increased (Table 2). As observed with falling number, the largest contributor to the total variation was among-collaborator variance. An unexpected result was the apparent increase of within-sample variance indicating that as sprout damage increases, the amylograph is less able to provide reproducible data.

Amylograph results reported by all collaborators for all levels of sprout damage showed considerable overlap in values for the different levels of sprout damage (Figure 2a). The very high mobility numbers for duplicate 3:1 samples and for the 2:1 sample are obtained from one collaborator and, again, these outlying values probably contributed greatly to the among-collaborator variance reported in Table 2.

Mobility number increased about 2-fold from the sound to the 2:1 blend. The average increase in mobility number mean, for each successive level of sprout damage, was 12.5%. This is not a large change, and Figure 2a suggests that the ability to differentiate levels of sprout damage was poor. However, data from randomly selected individual laboratories (Figure 2b–d) in-

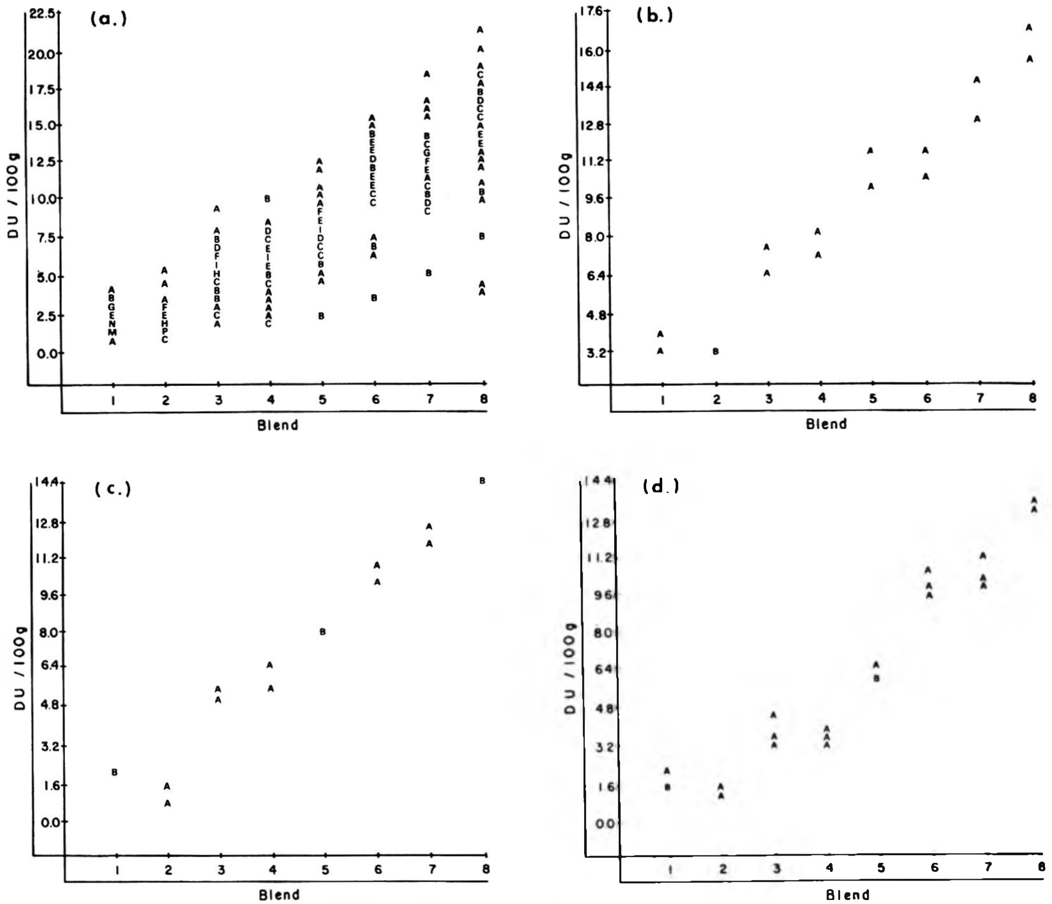

Figure 3. Reported results of colorimetric tests (expressed as DU/100 g). Designations same as in Figure 1.

dicated that most levels of sprout damage could be distinguished. It is of interest that for these laboratories the loss of precision at higher levels of sprout damage, as indicated by the within-sample variance, is not evident.

Colorimetric Test Results

As observed with falling number and amylograph results, the among-collaborator and between-duplicate variance components of the colorimetric data increased with increasing sprout damage (Table 2). There was considerable spread in values for each level of sprout damage (Figure 3a). The lowest values (in DU/100 g) for 6:1, 3:1 duplicate, and 2:1 blends were obtained by one collaborator and, as suggested for the preceding methods, these values undoubtedly contributed to the increasing among-collaborator variance. Nevertheless, there was still considerable overlap in values for the different levels of sprout damage. This spread can be attributed not only to the reasons discussed under sources of error, but also to an additional factor. Most collaborators with falling number and amylograph equipment had used them on a routine basis. They were, therefore, familiar with the sequence of events necessary to perform the tests properly. In addition, much work has been done to standardize the procedures, as evidenced by the publication of approved procedures for both tests (4). No such familiarity or established procedure exists for the colorimetric test. For most collaborators, this study was their first exposure to the colorimetric assay. This is a relevant point because the colorimetric test is a dynamic enzymatic assay and some precision is required, particularly in the timing of the reaction, if reproducible results are to be obtained. In our work with this test, experience did increase precision considerably.

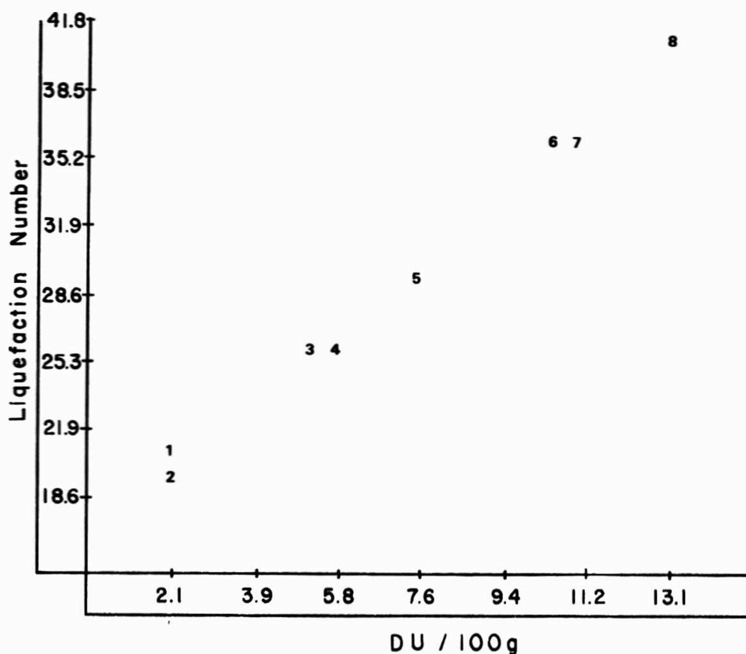

Figure 4. Relationship between means of reported values from all collaborators for colorimetric and falling number tests (expressed as DU/100 g and liquefaction number). Designations same as in Figure 1.

The large range in reported values for each level of sprout damage was likely because of differences among collaborators. This is supported by data from Table 2 which illustrates that, as with falling number and amylograph results, the among-collaborator variance was the major contributor to the total variance. Although the within-sample variance increased from sound to the 10:1 blend, it did not significantly increase with further increases in the amount of sprout damage. This means that for all the sprouted blends tested, the reproducibility of the method was essentially equal. This is supported by graphical data (Figure 3b–d) which show that the spread in values within randomly selected laboratories did not increase as sprout damage increased. The significantly lower within-sample variance for sound wheat probably resulted more from low colorimeter response rather than an actual increase in reproducibility. A sound sample gives a very low result for the CBA test, whereas it would give the highest readings for the viscometric tests. These graphical data show that the different levels of sprout damage could be easily distinguished using the colorimetric test.

The increase in the absorbance as the blend ratio changed from 10:1 to 2:1 averaged 36.8%. In calculating this estimate, we did not include the increase in the absorbance from sound to 10:1 blend because we felt that this increase (~200%) unfairly inflated the estimate over the whole range. There was an 8-fold increase in the absorbance from sound (0.068) to 2:1 blend (0.530) compared with a 2-fold unit increase for falling number and amylograph data.

Relationships Among Methods

Figures 4–6, which were developed using the means of all reported values at each level of sprout damage, show that the results for all 3 tests were highly linearly interrelated, particularly between falling number and colorimetric data.

A decision as to which method will best measure sprout damage depends on acceptance of α-amylase content as an index of sprout damage. While we recognize that viscometric tests have some advantage in that they test the effect of sprout damage on the rheological properties of wheat (or flour) under conditions which approximate those of actual baking, the fact that α-amylase is directly related to baking properties indicates that a method which measures α-amylase may serve as a suitable alternative to viscometric methods. The fact that results from all 3 methods are linearly interrelated suggests that the primary effector of both viscometric tests is probably α-amylase. It is possible that α-amylase in sprouted wheat could become inactivated during storage. This would limit the effective-

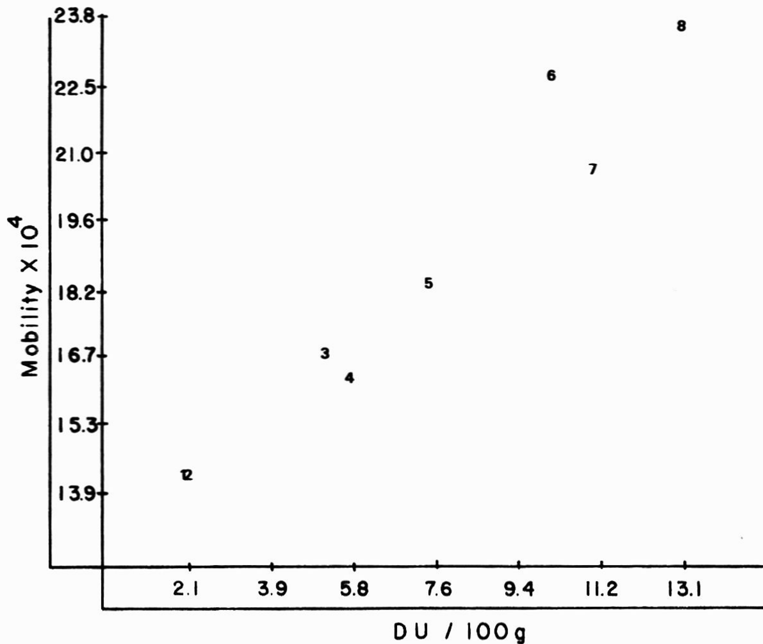

Figure 5. Relationship between means of reported values from all collaborators for colorimetric and amylograph tests (expressed as DU/100 g and mobility number). Designations same as in Figure 1.

ness of an evaluation based on α-amylase. However, such conditions would be relatively severe and probably unlikely, given normal storage conditions in the U.S. In addition, to eliminate all shortcomings would limit the possible tests to an actual baking test on the wheat flour. This would obviously be impractical at most grain evaluation facilities. We do not contend that an α-amylase assay is the ideal test, but rather that it can provide a simple, and, in

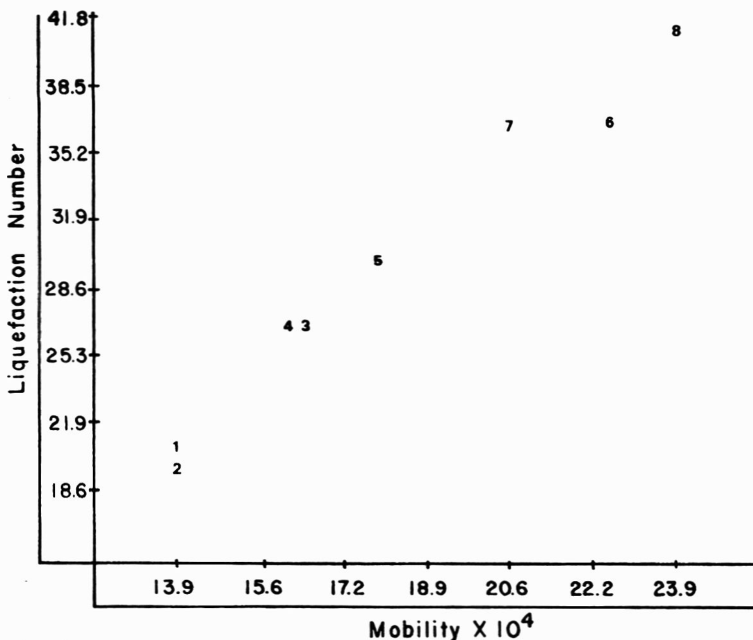

Figure 6. Relationship between means of reported values from all collaborators for falling number and amylograph tests (expressed as liquefaction number and mobility number). Designations same as in Figure 1.

most cases, accurate criterion for the initial evaluation of wheat for sprout damage.

Believing the colorimetric method to be a suitable means of assessing sprout damage, we wanted to determine how well the colorimetric test would predict the actual α-amylase content of the sprouted wheats. Therefore, α-amylase was determined at the USGMRL for each blend of sound and sprout-damaged wheat by both the micro-SKB method reported by Perten (5) and the colorimetric procedure (3).

There was a strong linear relation between the results for the 2 test methods ($r = 0.99$). A strong linear relation also existed between the values for the micro-SKB method and the mean colorimetric values obtained by the collaborators.

Conclusions

The major source of error in the 3 methods used in this study was variation among collaborators. Although each method could be made to perform adequately within one laboratory, there were large differences among laboratories. Considering the length of time that the amylograph and falling number methods have been in use, the results of this study indicate that standardization of these techniques is no easy task. We believe, however, that there is a potential for standardization of the colorimetric test. Common colorimeter and cell size and some experience should decrease differences among the collaborators. The absorbance change from one level of sprout damage to the next was considerably higher for the colorimetric method than corresponding changes for the falling number and amylograph methods. This indicates that, of the 3 tests, the colorimetric test is the most sensitive and provides the best capability to differentiate among levels of sprout damage. This capability is of particular importance because there is a real need for a highly sensitive method for measuring sprout damage, particularly for foreign markets.

High sensitivity, reproducibility, speed, and simplicity of analysis should make the colorimetric test an advantageous alternative to the present methods for evaluating sprout-damaged wheat in marketing channels.

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TECHNICAL COMMUNICATIONS

Ethylene Dibromide Residues in Biscuits and Commercial Flour

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Flour and biscuit samples from a school lunch program were analyzed for ethylene dibromide (EDB). Flour samples were extracted with hexane at room temperature with maximum extraction of EDB in 4 days. Biscuits were extracted by steam distillation with hexane; optimum recoveries were obtained by a triple extraction of the sample. Recoveries of EDB from flour and biscuits ranged from 85 to 103% as determined by gas-liquid chromatography on a 15% OV-17 column and a ^{63}Ni electron capture detector. Random samples were confirmed by gas chromatography-mass spectrometry. From <8 ppb to 4 ppm EDB were determined in flour and <0.5 ppb to 260 ppb in biscuits. Possible sources for the higher values are discussed.

Ethylene dibromide (EDB), commonly used as a grain fumigant, is presently on the Rebuttable Presumption Against Registration (RPAR) list. A model for predicting levels of EDB in flour and baked products had been formulated (1) and results given in this paper were used to confirm it. The levels of EDB in flour and biscuits were compared to determine if baking would remove EDB or if a relationship exists between the amount of EDB in flour with the amount remaining after baking.

EDB has been previously determined in air, water, soil, grain, and foodstuffs. Previous methods for determining EDB in grain include steam distillation (2), cold water-acetone extraction (3), sweep codistillation (4), and acid reflux (5). Many of the published methods were developed for grain, but flour is a more processed commodity and therefore more likely to pose problems with interferences. Berck (6) analyzed wheat, flour, bran, middlings, and bread for fumigants, using an acetone extraction and quantitation by ^{3}H electron capture gas-liquid chromatography (GLC). Unlike Berck's flour samples, in which the grain was processed into flour in the laboratory, our flour samples were commercial. An acetone extraction of our samples resulted in interferences which were remedied by using hexane. Some loss of EDB was expected through baking; therefore, we needed a more sensitive method to analyze biscuits.

Malone (7) compared 3 methods for the determination of organic fumigants in grain: sweep codistillation, steam distillation, and acid reflux. The listed sensitivities of each method for EDB in grain were 0.2 ppm (parts per million) for sweep codistillation, 0.15 ppm for steam distillation, and 0.03 ppm for acid reflux. Although it appears that the acid reflux would be the method of choice, Malone (7) states that the sensitivity of the steam distillation method could be increased 2.5 times if the total volume of the extraction solvent was limited to 10 mL instead of the 25 mL previously published. If, in addition, the sample size of the steam distillation method is increased to that of the acid reflux method (50 g to 100 g), sensitivities of the 2 methods would be equivalent. The steam distillation method was selected over the acid reflux method because of the simplicity of the apparatus.

Gas chromatography has been used often for the determination of EDB, with both flame ionization and electron capture detection. Clower (8) reported improved resolution and lower limits of detection (4.3 ng gave >50% recorder response) for EDB, using a 15% OV-17 GLC column and a ^{63}Ni electron capture detector. Under Clower's chromatographic conditions and a room temperature hexane extraction of the flour, samples varied from <6 ppb up to 4.2 ppm. The biscuit samples, extracted by steam distillation, and analyzed in the same manner resulted in samples with levels of EDB from <0.5 ppb to 260 ppb.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph* (GC).—Varian Model 3700, or equivalent, equipped with ^{63}Ni detector. Conditions: detector 260°C, column 90°C, injector 160°C; nitrogen flow 120 mL/min; 150 pg EDB gave half scale deflection with 1 mV recorder.

(b) *Chromatographic column.*—6 ft × 1/8 in. id

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stainless steel, packed with 15% OV-17 on 80-100 mesh Chromosorb W.

(c) *Gas chromatograph-mass spectrometer (GC/MS).*—Hewlett-Packard Model 5990. Conditions: injector 200°C, column 96°C; electron multiplier volts, 1800.

(d) *Chromatographic column.*—3 ft × 1/8 in. OV-101 (HP test column).

(e) *Barrett distilling receiver.*—SGA Scientific, Inc., Bloomfield, NJ 07003, or equivalent.

(f) *Hexane.*—Pesticide residue grade, or equivalent.

(g) *Ethylene dibromide standard.*—Prepare standards in hexane. Stock standard: Pipet 1 mL Baker grade EDB (mp 9-10°C) (98%) into 100 mL volumetric flask, dilute to volume with hexane (2.18 g/100 mL). Serial dilutions are made until working standards of 22 and 218 ng/mL are obtained.

Procedure

Samples of biscuits and flour, destined for the school lunch program, were obtained from USDA, Food and Grain Inspection Service. Biscuits had been baked from each of the flour samples 12 min at 425°F. After baking, samples were sealed in plastic bags and frozen to prevent any further loss of EDB. Flour samples were also sealed in plastic bags and frozen. All samples were analyzed at the Chemistry Laboratory of the Chemical and Biological Investigations Branch, Beltsville, MD.

Flour.—Flour samples were removed from the freezer, and allowed to warm to room temperature before weighing. Twenty grams of flour and 50 mL hexane were mixed together in an Erlenmeyer flask and sealed with a Teflon-lined screw-cap. Samples were kept at room temperature and shaken about once a day. The supernate was injected into the GLC system without any further cleanup or concentration.

Biscuits.—The biscuit samples were extracted by codistillation with hexane. Frozen biscuit samples, weighing between 30 and 50 g, were chopped into small pieces and weighed into 1 L round-bottom flask equipped with heating mantle, Barrett distilling receiver, and condenser. In addition to sample, 300 mL deionized water and 10 mL hexane were added. Enough heat was applied to cause boiling, and hexane was collected in distilling receiver, immersed in an ice water bath to prevent loss of EDB or hexane. The heating was stopped when the first drops of water condensed into the receiver tube. The hexane was transferred to 15 mL glass-stoppered test tube, and 2-3 g sodium sulfate was added to remove water. Samples were injected onto a gas chromatograph without any further cleanup or concentration.

Chromatographic Analysis

An 8 μL aliquot of hexane supernate was injected into the gas chromatographic system. A suitable amount of standard was injected to give a similar size peak as that of sample. Samples and standards were injected alternately and quantitated by peak height. Detector temperature was set at 260°, not 350°C as published by Clower, for maximum sensitivity. Individual instruments may vary and the detector temperature may have to be selected for each instrument for best results. Confirmation was based on retention times and peak ratios of the 2 masses, obtained by injection into the GC/MS system operated in the select ion monitoring program set on 107 and 109 mass units.

Results and Discussion

Flour samples determined to be free of EDB were spiked in triplicate by pipetting 0.5-1.0 mL standard directly into the flour sample. Recoveries were 5 ppb (93%), 20 ppb (103%), 30 ppb (102%), 40 ppb (90%), and 100 ppb (104%). Samples were analyzed daily for 10-14 days. Maximum levels of EDB were obtained at day 4. The biscuit extraction method was a modification of Bielorai and Alumot's steam distillation procedure (2). Hexane was satisfactorily substituted for toluene because of the GLC temperature conditions. Recovery data for triplicate spiked biscuit samples were 0.5 ppb (80%), 1 ppb (100%), 2 ppb (96%), 5 ppb (90%), and 20 ppb (99%). Biscuit samples, portions of which had been previously analyzed as negative for EDB, were spiked by pipetting 0.5-1.0 mL of a standard solution into a 1 L round-bottom flask. Some initial biscuit samples were analyzed by extracting only once with hexane. Further work indicated that one extraction was insufficient and 3 extractions appeared optimum. Each of the extracts was analyzed separately. Although with most samples 2 extractions were sufficient, 3 extractions are indicated to ensure complete EDB recovery. Various methods were tried but did not improve the recovery of the extraction of EDB: Iso-octane was substituted for hexane to increase the temperature of the distillation; the hexane, water, and bread were mixed together and left overnight at room temperature before steam distilling. This incomplete extraction appears to be due to the extraction efficiency of hexane and water.

Table 1. Concentrations of EDB in flour and biscuit samples

No.	EDB, ppb	
	Flour[a]	Biscuits[a]
14648	ND	ND[b]
14647	6	0.5[b]
19507	9	1
19509	11	3
14178	11	14
19506	13	3
14623	14	ND[b]
17108	14	15
19510	18	6
14185	21	1[b]
17103	27	9
17100	28	12
13486	30	16[b]
14650	34	27
13411	53	69
19512	67	3
14645	98	2[b]
7761	1.7 ppm	29[b]
13410	3.4 ppm	143
13409	4.0 ppm	260[b]
17141	4.0 ppm	66
14165	4.2 ppm	106

[a] ND = not detected. ND flour = <5 ppb; ND biscuits = <0.5 ppb. The limit of detection was calculated as 2.5 times the signal-to-noise ratio on the chromatograph.
[b] Extracted once only.

Results for the flour and biscuit samples are shown in Table 1. Our results show little correlation between levels of EDB in flour and biscuits. EDB is currently registered for use in post-harvest fumigation and fumigation spot-treatment of grain milling machinery. There are innumerable variables which affect adsorption and desorption of EDB on grain (9) (i.e., humidity, temperature, length of time, etc.); therefore, it is unlikely that enough documentation can be obtained to relate the final levels to EDB treatment. Use of commercial samples is necessary to determine final EDB levels for evaluation of potential dietary intake.

The Food and Agriculture Organization (FAO) (10) has set recommended guidelines of 0.1 ppm (10 ppb) for EDB in cooked cereal products. Eleven samples were above this suggested limit. Further work is needed to establish the statistical significance of such findings.

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Anomalies in the Mass Spectrum of *N*-Nitrosopyrrolidine

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Several anomalies in the published standard mass spectrum of *N*-nitrosopyrrolidine (NPYR) were pointed out and a new standard spectrum was presented. It was suggested that the published standard was partially thermally degraded, perhaps by an overheated inlet line or interface to the mass spectrometer. Specific anomalies thought to be due to overheating included a marked decrease in the relative intensity of m/z 100 (M^+) and a concomitant increase in m/z 30 and m/z 69. The large ion at m/z 28 could not be attributed to overheating, nor to an air leak; rather it is part of the normal spectrum for NPYR.

The legal mandate requiring mass spectral evidence for the confirmation of *N*-nitrosamines in cooked bacon (1) requires that reliable standard mass spectra be readily available. The standard spectrum recently published (2) under the aegis of the National Bureau of Standards (NBS) for *N*-nitrosopyrrolidine (NPYR), a carcinogen in experimental animals and often found in trace (ppb) levels in cooked bacon, is, in our view, markedly atypical of what is generally observed in gas chromatographic/mass spectrometric (GC/MS) analyses. It is well known that mass spectra can vary between instruments, and even with operational parameters (such as temperatures of inlet systems, etc.) on the same instrument (3, 4). Nevertheless, the standard spectrum referred to above may be atypical to the degree that confirmation of NPYR in foods and other products is made difficult.

For comparative purposes, 2 mass spectra of NPYR are given in Figure 1. On the right (B) is the spectrum of the NBS standard referred to above (2); on the left (A) is the spectrum obtained on our Finnigan Model 1015C GC/MS system. The gas chromatograph used was a Varian Aerograph 1400; the column was a stainless steel capillary 0.75 mm id × 152.4 m wall-coated with SF-96. The injection port temperature was 180°C and the column was isothermal at 125°C. The carrier gas was helium at 15 mL/min, and the column effluent was directed to a glass jet separator (interface) held at 165°C. The transfer line to the ion source was 140°C and the manifold was 115°C. The MS ion source was operated at 400 μA ionizing current and an electron energy of 70 eV; the electron multiplier was operated at 2.7 kV. Approximately 150 ng NPYR in hexane was injected, and MS data were collected and processed using a Riber 400 data system. The spectrum shown in Figure 1A was obtained under the stated conditions. Different conditions, such as excessive interface or inlet temperatures, may give rise to markedly different spectra.

Although both Pensabene et al. (5) and Lijinsky et al. (6) have published collections of standard spectra for *N*-nitroso compounds including

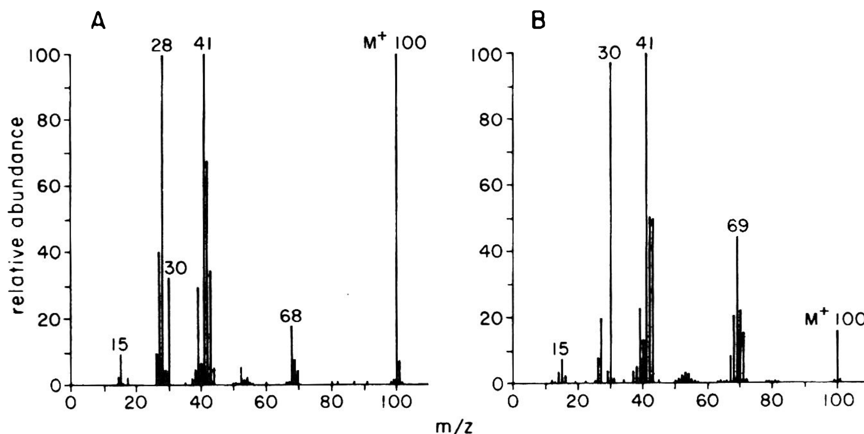

Figure 1. Mass spectra of *N*-nitrosopyrrolidine: A, present study; B, Heller and Milne (2).

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NPYR, to our knowledge the discrepancies in the NBS standard have not been pointed out.

In the trace analysis of foods for NPYR, we routinely find that m/z 28 is very large, if not the base peak. Furthermore, we suspect that thermal decomposition of NPYR in heated inlet systems may yield artificially high relative abundances for m/z 30 and m/z 69 and a lower abundance for M^+ (m/z 100). A recent study of the thermal dehydrogenation during GC/MS of 2-hexyl-5-pentylpyrrolidine leading to the 2 corresponding dialkylpyrrolines (4) gives credence to this idea. In conclusion we feel that Figure 1A represents much more accurately the electron impact fragmentation pattern of undegraded NPYR as usually observed by GC/MS.

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Jack Bean Meal as Source of Urease in Determination of Urea

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Ground jack bean was added to feed samples as a source of urease in urea determinations. Jack bean meal is much less expensive and is more stable in the presence of soluble salts than is refined urease powder. Percent recoveries in terms of protein equivalent for 3 types of feed ranged from 96.1 to 98.9, using the proposed method. Jack bean meal was equal or superior to refined urease powder in converting urea to ammonia in all samples analyzed.

The present AOAC urea method **7.033** (1) has remained unchanged since 1941 (2), even though the method was reviewed in 1943 and 1944. In 1944, the reviewers recommended that study be discontinued (3).

Our interest in improving this method was motivated by the high price of urease powder and the poor accuracy and precision in determinations on liquid feeds. Since many liquid feeds and mineral mix feeds have high concentrations of soluble salts, the resulting pH after adding water may inactivate the commercially available urease. We tried with some success to adjust the sample plus water to pH 5 before adding urease; however, the pH adjustment required considerable time and urease powder was still inactivated by some samples that had high concentrations of soluble salts.

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Ground jack beans containing urease can be purchased relatively inexpensively from several chemical companies. Therefore, we decided to investigate jack bean meal as a urease source.

METHOD

Reagents

(a) *Defoaming solution*.—Antifoam B emulsion (Dow Corning Corp.).

(b) *Jack bean meal*.—To pass 1.5 mm screen.

(c) *Calcium chloride solution*.—Dissolve 25 g $CaCl_2$ in 100 mL water.

(d) *Phenolphthalein solution*.—Dissolve 0.1 g phenolphthalein in 100 mL 50% alcohol.

(e) *Hydrochloric acid* (*1 + 1*).—Add 1 part HCl to 1 part water.

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Table 1. Recovery of nitrogen from urea standard by proposed method[a]

Urea, g	Nitrogen, %	Std dev.	Rec., %
0.2	45.78	1.31	99.1
0.3	45.89	1.28	99.3
0.4	45.71	1.29	98.9

[a] Urea standard contained 46.2% N (288.8% protein) from quantitation by AOAC method **7.015**. Means and standard deviations from 10 analyses are shown.

Table 2. Determination of urea blank in selected feeds

Sample	Sample wt, g	Blank (equivalent protein, %)[a]
Cottonseed meal 41% protein	1	0.27
	3	0.24
	5	0.26
	7	0.25
Mean		0.26
Milo 9.5% protein	1	0.05
	3	0.06
	5	0.09
	7	0.07
Mean		0.07

[a] Values include urea blank from 0.5 g jack bean meal.

Procedure

Place 1–5 g feed sample (to contain ca 0.2 g protein equivalent) in Kjeldahl flask with ca 250 mL water. Add 3–5 drops phenolphthalein solution and 1 scoop (0.5–1 g) jack bean meal, stopper tightly, mix by swirling flask, and let stand 1 h at room temperature. If mixture turns pink, immediately add HCl (1 + 1) dropwise until pink color disappears. Rinse stopper and neck of flask with 3–4 mL water. Add 2 g MgO (heavy type), 5 mL $CaCl_2$ solution, and 3 mL defoaming solution. Connect flask to condenser by Kjeldahl connecting bulb. Distill 100 mL into measured volume standard acid, **2.055(j)** (1), and titrate with standard alkali, **2.055(k)**, using methyl red, **2.055(i)**. Determine blank for each lot of jack bean meal, using above sample procedure. If jack bean blank is significant, subtract blank from final result.

Results and Discussion

A urea standard was analyzed 10 times by the Kjeldahl method for crude protein in animal feed, **7.015 (1)**. The resultant mean was 46.2% N (288.8% crude protein from N × 6.25). Recoveries ranged from 98.9 to 99.3% when N was determined by the proposed urea method and compared with total N determined by AOAC method **7.015** (Table 1). Standard deviations of less than 1.4 at 3 different weights indicate that analysis of the urea standard by the proposed method was precise.

There is a urea blank in some feeds due to the breakdown of the feed protein to ammonia. Values in Table 2 indicate that the urea blank observed using the described method was small and relative to the total protein of the feed.

Recoveries were determined on 2 g samples of feed spiked with 0.2 g urea. Calculated on the basis of a 2 g sample, the added urea should produce 28.88% protein. The values in Table 3 indicate that recoveries for the proposed method ranged from 96.1 to 98.9% on the feed materials tested. The percent recovery for cottonseed meal and milo was approximately the same with the proposed method (jack bean meal) as with method 7.033 (urease powder). However, the method using jack bean meal gave better recovery in a mineral mix.

The recovery values indicate that jack bean

Table 3. Recovery of protein equivalent from urea in feed samples

Sample (2 g)	Urea blank, % protein[a]	Urea added, g	Urea − blank, % protein[a]	Rec., %
Proposed Method (Jack Bean Meal)				
Cottonseed meal	0.26	0.2	28.43 ± 0.37	98.5
Milo	0.07	0.2	28.57 ± 0.36	98.9
Mineral mix	0.01	0.2	27.75 ± 0.41	96.1
AOAC Method **7.033** (Urease Powder)				
Cottonseed meal	0.20	0.2	28.51 ± 0.45	98.7
Milo	0.03	0.2	28.50 ± 0.38	98.7
Mineral mix	0.01	0.2	26.12 ± 0.39	90.4

[a] Results are means and standard deviations of 5 determinations.

meal is a satisfactory substitute for refined urease powder for determining urea in feeds. The major benefits are that jack bean meal is much cheaper than refined urease powder, and a special urease solution does not have to be prepared and neutralized for each set of samples.

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Care of Reverse Phase High Performance Liquid Chromatographic Columns When Injecting Extracts of Fat- or Oil-Based Preparations

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When analyzing extracts of pharmaceutical preparations, the chromatographer may place some of the base fats or oils onto the reverse phase analytical column with each injection. This is not a problem if they are washed free from the column periodically. If not cleaned off the column, column efficiency and selectivity can be lost forever. A simple procedure is described for washing columns.

Recently, Van Dame (1) described the quantitative determination of steroid acetates in pharmaceuticals by high performance liquid chromatography (HPLC). This procedure and others requiring similar separations can present a problem. While the procedure may seem to be quite satisfactory, the acetonitrile extraction may also extract oil from the formulation. The oil is then injected onto the LC column along with the sample extract. With time, the oil can alter column performance.

Usually such oils are highly retained on a C_{18} or C_8 bonded phase, particularly with high water content mobile phases such as that used by Van Dame. These oils (which can be eluted with nonpolar mobile phases) slowly coat the C_{18} or C_8, forming a modified bonded phase which eventually will affect the separation. How rapidly this occurs depends on the amount of oil in the formulation, the composition of the extracting solvent, the number of injections made, and the composition of the mobile phase. When and if these oils elute, they are not seen in the 230-280 nm UV region most used for detection, but the separation may be unsatisfactory.

In addition, we have found that if an oil-modified column is not frequently cleaned, the oil migrates into the inner pores of the bonded phase support to permanently change its selectivity, i.e., probably rendering the column useless.

If oil-containing solutions are injected onto a bonded reverse phase column, it is recommended that after each 10 samples or at the end of each day, the column be washed with 20 column volumes (50 mL for a 4.6 mm × 25 cm column) of pure tetrahydrofuran or methylene chloride followed by acetonitrile or methanol, whichever is the organic component of the mobile phase to be used for the separation. This will wash the column of any of the cleaning solvents. The extra time taken for cleaning will increase reproducibility and column life. Although guard columns can be used ahead of the analytical column (2), these do not have the capacity to effectively remove the oils from samples. Thus, even if a guard column is used as a final filter of particulate and impurities, the washing procedure is recommended. Column washing is suggested not only in reference to the above article, but should be related to any similar HPLC procedure.

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Gas Chromatographic/Mass Spectrometric Study of Isoequilin A

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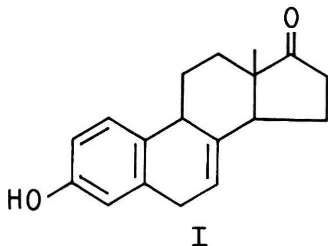
Isoequilin A, which is produced by the acid-induced isomerization of equilin, has been resolved into 3 major components by gas chromatography. Tentative identification of the 3 compounds was made by using combined gas chromatography/mass spectroscopy. One of these compounds, identified as 8-dehydroestrone, closely resembled a component previously identified in pregnant mares' urine.

A number of pharmaceutical products used for estrogen replacement therapy are derived from the urine of pregnant mares. Equilin (I), present as a sulfate ester, is one of the major estrogens excreted by the pregnant mare (1) and one of the required components in both conjugated and esterified estrogens (2). The existence of an equilin isomer as a naturally occurring minor constituent of pregnant mares' urine, and hence pharmaceutical products, is now generally accepted (3–5). However, the chemical structure of such an isomer has not been conclusively established.



Because of the low concentration of the equilin isomer in urine, early workers attempted to synthesize compounds with similar properties for use as an identification tool. In one such attempt (6), equilin was refluxed in acid to produce a compound, termed isoequilin A, to which the authors assigned the structure 8-dehydro-14-isoestrone. This structure was unusual because the authors concluded that, in addition to rearrangement of the equilin double bond to a position of conjugation with the aromatic A ring, epimerization had taken place at carbon 14. Later, Banes et al. (7) repeated the synthesis and resolved isoequilin A into 2 components identified as 8-dehydro-14-isoestrone and 9-dehydro-14-isoestrone. These authors isolated a compound with properties similar to 9-dehydro-14-isoestrone from urine of pregnant mares but concluded that the compound was an artifact produced by isomerization of equilin during the acid hydrolysis of the sulfate esters to the free steroids.

Later, using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS), Roman et al. (3) identified 8-dehydroestrone as a naturally occurring component of pregnant mares' urine. A variety of hydrolytic conditions were used to verify that the equilin isomer in the urine was not an artifact of hydrolysis. The compound was determined to have the naturally occurring α configuration at carbon 14 using the classic palladium-induced dehydrogenation devised by Hirschmann and Wintersteiner (6). Assignment of the double bond to the 8,9 position was based on mass spectral evidence.

Conversely, Roos (5) recently identified 9-dehydroestrone as a component in commercial conjugated estrogen tablets by using high performance liquid chromatography (HPLC). This author was also able to resolve isoequilin A into 2 components. Similarities in chromatographic mobility and UV spectra led to the conclusion that the main component in isoequilin A and the equilin isomer in the estrogen tablets were the same compound.

Because of the important role isoequilin A has played in the structural identification of equilin isomers derived from pregnant mares' urine, a study of the formation of this product was undertaken in our laboratory.

Experimental

Reagents

Estrone was obtained from Roussel Ltd, and equilin and equilenin from Syntex Ltd. 6-Dehydroestrone and 14-isoequilenin were purchased from Mann Research Laboratories and Steraloids Inc., respectively. All other chemicals were reagent grade.

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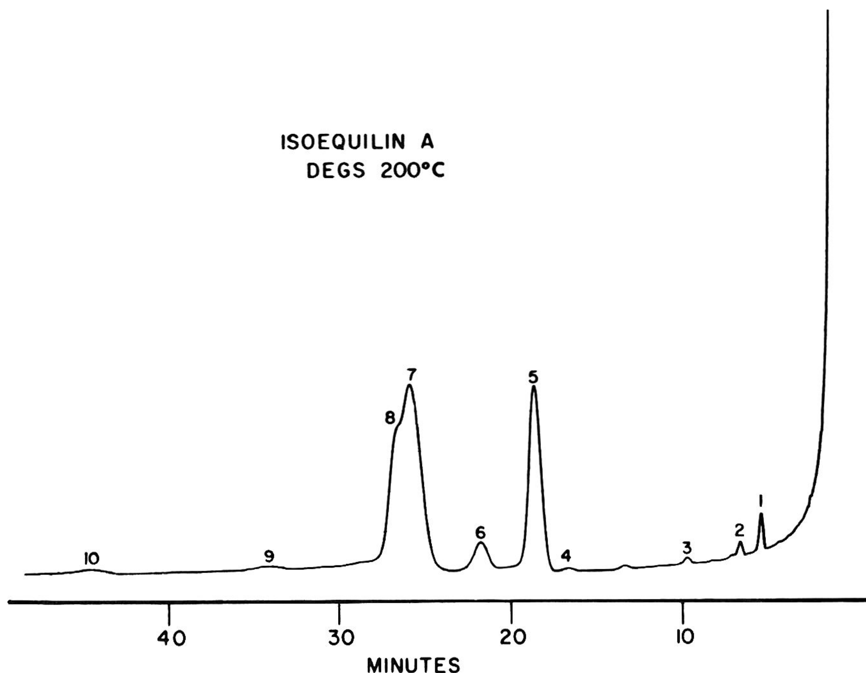

Figure 1. Gas chromatogram of trimethylsilyl derivative of isoequilin A on a 1% DEGS column at 200°C. See text for discussion of peaks.

Apparatus

(a) *Gas chromatograph.*—Perkin-Elmer Model 900 equipped with dual flame ionization detectors. Air flow 500 mL/min, hydrogen flow 40 mL/min, nitrogen carrier gas flow 15 mL/min. Peak areas were determined with Infotronics Model CRS-100 integrator.

(b) *DEGS column.*—8 ft × 2 mm id glass column packed with diethylene glycol succinate (DEGS) on 80–100 mesh Chromosorb G-HP (both from Chromatographic Specialties Ltd). Packing was prepared by solution-coating technique, using 1% (w/v) solution of DEGS in $CHCl_3$ followed by fluidized drying. Several batches of DEGS packing were prepared and repacking column with these materials resulted in same chromatographic characteristics. Column was conditioned at 200°C with nitrogen flowing at 15 mL/min overnight before use. Under normal operating conditions of column temperature of 200°C, injector temperature 260°C, manifold temperature 270°C, and nitrogen flow 15 mL/min, retention time for silyl ether of estrone on freshly packed column is ca 30 min. At this temperature, column bleed is appreciable and with continuous use there is a decrease in retention times of various components. By lowering column temperature to 150°C overnight, columns normally can be used from 2 to 4 weeks before loss in column efficiency necessitates replacing packing.

Gas Chromatography/Mass Spectrometry

Combined GC/MS was carried out with an LKB Model 9000 instrument. GC conditions were essentially the same as those described above, except that a 5 ft × 2 mm id column operated at 175°C was used to reduce GC bleed and hence MS background. MS conditions were as follows: electron energy, 70 eV; source temperature 270°C; accelerated voltage 3.5 kV; trap current 60 μA. Mass spectra of authentic samples of estrone, equilenin, and 14-isoequilenin as silyl ethers were determined, using same GC conditions. MS conditions were as described above.

Formation of Isoequilin A

Procedure of Hirschmann and Wintersteiner (6) was followed but on a much smaller scale. Twenty mg equilin was refluxed 80 min in mixture of 3 mL acetic acid and 2 mL concentrated hydrochloric acid. The same reaction was also performed with reflux time of 2 h, and reaction product was diluted with water and extracted with $CHCl_3$. Residual acid in $CHCl_3$ was neutralized with sodium bicarbonate, solution was

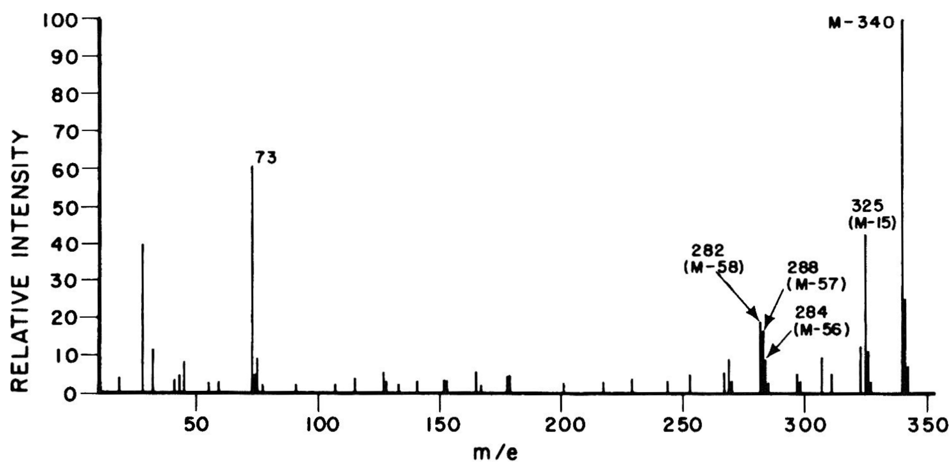

Figure 2. Mass spectrum of the trimethylsilyl ether of compound 7.

filtered, and solvent was removed. Residue was silylated and chromatographed as described previously (3).

Results and Discussion

Both Banes et al. (7) and Roos (5) reported that isoequilin A is comprised of 2 main components. Using the GC system previously reported for the identification of estrogens derived from pregnant mares' urine (3), isoequilin A was observed to have 3 major components. This is shown by the chromatogram in Figure 1.

The molecular ions of the 3 major peaks, 5, 7, and 8, indicate that all 3 compounds are isomeric with equilin. With the logical assumption that any isomer of equilin formed by acid-induced rearrangement would have a double bond conjugated to the aromatic A ring and the observation that none of the 3 isomers had the same relative retention time as an authentic sample of 6-dehydroestrone (3), we concluded that at least 1 of the 3 isomers must have the iso (β) configuration in the 14 position.

Although only partially resolved, it was pos-

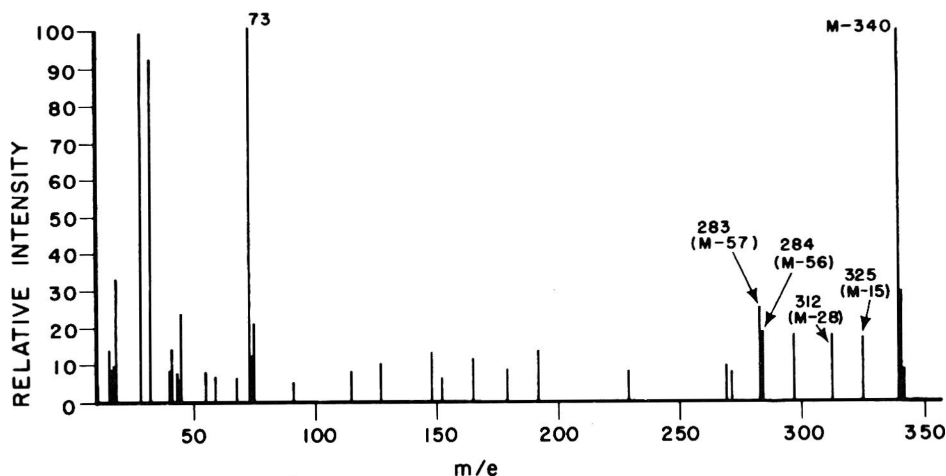

Figure 3. Mass spectrum of the trimethylsilyl ether of compound 8.

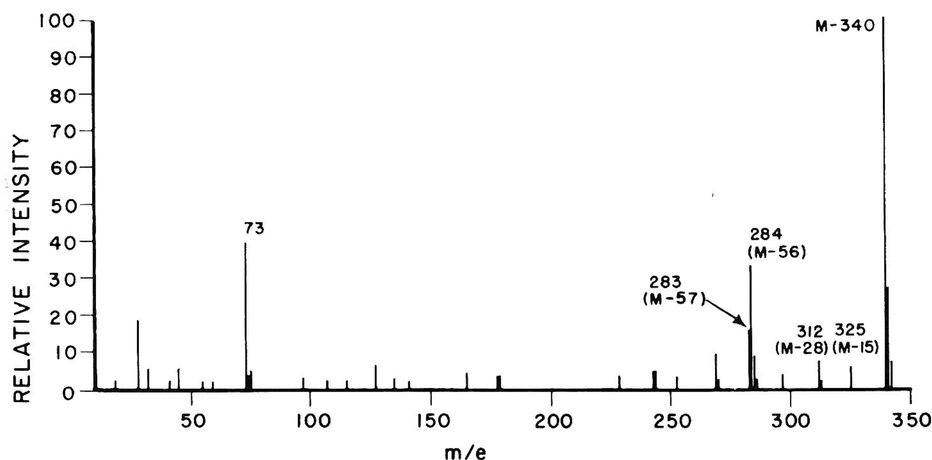

Figure 4. Mass spectrum of the trimethylsilyl ether of compound 5.

sible to obtain reasonable mass spectra for compounds 7 and 8 (Figure 1) by recording the spectra at the leading and trailing edges of the overlapping peaks. Mass spectra of compounds 7 and 8 are shown in Figures 2 and 3, respectively. Within experimental error, the relative intensity of major fragments in the mass spectrum of compound 8 is the same as reported for 8-dehydroestrone (3). Combined with the close agreement in relative retention times, 1.22 for compound 8 and 1.21 for 8-dehydroestrone (3), we concluded that the 2 are probably the same compound.

The mass spectrum of compound 7 is unique among spectra recorded for the components in isoequilin A as well as the spectra obtained for estrogens in pregnant mares' urine (3). The spectrum is the only one recorded containing an M-58 peak. In addition, the M-15 peak is much more intense (relative intensity 40%) than observed for any other spectrum. According to Djerassi et al. (8), both of these features are characteristic of an estrogen with a double bond in the 9,11 position. It was the absence of these mass spectral features that led Roman et al. (3) to conclude that the isomer of equilin in pregnant mares' urine was not 9-dehydroestrone. Based on the characteristic fragmentation pattern, compound 7 was tentatively identified as either 9-dehydroestrone or 9-dehydro-14-isoestrone. As indicated by Djerassi et al., it is not possible to predict the stereochemistry at position 14 from MS data alone.

With the exception of an intense M-56 peak, the mass spectrum of compound 5, shown in Figure 4, is similar to the spectra for equilin and 8-dehydroestrone (3). Since the mass spectrum does not have the characteristic fragmentation expected for an estrogen with a double bond in the 9,11 position and since the relative retention time (1.11) does not correspond to that for either 6- or 8-dehydroestrone, compound 5 must be either 8-dehydro-14-isoestrone or 6-dehydro-14-isoestrone.

Compounds 9 and 10 were established to be 14-isoequilenin and equilenin, respectively, by comparison of relative retention times and fragmentation patterns to authentic standards. Compound 6 is estrone, present as an impurity in the equilin (approximately 4%) used to prepare isoequilin A, and was used as an internal standard to calculate relative retention times. Compound 4 has a fragmentation pattern similar to estrone. Not present in the original equilin sample, compound 4 is possibly isoestrone formed by an acid-induced epimerization at the 14 position of estrone. Compounds 1 and 2 have molecular ions indicative of dihydroequilenins. However, the relative retention times of these compounds, 0.23 and 0.29, are much shorter than are those for 17α and β-dihydroequilenin in pregnant mares' urine, relative retention times 0.46 and 0.52, respectively (3). These compounds are probably the 14-epimers of 17α and β-dihydroequilenin. Compound 3 is a minor component of isoequilin but it is of interest be-

cause its molecular ion is 2 mass units lower than dihydroequilenin. Presumably, it is formed from dihydroequilenin, and has an additional double bond in conjugation with the aromatic A and B rings.

The GC and GC/MS data on isoequilin A are consistent with the reported structural assignment of 8-dehydroestrone as the principal equilin isomer in pregnant mares' urine (3). However, the complexity of isoequilin A, comprised of 3 isomers with both structural and stereochemical differences, makes it a poor model for assigning the unknown structures of naturally occurring equilin isomers.

In addition to the problem of relating equilin isomers in isoequilin A to those present in pregnant mares' urine, a second reason for the different structures suggested by Roman et al. (3) and Roos (5) may be the different chromatographic techniques used. The GC technique used by Roman et al. shows the same response factor for equilin and its isomers, whereas the HPLC technique with UV detection used by Roos is much more sensitive to the equilin isomers than to equilin itself. According to the UV spectra published by Banes et al. (7), the 8,9 and 9,11 isomers have absorptivities approximately 50 and 100 times, respectively, that of equilin at 254 nm. Thus, while the presence of a 9,11 isomer at 10% the level of 8-dehydroestrone in pregnant mares' urine would probably go undetected by GC, the same amount of material could yield an HPLC peak comparable in size to that for equilin.

Acknowledgment

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Novel Thin Layer Chromatographic Techniques for Aflatoxin Analysis

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Some procedures for improving thin layer chromatographic (TLC) spot size and fluorescence intensity are described. By using strong eluting solvents, diffuse TLC spots can be reduced in size and poorly resolved chromatograms returned to their original state for redevelopment. The blanketing of weak aflatoxin bands with an inert layer of gas results in an increase in UV fluorescence.

In the course of developing new methods of aflatoxin analysis, a number of interesting thin layer chromatographic (TLC) techniques of general applicability were discovered. To decrease the time required for elution and spotting, our laboratory began using high performance TLC plates with a preadsorbent spotting zone (Whatman LHP-K).

When using TLC for the detection of aflatoxin M_1 in milk, a problem arose. The lipid content of the milk extract exceeded the capacity of the TLC plates, resulting in a smearing of the aflatoxin spots whenever excessive extract was spotted on the plates. This may in part be overcome by eluting first with anhydrous ethyl ether, which elutes the lipids but leaves the aflatoxin position almost unchanged.

Another technique has proven useful where

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a chromatographic separation has been unsuccessful and there is no more sample available. By eluting with methanol in the opposite direction to the first elution, a chromatogram can be returned to its original state for another attempt at separation. If all the components have not quite returned to their original position, this can be corrected by a brief elution with methanol in the forward direction until all the components are merged.

An approach that is quite effective in reducing the size of individual TLC spots is spotting methanol with a microsyringe, a few millimeters on either side of the aflatoxin spot. Where the 2 expanding spots merge, the aflatoxin will be deposited as a line as the methanol evaporates. This line may be reduced to a point by applying the above procedure to the ends of the line. Caution: Have adequate ventilation when using this procedure; exposure to methanol vapor can result in blindness.

This technique has proven useful in correcting poor spotting and confirming the color of weak spots. In using this technique, exposure to UV light should be limited to avoid decomposition. When confirming the color of weak spots, caution should be used because undesired material may be concentrated as well. This can be checked by eluting the concentrated spot with a suitable solvent mixture. If 2-dimensional chromatography had been previously performed, it may be necessary to move spots that could interfere to one side with methanol from a microsyringe.

We have found that by blowing a stream of inert gas onto a weakly fluorescent spot, the quenching by the oxygen in the air is reduced and the fluorescent intensity is increased. Moving the stream of inert gas back and forth across a suspected spot makes its fluorescence particularly noticeable as the fluorescence fluctuates. This fluorescence enhancement by an inert atmosphere may prove useful in quantitative fluorodensitometry.

Acknowledgment

The author thanks Leonard Stoloff, Food and Drug Administration, Division of Chemistry and Physics, and Robert Stubblefield, U.S. Department of Agriculture, for their helpful advice in our work.

Template for Marking Thin Layer Chromatographic Plates for Two-Dimensional Development

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A compact template for marking 200 × 200 mm thin layer chromatographic (TLC) plates for 2-dimensional TLC is described. Directions are provided for use of the easily fabricated apparatus. The template permits rapid, accurate, and reproducible marking of plates for application of samples and standards.

Two-dimensional development is a technique used in thin layer chromatography (TLC) in which substances are separated, first by development in one direction and then by development in a direction perpendicular to the first. The technique is used to resolve a mixture of substances that are poorly separated by 1-dimensional TLC (1).

The layout of TLC plates with pencil and ruler in preparation for 2-dimensional development can be a tedious chore. In 1960, Mitchell and Mills (2) described a template for simplifying the marking of paper chromatograms for use in 2-dimensional chromatography. Since the template described for paper touches the surface during marking of TLC plates it may mar or contaminate the adsorbent layer. A more compact template and its use for accurately and neatly marking plates or sheets for 2-dimensional TLC is described here.

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This paper was presented at the 94th Annual Meeting of the AOAC, Oct. 20-23, 1980, at Washington, DC.

Design and Use of Template

The template (210 × 70 × 11 mm) is made of aluminum or other suitable material (Figure 1). The width was chosen so that the distances on a plate (Figure 2) from points 1, 2, 3, and 4 and 1, 5, 6, and 7 to lines f_1 and f_2, respectively, are exactly 100 mm (10.0 cm, or the standard development distance). Templates of the same general design but with different dimensions can be

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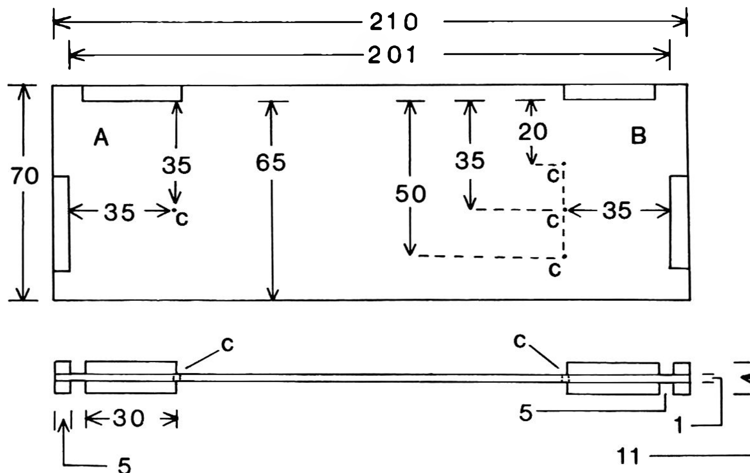


Figure 1. Template dimensions and construction. The template is made of aluminum. Dimensions are in mm. Holes at C are 2 mm in diameter. Each of the 4 pairs of supports (30 × 5 × 5 mm) is secured to the 1-mm thick base with 2 No. 256 flat-head screws (not shown). Top and front views are shown in the upper and lower drawings, respectively.

constructed to provide other development distances or numbers of sample points.

The supports (30 × 5 × 5 mm) on the template are designed to touch the edges of a 200 × 200 mm TLC plate or sheet during marking. The template may be turned over to facilitate laying out sample points. A sharp metal stylus of less than 1.5 mm diameter or a pencil is used to mark on or through the adsorbent layer.

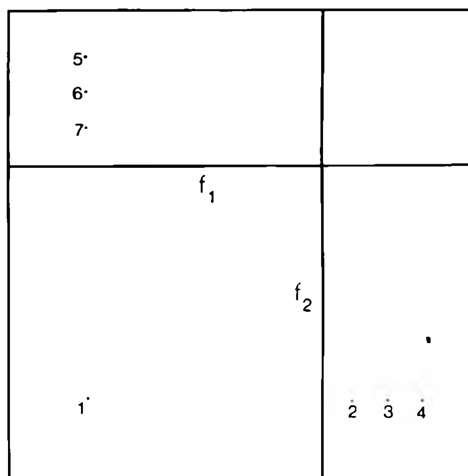


Figure 2. Layout of a TLC plate for 2-dimensional TLC. Lines f_1 and f_2 are 100 mm from point 1. Sample is applied at point 1 and standards at points 2-4 and 5-7.

Place the template over the right side of the plate with end A (Figure 1) over the upper corner and end B over the lower corner. Mark points 2, 3, and 4 (Figure 2). Using the left edge of the template as a straightedge, draw line f_2. Turn the template over and place it over the lower portion of the plate. Put end A over the left corner and end B over the right corner. Mark point 1. Put the template over the upper portion of the plate with end A over the right corner and end B over the left corner. Mark points 5, 6, and 7. Using the lower edge of the template as a straightedge, draw line f_1.

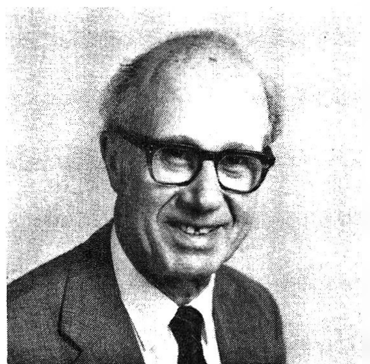
Discussion

Use of the template produces marked plates without contaminated or scratched surfaces because the adsorbent layer is not touched. Use of the template in the determination of organophosphorus pesticide residues (3) indicates that markings are accurate and reproducible from plate to plate. Each plate can be laid out in less than 30 s using the template (more than 90 s were required using a ruler). The template's compact size facilitates handling and storage.

References

(1) Stahl, E. (1969) *Thin-Layer Chromatography, A Laboratory Handbook*, 2nd Ed., Springer-Verlag, New York, NY, p. 88
(2) Mitchell, L. C., & Mills, P. A. (1960) *J. Assoc. Off. Agric. Chem.* **43**, 748–750
(3) Gardner, A. M. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 517–524

FOR YOUR INFORMATION



1981 Wiley Award Winner is Leonard Stoloff, FDA, for Work in Mycotoxins

Leonard Stoloff, Senior Chemist with the Food and Drug Administration's Bureau of Foods, Division of Chemistry and Physics, is the winner of the 1981 AOAC Harvey W. Wiley Award. Mr. Stoloff is receiving the award for his outstanding contributions to research and methods development for mycotoxins. Helen L. Reynolds, 1981 AOAC president, will present the award to Mr. Stoloff on Monday evening, October 19, during AOAC's 95th Annual Meeting.

The $750 award is given annually to a scientist who has made outstanding contributions to the development and validation of methods of analysis for foods, vitamins, food and color additives, pesticides, drugs, cosmetics, plants, feeds, fertilizers, forensic sciences, environmental contaminants, or other related areas. The award was established in 1956 in honor of Harvey W. Wiley, "Father" of the 1906 Pure Food and Drug Act, and a founder of the AOAC. Its primary purpose is to emphasize the role of the scientist in protecting the consumer and the quality of the environment.

Mr. Stoloff has been largely responsible for the development of the science of mycotoxicology as an important component of the agricultural and public health sciences on an international scale. He has been involved in mycotoxin research since its infancy, beginning with the isolation of aflatoxin B_1 for the structural elucidation studies at the Massachusetts Institute of Technology. In 1964, his research group at FDA was the first to develop a practical method for the analysis of peanuts and peanut products for aflatoxins.

As FDA's top agency specialist in the technology and chemistry of mycotoxins, Mr. Stoloff directs all FDA research on mycotoxin analytical methodology, coordinates interagency mycotoxin surveillance and survey programs, and maintains a comprehensive computerized mycotoxin literature data bank. He has been instrumental in the development and implementation of FDA programs of certification and regulation to ensure that contaminated foods and feeds do not enter commercial channels.

As General Referee on Mycotoxins for the AOAC since 1969, he has played the key role in orchestrating the development and interlaboratory validation of methods for aflatoxin sampling and analysis.

Mr. Stoloff is author or co-author of 86 publications, is a member of the American Chemical Society's Division of Agricultural and Food Chemistry, and has served in various positions, most notably as editor of the newsletter, *The Cornucopia*, from 1962 to 1980. He is also a member of the Institute of Food Technologists, American Association for the Advancement of Science, and the Washington Chromatography Group. He received an FDA Group Commendable Service Award in 1974 and was named a Fellow of the AOAC in 1977.

Before coming to FDA in 1963, Mr. Stoloff served as Assistant Technical Director with Marine Colloids, Inc., in Rockland, ME, from 1959 to 1963. Before that, he was Director of Research of Seaplant Corp., New Bedford, MA, from 1951 to 1959, and was Chief Chemist with the Krim-Co Corp., New Bedford, from 1944 to 1951. He also served as a chemist with the U.S. Fish and Wildlife Service (1942–1944).

Mr. Stoloff holds a B.S. from Massachusetts Institute of Technology. He lives in Silver Spring with his wife, Pauline.

1981 Fellows of the AOAC Announced

Helen L. Reynolds, AOAC president, will present Fellow of the AOAC Awards to 8 accomplished scientists at AOAC's 95th Annual Meeting in Washington, DC, on Monday morning, October 19, 1981.

The recipients of the awards are Charles C.

Clark, Drug Enforcement Administration, Miami, FL; Louis L. Gershman, Food and Drug Administration, Boston, MA; Kenneth Helrich, Rutgers University, Cook College, New Brunswick, NJ; Arthur R. Johnson, Food and Drug Administration, Washington, DC; Valva C. Midkiff, University of Kentucky, Lexington, KY; James P. Minyard, Jr, Mississippi State Chemistry Laboratory, Mississippi State, MS; Robert D. Stubblefield, U.S. Department of Agriculture, Peoria, IL; and Sidney Williams, Food and Drug Administration, Washington, DC (retired).

The Fellow of the AOAC Award was established in 1961 to recognize those persons giving meritorious service to the Association. Winners of the Award have performed notably for 10 years or more, usually as officers, referees, or committeemen. Nominations are made by AOAC members, reviewed by the Committee on Fellows, and finally approved by the Board of Directors.

Charles C. Clark has held a total of 5 Associate Refereeships. At present, he is Associate Referee for 3 topics—Ephedrine, Cocaine, and Phencyclidine—and General Referee for Narcotic and Dangerous Drugs.

Louis L. Gershman has held 3 Associate Refereeships and is General Referee for Fish and Other Marine Products.

Kenneth Helrich has been an Associate Referee. At present, he is Chairman of Committee E, and a member of the Long Range Planning Committee.

Arthur R. Johnson has held 3 Associate Refereeships and is General Referee on Sugar and Sugar Products and Secretary to Committee C.

Valva C. Midkiff has been a General Referee and a member of the Committee on Automated Methods. At present, he is a member of Committee G and is Associate Referee on Sampling and Sample Preparation of Feeds.

James P. Minyard, Jr., AOAC's President-Elect, has been an Associate Referee, and a member of 2 Committees. At present, he is a member of the Board of Directors, the Committee on Classification of Methods, the Committee on International Cooperation, and a member and past Chairman of the Editorial Board.

Robert D. Stubblefield is Associate Referee for Aflatoxin M and is a member of the Joint Mycotoxins Committee.

Sidney Williams has held 4 Associate Refereeships and been a member and Chairman of Committee E. At present, he is Editor of *Official Methods of Analysis*, 14th Edition.

AOAC to Hold 95th Annual Meeting October 19-22

The Association of Official Analytical Chemists (AOAC) will hold its 95th Annual Meeting October 19-22, 1981, at the Marriott Twin Bridges Hotel, Washington, DC. Current developments in analytical methodology pertaining to agricultural, environmental, and public health areas will be presented and discussed.

Five symposia planned for this meeting are: AOAC Methods Development—Challenge of the Next Decade, Monday, October 19; Analytical Methodology for Lead in Foods, Monday and Tuesday, October 19 and 20; Computers in the Laboratory, Tuesday, October 20; Infant Formula Regulation and Infant Food Problems, Wednesday, October 21; and Problems and Solutions in Trichothecene Methodology, Wednesday, October 21.

About 200 papers will be given on new techniques, methods and instrumentation for analysis of foods, drugs, pesticides, cosmetics, feeds, fertilizers, mycotoxins, beverages, colors, forensic science materials, hazardous substances, vitamins, water and air pollutants, microbiological and extraneous material contamination of foods, and related subjects.

October 17-18, immediately preceding the AOAC Meeting, the American Chemical Society will sponsor a course on Laboratory Automation: Micro-, Mini-, or Midi-Computers. Instructor will be Dr. Raymond E. Dessy, Virginia Polytechnic Institute and State University.

For further details, contact Kathleen M. Fominaya, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; phone 703/522-3032.

Workshop Targets New SRMs to Improve Nutrition Measurement

Almost all processed food products bear a detailed label proclaiming levels of vitamins, minerals, and other nutrients. However, the sheer complexity of nutrient analysis has made providing the public with accurate food labeling extremely difficult.

In an effort to solve some of the problems, about 90 industry, government, and university representatives met last fall at the National Bureau of Standards (NBS).

The focus of the meeting was the need for

Standard Reference Materials (SRMs) with certified values for the concentration of organic nutrients such as cholesterol, vitamins, and sugars.

SRMs are materials which have concentrations of certain compounds or elements carefully measured and certified by NBS. These standards are then used by manufacturers, scientists, and laboratory technicians to calibrate the instruments and methods used in chemical analysis, physical properties determinations, and other types of measurements.

Currently, the Bureau produces only a small number of food SRMs, and these are certified solely for a limited number of trace metals.

"The only kinds of standards for organic nutrient measurement," says Sam Margolis, an NBS research chemist who coordinated the workshop, "are put out by industry associations and are only available in limited quantities." There are noteworthy differences between industry standards and SRMs produced by NBS. SRMs provide more assurance that instruments can be calibrated to "true" values rather than to values which may reflect systematic measurement errors.

The meeting participants reached general agreement on the following points: (1) the immediate need for SRMs consisting of food matrices with specific nutrients at certified concentrations such as: corn syrup certified for glucose, maltose, maltotriose, and moisture; partially hydrogenated vegetable oil certified for cholesterol; fortified nonfat dry milk certified for niacin, thiamin, and riboflavin; either nonfat dry milk or a sugar-containing dry drink mix certified for vitamin C; a nonfat dry milk certified for galactose and lactose (lower priority); (2) with the exception of vitamin B_{12} and folacin, the minimal need for pure compound reference materials of higher purity than the quality reference materials available from the U.S. Pharmacopeia; (3) despite the need for improvement in the measuring of "bound" nutrients like the B vitamins, the inadvisability of producing such SRMs at this time because current technology cannot produce materials with sufficient stability.

The workshop was sponsored by NBS, the Food and Drug Administration, the Department of Agriculture, and the National Food Processors Association. For further information or to offer suggestions on particular nutrient SRMs, contact Dr. Sam Margolis, Chemistry Bldg., Room 327, National Bureau of Standards, Washington, DC 20234; phone 301/921-2867.

New Private Sustaining Members

AOAC would like to welcome our new private sustaining members: Allergan, Irvine, CA; E. I. du Pont de Nemours & Co., Wilmington, DE; General Mills, Inc., Minneapolis, MN; Heinz U.S.A., Pittsburgh, PA; Hershey Foods Corp., Hershey, PA; Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ; Kraft, Inc., Glenview, IL; Mead Johnson and Co., Evansville, IN; Ortho Pharmaceutical Corp., Raritan, NJ; Pfizer Inc., New York, NY; Procter and Gamble Co., Cincinnati, OH; Rhone-Poulenc Chemical Co., Agrochemical Div., Monmouth Junction, NJ; SmithKline Corp., Philadelphia, PA; and Velsicol Chemical Corp., Chicago, IL.

Interim Methods Adopted

The following methods have been approved by the respective General Referees and Committees for adoption as interim official first action: Gas-Liquid Chromatographic Determination of Pirimicarb in Formulations, Peter D. Bland, ICI Americas, Inc., Goldsboro, NC; Determination of Polar Components in Frying Fats, IUPAC Commission of Oils, Fats and Derivatives Working Group No. 7; Gas-Liquid Chromatographic Determination of Sulfamethazine in Swine and Cattle Tissues, Arthur J. Manuel and William A. Steller, American Cyanamid Co., Princeton, NJ; Confirmation/Quantitative Determination of Sulfamethazine in Swine Tissue by Gas-Liquid Chromatography/Mass Spectrometry, R. M. Simpson, F. B. Suhre, and J. W. Shafer, Food Safety and Quality Service, Beltsville, MD; Mineral Oil Vacuum Distillation–TEA Method for Nitrosamines in Bacon, E. L. Greenfield et al., U.S. Department of Agriculture, Beltsville, MD. The Association will vote on adoption as official first action at the 95th Annual Meeting, Oct. 19–22, 1981.

Meetings

October 19–22, 1981: 95th Annual AOAC Meeting, Marriott Hotel, Twin Bridges, Washington, DC. (For details, see article above.) Contact is Kathleen Fominaya, AOAC, 1111 N. 19th St, Arlington, VA 22209; telephone 703/522-3032.

November 1–5, 1981: 109th American Public Health Association Annual Meeting, Los

Angeles, CA. The general theme of the meeting will be "Energy, Health, and the Environment." For more information, contact Jon M. Counts, Bureau of Laboratory Services, Arizona Department of Health Services, 1520 W. Adams St, Phoenix, AZ 85007; telephone 602/255-1188.

April 13-15, 1982: 7th Annual AOAC Spring Training Workshop and Exposition, Fairmont Hotel, New Orleans, LA. Planned for this workshop are sessions on: drugs and antibiotics in feeds, food toxicology, forensic chemistry, pesticide residues, seafood quality, mycotoxins, environmental monitoring, fertilizers, sugars, laboratory automation, pesticide formulations, quality assurance, veterinary toxicology, hazardous waste monitoring, and analysis of toxicological substances. For additional information, contact co-chairmen: Nicole F. Hardin, U.S. Food and Drug Administration, 4298 Elysian Fields Ave, New Orleans, LA 70122; telephone 502/589-2471, or Hershel Morris, Louisiana Department of Agriculture, PO Box 16390-A, University Station, Baton Rouge, LA 70893; telephone 504/388-2755.

May 11-14, 1982: Fourth International Symposium on Quantitative Mass Spectrometry in Life Sciences. Rijksuniversiteit, Gent, Belgium. For information, contact Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Gent, Belgium.

CORRECTIONS

J. Assoc. Off. Anal. Chem. (1981) 64, 628-634, "High Pressure Liquid Chromatographic Analysis of Chlorpyrifos-Containing Insecticidal Formulations: Collaborative Study," by Norman E. Skelly, David J. Jackson, and Phyllis K. Anderson, p. 628. Change second author's name to read: David J. Jensen.

J. Assoc. Off. Anal. Chem. (1981) 64, 841-843, "Portable and Sensitive Detector Strips for Rapid Detection of Organophosphorus, Mercury, Copper, Cadmium, and Silver Compounds," by Nanguneri V. Nanda Kumar and Yalavarthi Prameela Devi, p. 841, right column, (j) *Detector strip A*, add as a second paragraph:

Note: For detection of oxygen analogs of organophosphorus pesticides, dilute 2% enzyme suspension 1:8 with acetone to prepare detector strips A for use in AB combination. Use of diluted enzyme suspension overcomes masking of white inhibition spots (stable for 5 min) by magenta color. Do not change preparation procedure for A strips to be used for detection of heavy metals.

J. Assoc. Off. Anal. Chem. (1981) 64, 991-998, "Gas Chromatographic Determination of Trialkyl/Aryl Phosphates in Drinking Water, Following Isolation Using Macroreticular Resin," by Guy LeBel, David T. Williams, and Frank M. Benoit, p. 991, Abstract, last line. Change to read "ranging from 0.2 to 75 ng/L."

BOOK REVIEWS

Food Microbiology. A Framework for the Future. By Anthony Nelson Sharpe. Published by Charles C Thomas, Springfield, IL, 1980. 217 pp. Price $24.75.

Microbiologists have traditionally focused their attention on the activity and properties of microorganisms. Their physical appearances under a microscope, their ability to ferment glucose, and the number which grow on artificial media at carefully controlled times, temperature, pH, etc. have been minutely studied and reported. This old framework has not changed appreciably in 100 years. Thus, problems of spoilage, bacterial toxins, and general quality of food have each been related directly to the organism(s). Methods for analysis for shelflife and safety standards are predicated on the detection of one or more species of microorganisms.

This book is not a text of food microbiology, but a discussion of a way of dealing with microbiological problems associated with foods. The author frankly states, "I hope it will be controversial, for the subject is in need of stirrin' up." He notes that food microbiology is a "scientific backwater in which one could mill around for many years," mainly because little of the laboratory routine has been mechanized. In fact, the emphasis on enumerative microbiology is the chief drawback to the introduction of machines. An alternative way of investigating food microbiology problems is presented, one that lends itself to the use of time-saving instruments.

The author notes that bacterial counts are currently used to monitor spoilage, presence of toxins, and unacceptable organoleptic properties. Why not build a machine to incubate a food sample (rather than use artificial media) for various times and temperatures? The presence of toxin, hydrogen sulfide, and other by-products can be measured directly. With instruments, sequential measurements could be made over time and a suitability standard expressed in terms of the amplitude of the measurement. These measurements would be related directly to, possibly, the acceptability of a product by a taste panel. The measurement and result would be credible in terms of product quality. Knowledge of the aerobic plate count at some particular time does not always relate to product quality. For example, the distribution of species of organisms is not known nor is the potential metabolic activity of the organisms in the food in question. The author suggests new measures of wholesomeness and the application of current mechanical and computer technology to this concept.

The author is aware of the problems presented by the need for setting tolerances. Humans have a distribution of responses to taste, toxins, etc. The zero tolerance concept is rejected. Another major problem is acceptance of the new technology. Traditionally the machines are tested against enumerative procedures now standard. There is often little correlation between the two measurements. As the author notes, "the successful popularization of a radically new approach to food microbiology may depend not only on its ability to challenge an overwhelmingly greater body of scientific data and experience built up over the years by existing approaches but also its ability to exploit the emotional response of scientists, laymen, lawyers, and politicians alike."

The author presents a plausible alternative to certain current views in food microbiology. Only future research will determine if these concepts are workable.

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JAMES E. GILCHRIST

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The Pesticide Manual: A World Compendium, 6th Ed. Edited by Charles R. Worthing. Published by the British Crop Protection Council, Worcestershire, UK. Also available from International Scholarly Book Services, Inc., PO Box 1632, Beaverton, OR 97075. 1979. ix + 655 pp. Price $65.00.

A compendium gathers in brief, orderly, and intelligible form the important features of a field of knowledge or subject category. These features or facts are concisely presented in this manual by grouping the information about each compound in separate sections. These are heading, nomenclature and development, manufacture and properties, uses, toxicology, formulations, and analysis. The heading contains the Wiswesser Line-Formula Notation in addition to common

name, chemical structure, molecular formula, and molecular weight. The section on nomenclature and development will be of little interest to a regulatory chemist; but the properties, uses, and formulations sections furnish a good overall picture of the pesticides discussed. Also, the information on toxicology is more extensive than are usually found in handbooks; for example, data on fish are included for many of the pesticides.

A most important section, from the standpoint of the regulatory chemist, is the one referencing the methods of analysis. Unfortunately this section fails to adequately reference the official methods of the AOAC. The listing of AOAC methods for pesticide formulations varies from complete omission, as for atrazine and captan, to excellent references, as for heptachlor. In addition, the AOAC multiresidue method receives little attention under the various pesticides to which it is applicable. This manual does, however, contain a number of good analytical references, including many found in *Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives.*

The manual includes four indices. They are based on the Wiswesser Line-Formula Notation, molecular formula, code numbers, and names.

The Pesticide Manual furnishes information on all pesticides currently in use including a significant amount of analytical data. Pesticide chemists will find this to be a useful reference book.

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The Particle Atlas, Edition Two. Vol. V. Light Microscopy Atlas and Techniques. By W. C. McCrone, J. G. Delly, and S. J. Palenik. **Vol. VI. Electron Optical Atlas and Techniques.** By John A. Brown and Ian M. Stewart. Edited by W. C. McCrone. Published by Ann Arbor Science Publishers Inc., Woburn, MA. Vol. V, 1979, pp. 1139–1454. Vol. VI, 1980, pp. 1455–1703. Price $90.00 each.

These two volumes of *The Particle Atlas* update and enlarge upon the previous volumes of this set. Volume V features new techniques and applications of light microscopy and contains photomicrographs and descriptions for 412 new particle types. Volume VI features articles on electron optical methods and contains scanning electron microscope pictures of the new particles as well as energy dispersive X-ray patterns for most of them.

Included in Volume V are articles on the laser Raman microprobe, Hoffman modulation contrast, dispersion staining, and microchemical reactions for particle identification. Excellent sections on the determination of the geographical origin of dust samples, the microscopical examination of air pollutants, particle analysis in the crime laboratory, and the application of particle study in art and archaeology illustrate how the examination of particles with the light microscope can yield considerable information in these diverse fields.

Volume VI illustrates how the advances in the various electron optical methods and the automation of these methods permit in-depth analysis of smaller and smaller particles. Information is provided on techniques for the collection and preparation of these minute particles.

Those who have found previous volumes of *The Particle Atlas* useful in their work will probably want these new volumes because the increased coverage of common particles should help them to identify more of the particulate matter they examine.

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Molecular and Cellular Aspects of Carcinogen Screening Tests. Edited by R. Montesano, H. Bartsch, and L. Tomatis. IARC (International Agency for Research on Cancer) Scientific Publications No. 27. Published by World Health Organization, Geneva, Switzerland. Also available from WHO Publications Centre USA, 49 Sheridan Ave., Albany, NY 12210. 1980. 371 pp. Price $33.00.

Molecular and Cellular Aspects of Carcinogen Screening Tests is a compilation of 23 contributed papers. The papers cover 4 areas: carcinogenic mechanisms, scientific rationale for short term assays, validation of test systems, and a summary of international testing programs. The content and quality of the papers are generally good. Several of the research papers contained in the sections on carcinogenic mechanisms and on scientific

rationale for short-term assays are timely reports describing interesting and novel experimental approaches. Unfortunately, a number of the better papers were rather short.

Of special note are papers dealing with genetic and environmental factors affecting carcinogen metabolism, enzymology of DNA repair, and an analysis of reversible and irreversible lesions in cancer development. These 3 topics remain important areas for mechanism research. Some of the manuscripts reviewing international testing programs are somewhat out-of-date because of the numerous shifts which have occurred in test strategies since 1979.

Among the papers describing test system validation, a manuscript by Griesemer and Cueto describing a scheme for the classification of evidence defining the carcinogenicity of chemicals in animals, is a much needed review of animal bioassay data with recommendations for the interpretation of results into 5 categories of positive responses and 4 categories of equivocal or negative responses. Another excellent review of in vitro assays was contributed by Bartsch et al. This paper is a very comprehensive analysis of the results of 180 chemicals in microbial and mammalian in vitro tests. Some of the other reviews of validation studies are descriptions of testing strategies and do not actually review comparative results. Good features of the book are its coverage of both in vitro and in vivo data and its attempt to integrate the interpretations from both research areas.

Like other IARC publications, the papers are generally of high quality. The book should be a useful reference work for investigators dealing with carcinogenesis mechanisms.

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An Introduction to Synthesis Using Organocopper Reagents. By Gary H. Posner. Published by Wiley-Interscience, John Wiley and Sons, 605 3rd Ave., New York, NY, 1980. 140 pp. Price $23.50.

Two major themes run throughout this timely and nicely written monograph. The first is a description of the use of organocopper reagents in organic syntheses. The second consists of examples and explanations of retrosynthetic analysis in planning complicated organic synthetic research. In this procedure, the individual bonds of a model of the molecule to be synthesized are disconnected one by one and methods of synthesis are proposed for reforming the broken bonds.

The foreword to the book was written by E. J. Corey and gives a brief early history of organocopper chemistry outlining the contributions from Gilman (1936), Kharasch (1941), House (1966), Corey (1967), and Posner (1968).

Reactions of organocopper compounds are covered under 2 principal types, addition reactions and substitution reactions. Addition reactions are usually additions to α,β-unsaturated carbonyl compounds. Besides the products associated with Michael reactions, the enolate intermediate generated by the initial organocopper addition can undergo further reactions such as oxidation or alkylation. The substitution reactions illustrate enhanced stereoselectivity and specificity. Further, organocopper reagents have the advantage over other metal reagents because of their ease of preparation and lower toxicity.

Numerous examples were taken from the literature such as one by Stork and Isobe for a prostaglandin synthesis. At the ends of the sections are references to journals and to recent books on organic syntheses of value to many chemists.

The final chapter contains a few laboratory procedures to illustrate reagent preparation and use.

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NEW PUBLICATIONS

Chemical Technicians' Ready Reference Handbook, Second Edition. Edited by Gershon J. Shugar, Ronald A. Shugar, Lawrence Bauman, and Rose Shugar Bauman. Published by McGraw-Hill Book Company, 1221 Avenue of the Americas, New York, NY 10020, 1981. 867 pp. Price $39.50. ISBN-0-07-057176-7.

Formulas, equations, methods, technologies, and principles needed for laboratory operations are presented in this book. It provides simplified explanations, diagrams, and step-by-step procedures. The new edition features more than 90% revised and expanded coverage and hundreds more topics and procedures than the first edition. Safety measures in the laboratory are emphasized. The editors specify the equipment needed for a particular operation, illustrate this equipment, tell how to assemble it into a working unit, and give the sequential steps to be performed. Some topics examined are air and toxic vapors, gas sensors, water purity specifications, vacuum pumps, electric motor connections, compressed gases, chromatography, the electromagnetic spectrum, flammable organic compounds, fire handling procedures, ion exchange resins, handling reagents and solutions, pH values of acids and bases, the use and care of plastic ware, refractometry, and radioactivity.

Structural Crystallography in Chemistry and Biology. Volume 4. Benchmark Papers in Physical Chemistry and Chemical Physics. Edited by Jenny P. Glusker, Joyce J. Kaufman, and Walter S. Koski. Published by Academic Press, Inc., 111 Fifth Ave., New York, NY 10003, 1981. 448 pp. Price $50.00. ISBN-0-12-786523-9.

The importance of X-ray crystallographic studies to our knowledge of structure in the fields of chemistry and biology is the emphasis of this book. The book's material is divided into 4 parts: early history of structure determination by diffraction, methods of determining the structure from the measured diffraction pattern, the role of X-ray structure determination in chemistry, and the role of X-ray structure determination in biochemistry and biology.

Advances in Carbohydrate Chemistry and Biochemistry. Volume 38. Edited by R. Stuart Tipson and Derek Horton, Published by Academic Press, Inc., 111 Fifth Ave., New York, NY 10003, 1981. 608 pp. Price $64.00. ISBN-0-12-007238-6.

Volume 38 of this continuing publication for review articles in carbohydrate chemistry and biochemistry contains the following 8 articles: Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Polysaccharides; Photochemical Reactions of Carbohydrates; Fluorinated Carbohydrates; The Gulono-1,4-Lactones: A Review of Their Synthesis, Reactions, and Related Derivatives; The Chemistry and Biological Significance of 3-Deoxy D-Manno-2-Octulosonic Acid (KDO); Methylation Techniques in the Structural Analysis of Glycoproteins and Glycolipids; Bibliography of Crystal Structures of Carbohydrates, Nucleosides, and Nucleotides: 1977 and 1978; and Emil Hardegger (1913–1978).

Spectrometric Techniques. Volume 2. Edited by George A. Vanasse. Published by Academic Press, Inc., 111 Fifth Ave., New York, NY 10003, 1981. 320 pp. Price $43.00. ISBN-0-12-710402-X.

Volume 2 of *Spectrometric Techniques* supplements Volume 1 with an expanded treatment of the techniques of Fourier spectroscopy. Emphasis is given to applications of these techniques. Topics discussed include: distorted spectral features in Fourier spectroscopy and methods for their suppression, design considerations for Fourier spectrometers, problems of nonlinearity of drive, present capabilities of tunable laser spectroscopy, and absolute photon counting in the ultraviolet. Although the level of presentation is such that newcomers in the above-mentioned areas should be able to begin an experimental program, its detailed treatment and state-of-the-art coverage recommends this volume to researchers in the general field of spectroscopy.

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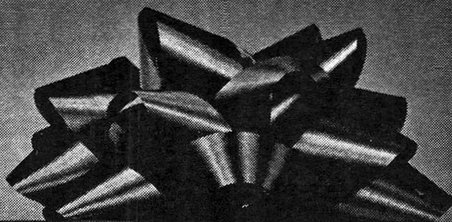

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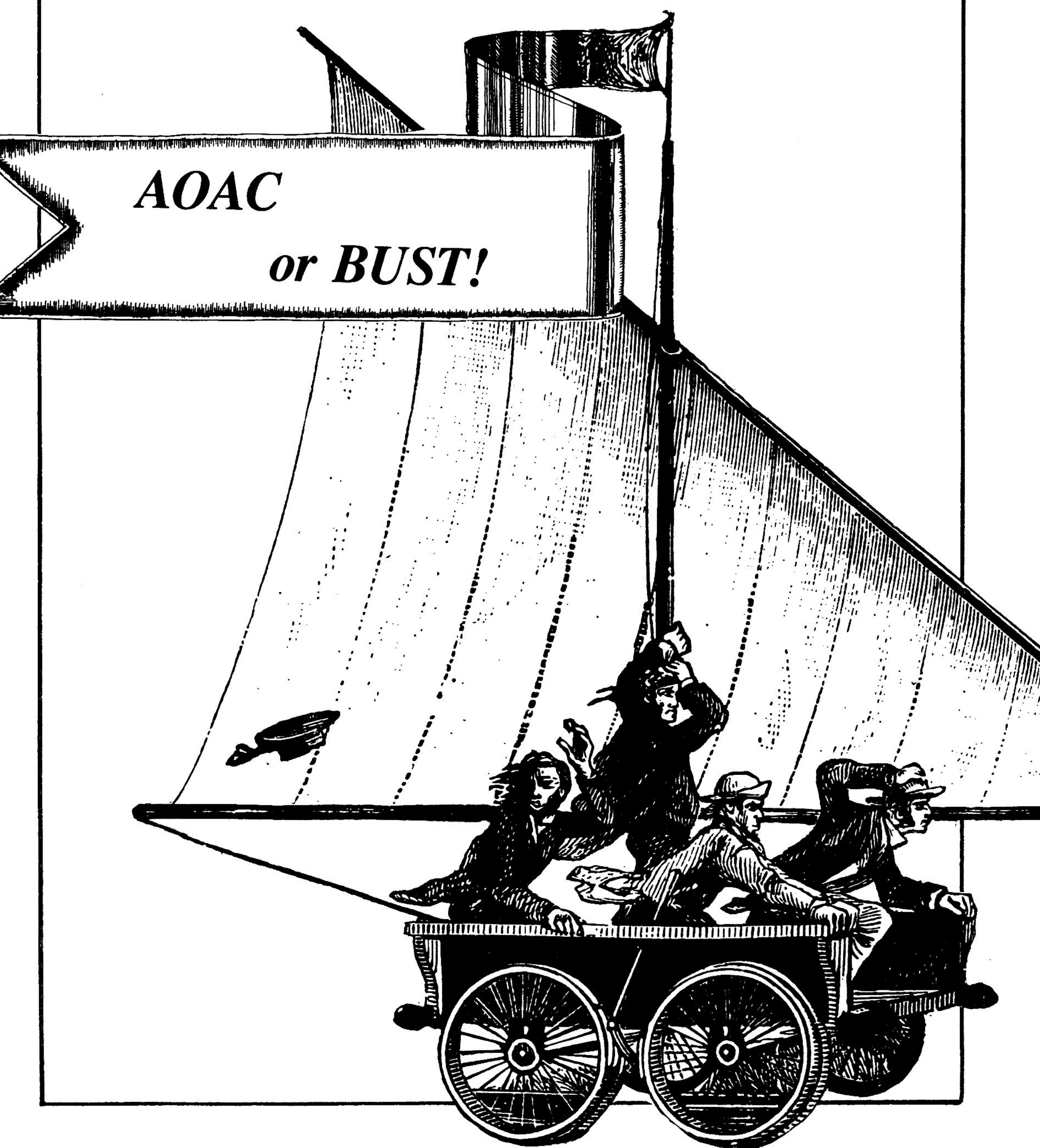

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