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401: METHOD FOR N-METHYLCARBAMATES

BASIC REFERENCES

- Krause, R.T. (1979) *J. Chromatogr.* **185**, 615-624
 Krause, R.T. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1114-1124
 Krause, R.T. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 734-741

GENERAL PRINCIPLES

N-methylcarbamate insecticide residues, including carbamate metabolites, are extracted with methanol. The extract is cleaned up by partitioning and column chromatography on a charcoal/Celite column. Residues are selectively determined with an HPLC system consisting of a reverse phase (RP) column, post-column hydrolysis and derivatization, and detection of the resultant derivative with a fluorescence detector.

Variations in the determinative step may be used for additional residues not of the N-methylcarbamate structure.

APPLICABILITY

Consult Guide to PAM I for additional information pertinent to the appropriate application of multiresidue methodology.

Method is applicable to residues with an N-methylcarbamate structure in either nonfatty or fatty foods when determinative step includes post-column hydrolysis and derivatization. See Table 401-a, following method description, for chemicals tested through the method.

Method is applicable to naturally fluorescent residues when post-column hydrolysis and derivatization are not performed. See Table 401-b, following Table 401-a.

Certain commodities, *e.g.*, oranges, contain naturally fluorescent co-extractives that interfere with analysis.

Limit of quantitation is 0.01 ppm carbofuran in high moisture products (fresh fruits and vegetables) and about 0.02 ppm in dry products.

REFERENCE STANDARDS

Dissolve reference standards of N-methylcarbamates in methanol to produce concentrations of 1 µg/mL. Store solutions in actinic glassware, and keep in refrigerator when not in use. Most carbamate standards stored in this manner are stable for several months. However, methiocarb sulfone and sulfoxide degrade within hours and days, respectively, even with stated storage precautions.

STEPS OF THE METHOD

Extraction (E)

- E1** (p. 401-3) Extraction with methanol
E2 (p. 401-4) Extraction with methanol, reduced sample size

Recommended Use

- high moisture products
 low moisture products



Cleanup (C)

- C1** (p. 401-5) Two stage liquid-liquid partitioning and charcoal/Celite column cleanup

all products



**Determination (D)**

DL1 (p. 401-9) HPLC, post-column hydrolysis and derivatization, fluorescence detection

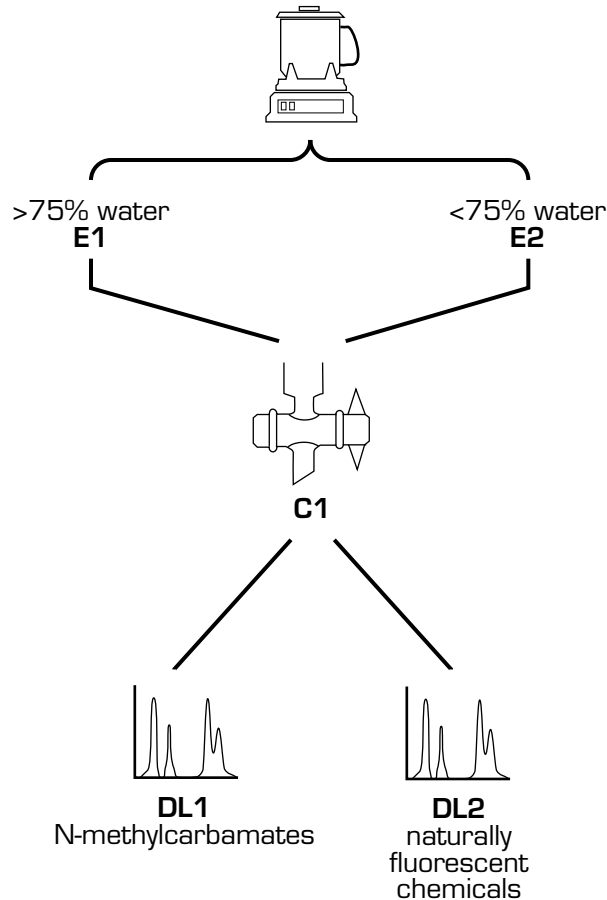
DL2 (p. 401-13) HPLC, fluorescence detection

Recommended Use

N-methylcarbamate residues

naturally fluorescent residues

Figure 401-a
Method for N-Methylcarbamates

**VALIDATION**

The following combination has undergone interlaboratory validation and is recommended for use:

E1 + C1 + DL1

Validation report:

Krause, R.T. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 726-733. Collaborative study leading to AOAC official final action status for aldicarb, aldicarb sulfone, bufencarb, carbaryl, carbofuran, 3-hydroxycarbofuran, methiocarb, methomyl, and oxamyl in grapes and potatoes.

AOAC official method reference: *Official Methods of Analysis of the AOAC* (1990) 15th ed., 985.23.

E1 EXTRACTION WITH METHANOL

**Reference**

Krause, R.T. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1114-1124

Principles

Residues are extracted from high moisture products (>75% water) with methanol, found to be the most effective extractant for N-methylcarbamates in tests using radiolabeled materials. The filtered extract is concentrated with a system that permits evaporation of the relatively high boiling point methanol without destroying heat-labile residues.

Apparatus

Buchner funnel (Buchner), porcelain, 12 cm diameter

evaporator, vacuum rotary with circulating chilled liquid (see Figure 401-b). Maintain 1+1 water/ethylene glycol solution in condensing coils and around receiving flask at -15°C with 1/2 horsepower cooling unit. Insulate condenser with Styrofoam or other material. Control evaporator vacuum with vacuum pump and gauge; manometer may be used but is not preferred.

filter paper, Sharkskin, or 597 S&S, to fit Buchner

flask, round-bottom (r-b), 2 L, T 24/40

homogenizer, Polytron Model PT 10-35, with PT 35K generator containing knives, head equipped with metal (not Teflon) bushing

homogenizer jar, four-side, glass, 1 qt

magnetic stirrer, star, 10 mm diameter \times 8 mm height

vacuum filtration flask, 500 mL

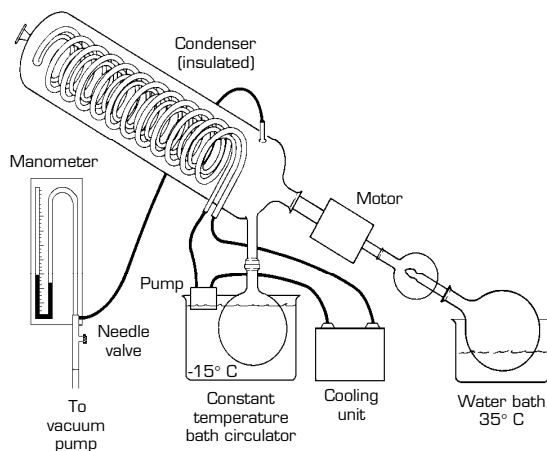
Reagents

methanol, distilled from all-glass apparatus

Directions

- Add 150 g chopped high moisture product and 300 mL methanol to homogenizer jar.
- Homogenize mixture 30 sec at about half speed (setting of 7) and then 60 sec at full speed.
- Vacuum filter homogenate through Buchner fitted with filter paper and collect filtrate in 500 mL vacuum filtration flask. Reduce vacuum during filtration if filtrate begins to boil.
- Transfer portion of filtrate equivalent to 100 g sample to 2 L r-b flask.

Figure 401-b
Vacuum Rotary Evaporator



To remove higher boiling solvents from solutions containing heat-labile residues. Water/ethylene glycol at -15°C cools receiving flask of rotary evaporator and (insulated) evaporator condenser.

$$\text{volume 100 g sample} = \frac{\text{mL water}}{100 \text{ g sample}} + 200 \text{ mL methanol} - 10 \text{ mL contraction factor}$$

- Add enough water to r-b flask to total 100 mL water.
- Add star magnetic stirrer to r-b flask. Place 250 mL trap on 2 L r-b flask and attach to vacuum rotary evaporator.
- Circulate refrigerated (-15°C) (1+1) water/ethylene glycol through evaporator condensing coils; maintain receiving flask at -15°C by immersion in refrigerated bath.
- Apply vacuum slowly to minimize frothing by regulating with needle valve. After full vacuum is applied, slowly place flask in 35°C water bath. Concentrate extract to 75 mL.

ALTERNATIVE:



E2 EXTRACTION WITH METHANOL, REDUCED SAMPLE SIZE

Principle

Reduced sample size permits same amount of solvent to extract residues effectively from low moisture products (<75% water).

Directions

- Proceed as in E1, except extract 75 g ground low moisture product with 300 mL methanol.
- Transfer portion of filtrate equivalent to 50 g sample to 2 L r-b flask.

$$\text{volume 50 g sample} = \frac{\text{mL water}}{50 \text{ g sample}} + 200 \text{ mL methanol}$$

- Continue as in E1, "Add enough water to r-b flask to total 100 mL water."

C1 LIQUID-LIQUID PARTITIONING AND CHARCOAL/CELITE COLUMN CLEANUP**Reference**

Krause, R.T. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1114-1124

Principles

Residues in aqueous extract are transferred to acetonitrile by liquid-liquid partitioning in the presence of sodium chloride. Co-extractives are removed from the acetonitrile solution by partitioning them into petroleum ether, which is discarded. Residues are partitioned from acetonitrile into methylene chloride. Methylene chloride solution is cleaned up on a charcoal/Celite column, and residues are eluted with toluene/acetonitrile.

Apparatus

chromatographic column, 22 mm id × 300 mm, Teflon stopcock, coarse porosity fritted disc

chromatographic column, 25 mm id, plain

evaporator, vacuum rotary, as described in E-1

flasks, round-bottom (r-b), 250 and 500 mL, 1 L, $\text{T} 24/40$

magnetic stirrer, star, 10 mm diameter × 8 mm height

separatory funnel (separator), 250 and 500 mL

vacuum adapter, side arm, with $\text{T} 24/40$ joints

Reagents

acetonitrile, distilled from all-glass apparatus; see Section 204 for distillation directions

Celite 545

charcoal (Nuchar S-N), produced by Westvaco Corp. and available from Eastman Kodak, Cat. No. 118 0454

1+4 (w/w) charcoal/Celite, combined after each is prepared as directed below; mix thoroughly and store in sealed container

dichlorodimethylsilane

glass wool, Pyrex

hydrochloric acid, concentrated, reagent grade

isopropanol, distilled from all-glass apparatus

methanol, distilled from all-glass apparatus

methyl red

methylene chloride, distilled from all-glass apparatus

petroleum ether, distilled from all-glass apparatus

sodium chloride, reagent grade

2% (w/v) sodium chloride/water

20% (w/v) sodium chloride/water

sodium sulfate, anhydrous, granular, reagent grade; see Section 204 for handling directions

toluene, distilled from all-glass apparatus

eluant: 25% (v/v) toluene/acetonitrile

Preparation of Silanized Celite 545

- Slurry 150 g Celite 545 with 1 L (1+1) hydrochloric acid/water in 2 L beaker, cover with watch glass, and stir magnetically while boiling 10 min.
- Cool slurry, filter, and wash with distilled or HPLC grade water until filtrate is neutral.
- Wash Celite with 500 mL methanol followed by 500 mL methylene chloride, and then air dry Celite in hood on watch glass to remove solvent.
- Transfer Celite to 1 L glass-stoppered (g-s) Erlenmeyer flask. Heat unstoppered flask in 120° C oven overnight and then cool flask in desiccator.
- Place flask in hood and carefully pipet 3 mL dichlorodimethylsilane onto Celite. Stopper flask, mix well, and let flask remain at room temperature 4 hr.
- Add 500 mL methanol to flask, mix, and let stand 15 min.
- Filter silanized Celite and wash with isopropanol until neutral.
- Air dry silanized Celite in hood to remove isopropanol.
- Dry silanized Celite in 105° C oven 2 hr and cool in desiccator. Store silanized Celite in g-s container.
- Test Celite for total silanization with two tests. Place about 1 g Celite in 50 mL water; silanized Celite will float. Place second 1 g Celite in 20 mL toluene saturated with methyl red; silanized Celite will appear yellow. If particles of Celite are dispersed in water and/or appear pink with methyl red/toluene solution, active sites still exist on Celite; repeat silanization.

Purification of Charcoal

- Slurry 100 g Nuchar S-N with 700 mL hydrochloric acid, cover with watch glass, and stir magnetically while boiling 1 hr.
- Add 700 mL water, stir, and boil additional 30 min.
- Cool slurry, filter, and wash with water until neutral.
- Wash Nuchar S-N with 500 mL methanol followed by 500 mL methylene chloride, and air dry Nuchar S-N in hood to remove solvent.
- Dry Nuchar S-N in 120° C oven 4 hr. Cool in desiccator. Store Nuchar S-N in g-s container.

Testing of Charcoal/Celite

- Prepare cleanup column of (1+4) (w/w) charcoal/Celite as described below.
- Prepare methanol solution of 5 µg/mL each carbaryl, methiocarb, methiocarb sulfoxide, and methomyl. Use freshly prepared mixed standard solution; methiocarb sulfoxide degrades in solution.

- Pipet 5 mL solution into 250 mL r-b flask and 5 mL into 25 mL actinic volumetric flask.
- Dilute solution in volumetric flask to 25 mL with methanol; use as HPLC reference standard.
- Evaporate standard solution in r-b flask just to dryness with vacuum rotary evaporator as described below. After last trace of methanol has evaporated, remove r-b flask from evaporator and dissolve carbamate residue in 10 mL methylene chloride.
- Transfer methylene chloride solution in r-b flask to prepared adsorbent column and elute as described below.
- After evaporation of eluate in r-b flask, dissolve residue in 25 mL methanol, filter aliquot through filtration device, and quantitate recovery of carbamates as in DL1. Nuchar S-N is considered satisfactory if recovery is $\geq 95\%$.

Directions

Partitioning

- Transfer concentrated extract from E1 or E2 to 500 mL separator containing 15 g sodium chloride. Shake separator until sodium chloride is dissolved.
- Wash r-b flask with three 25 mL portions acetonitrile, transferring each to 500 mL separator; shake separator 30 sec, and let layers separate 5 min.
- Drain aqueous phase into 250 mL separator containing 50 mL acetonitrile, shake 20 sec, let layers separate, and discard aqueous layer.
- Add 25 mL 20% aqueous sodium chloride solution to acetonitrile in 500 mL separator, shake 20 sec, let layers separate, and transfer aqueous solution to 250 mL separator.
- Shake 250 mL separator 20 sec, let layers separate, and discard aqueous layer.
- Add 100 mL petroleum ether to 500 mL separator, shake 20 sec, let layers separate, and drain acetonitrile layer into second 500 mL separator.
- Transfer acetonitrile in 250 mL separator to first 500 mL separator which contains petroleum ether, shake 20 sec, let layers separate, and transfer acetonitrile to second 500 mL separator.
- Add 10 mL acetonitrile to first 500 mL separator, shake, let layers separate, and transfer acetonitrile to second 500 mL separator. Discard petroleum ether layer.
- Add 50 mL 2% aqueous sodium chloride solution to acetonitrile in second 500 mL separator. Extract mixture successively with 100, 25, and 25 mL methylene chloride, shaking each 20 sec (shake 25 mL portions gently).
- Drain lower methylene chloride/acetonitrile layers through 22 mm id column containing about 5 cm sodium sulfate. Collect eluate in 1 L r-b flask.
- Add star magnetic stirrer to r-b flask. Place 250 mL trap on 1 L r-b flask and attach to vacuum rotary evaporator.
- Circulate refrigerated (-15°C) (1+1) water/ethylene glycol through evaporator condensing coils; maintain receiving flask at -15°C by immersion in refrigerated bath.

- Apply vacuum slowly to minimize frothing by regulating with needle valve. After full vacuum is applied, slowly place flask in 35° C water bath.
- Remove r-b flask from evaporator immediately after last traces of solution have evaporated and add 10 mL methylene chloride to r-b flask.

Charcoal/Celite Cleanup

- Fit one-hole No. 5 rubber stopper onto tip of chromatographic column with stopcock, add side-arm vacuum adapter and 500 mL r-b flask, open stopcock, and connect apparatus to vacuum line.
- Place 0.5 g silanized Celite in chromatographic column, tamp, add 5 g charcoal/Celite mixture, and tamp again. Add 1-2 cm glass wool plug on top of adsorbent.
- Prewash column with 50 mL 25% toluene/acetonitrile eluant. Close stopcock when prewash solution is about 0.5 cm from top of glass wool.
- Disconnect vacuum, discard solution in r-b flask, and reconnect flask to apparatus.
- Transfer 10 mL methylene chloride extract from partitioning steps to column and elute column at 5 mL/min.
- Wash 1 L r-b flask with 10 mL methylene chloride and then with 25 mL eluant. Transfer each separately to column and elute each to top of glass wool before adding next solution.
- Add 100 mL eluant and elute column at 5 mL/min. Turn off stopcock when top of eluant reaches top of glass wool.
- Evaporate solution in 500 mL r-b flask just to dryness using vacuum evaporator as above. Remove flask from evaporator immediately after all solution has evaporated.
- Immediately pipet 5 mL methanol into 500 mL r-b flask to dissolve residue.
- Cleaned up extract contains 20 g sample equivalent/mL solution for high moisture products and 10 g sample equivalent/mL solution for low moisture products.

DL1 HPLC, POST-COLUMN DERIVATIZATION, FLUORESCENCE DETECTION

**Reference**

Krause, R.T. (1978) *J. Chromatogr. Sci.* **16**, 281-288

Principles

Residues in methanol solution are separated on a C-8 reverse phase HPLC column using acetonitrile/water gradient mobile phase. Residues eluting from the column are hydrolyzed in-line to methylamine under alkaline conditions. Methylamine is reacted, also in-line, with o-phthalaldehyde and 2-mercaptoethanol to form a fluorophore that is measured by a fluorescence detector. This post-column derivatization-fluorescence detection determinative step is very selective for residues containing the N-methylcarbamate structure.

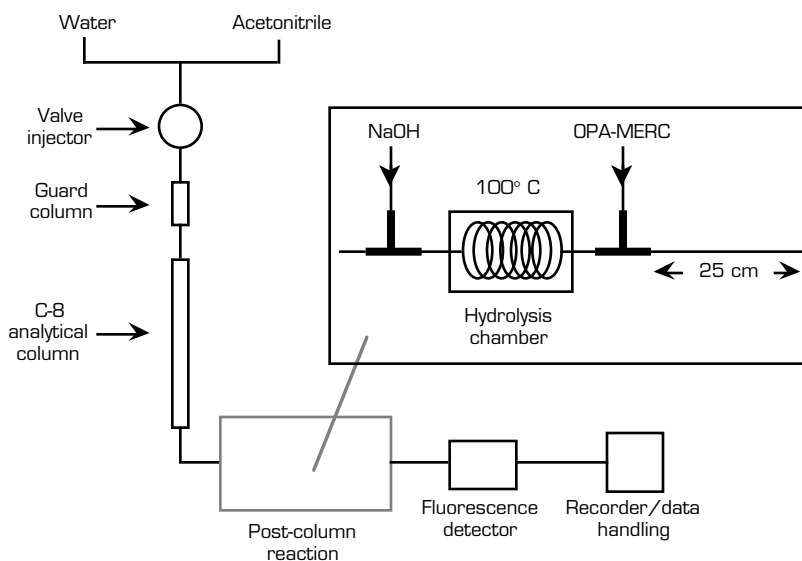
Apparatus

filtration device for solutions, 10 mL syringe with Luer-Lok tip, fitted with either (a) 13 mm diameter Swinny stainless steel filter holder and 13 mm diameter filters, 5.0 μm LS-type, or (b) disposable membrane filters, 13 mm diameter, 0.22 μm nylon membrane, encased in polypropylene. (Preassembled devices that do not require a syringe are also available.)

HPLC system (Figure 401-c) must meet system suitability test below. Complete system consists of:

- 1) mobile phase delivery system, programmable HPLC gradient system
- 2) injector, automatic sampler with 10 μL injection loop
- 3) guard column, stainless steel, containing 25-37 μm pellicular C-8 or C-18 packing
- 4) column oven or heater

Figure 401-c
HPLC System for Determination of N-Methylcarbamates



- 5) analytical column, 25 cm × 4.6 mm id, containing 6 μm Zorbax C-8 spherical particles. Column packing should consist of 5 or 6 μm spherical silica particles bonded with monofunctional octyl silane reagent to form monomolecular bond.
- 6) connecting tubing, No. 304 stainless steel (1.6 mm od × 0.18 mm id) to connect injector, column, and first tee
- 7) post-column derivatization unit, as shown in telescoped portion of Figure 401-b. Units used during collaborative study of method were assembled from the following parts:
 - a) reservoirs for sodium hydroxide and OPA-MERC reaction solutions, 60 cm × 25 mm id glass columns with Teflon fittings; pressurize reservoirs with nitrogen gas, adjusted to create appropriate reagent flow. (Pumps can be substituted for nitrogen gas pressure.)
 - b) 6 m × 0.5 mm id Teflon restriction coil, to connect each reservoir to 15 cm × 0.18 mm id stainless steel tubing, which in turn is connected to 0.74 mm id stainless steel mixing tee (Valco Instruments Co., Cat. No. ZVT-062) for connection to flow of mobile phase
 - c) carbamate hydrolysis chamber, stainless steel tubing, 3 m × 0.48 mm id No. 321, coiled to fit in small oven capable of maintaining constant, uniform 100° C
 - d) reaction tube, 25 cm stainless steel tubing between tee that delivers OPA-MERC solution and 1.5 cm × 0.3 mm id detector cell tubingCommercial post-column derivatization units that replace these components are now available from several manufacturers (ABI, Pickering Instruments, Waters). Systems with dual piston pumps are preferred.
- 8) fluorescence detector, dual monochromator, equipped with ≤20 μL cell
- 9) recorder, strip chart recorder or computing integrator compatible with detector

Reagents

acetonitrile, UV grade distilled from all-glass apparatus. Before use, degas acetonitrile in glass bottles by applying vacuum and slowly stirring with magnetic stirrer 5 min. Acetonitrile other than HPLC grade may cause broad, nonreproducible peaks in chromatograms.

2-mercaptoethanol (MERC), 98+%

methanol, distilled from all-glass apparatus

o-phthalaldehyde (OPA), chromatographic grade

sodium borate buffer solution, 0.05 M. Add 19.1 g ACS grade sodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) and about 500 mL degassed HPLC grade water to 1 L volumetric flask. Heat flask in steam bath to dissolve sodium tetraborate, cool to room temperature, and dilute to volume with degassed HPLC grade water. Mix well, but gently, to minimize re-incorporation of air into solution.

sodium hydroxide solution, 0.05 N. Prepare clear supernate sodium hydroxide as follows: to one part sodium hydroxide (reagent quality containing <5% sodium carbonate), add one part water and swirl until solution is complete. Stopper and set aside until sodium carbonate has settled, leaving clear liquid

(about 10 days). Pipet 27 mL clear supernate sodium hydroxide into 100 mL volumetric flask. Dilute to volume with water and mix (5 N sodium hydroxide).

Pipet 10 mL 5 N sodium hydroxide into 1 L volumetric flask. Dilute to volume with degassed HPLC grade water and mix well, but gently, to minimize re-incorporation of air into solution.

water, HPLC grade, commercial product or prepared from water purification equipment that produces distilled, deionized water. For HPLC, degas water as described for acetonitrile. Water must be adequately purified to prevent plugging HPLC column and extraneous peaks in chromatograms. All water used in HPLC procedure must be HPLC grade. ("Water" that does not specify HPLC grade means distilled water.)

OPA-MERC reaction solution. Weigh 500 mg OPA, transfer to 1 L volumetric flask, add 10 mL methanol, and swirl to dissolve OPA. Add about 500 mL 0.05 M sodium borate buffer solution and 1 mL 2-mercaptoethanol. Dilute to volume with sodium borate buffer solution. Mix well, but gently, to minimize re-incorporation of air into solution. (Borate solution purchased in plastic bottles or low purity grades of OPA may cause excessively high background fluorescence.) Solution is acceptable for about 2 days when stored at room temperature, about 1 week when stored in refrigerator or under helium.

System Operation

- Adjust mobile phase flow rate to 1.50 ± 0.02 mL/min with 50% acetonitrile/HPLC grade water.
- Adjust flow rates of 0.05 N sodium hydroxide and OPA-MERC reaction solution to 0.50 ± 0.02 mL/min each. Operate column oven at 35° C and hydrolysis chamber at 100° C.
- Set fluorescence detector excitation and emission wavelengths to 340 and 455 nm, respectively, and slit widths to 15 and 12 nm, respectively. Set detector photomultiplier tube gain to low and time constant to 1 sec.
- Equilibrate system 10 min with 12% acetonitrile/HPLC grade water, inject sample, and begin 30 min linear gradient to 70% acetonitrile/HPLC grade water.
- Adjust sensitivity so that 10 ng carbofuran produces $50 \pm 5\%$ full scale deflection on printer-plotter. Baseline noise should be $<2\%$, and carbamates should elute as shown in Figure 401-c.
- If system will not be used for several days, replace aqueous mobile phase with methanol and pump through system. Drain sodium hydroxide and OPA-MERC reaction solutions from their reservoirs, and wash reservoirs and associated tubing with water, then methanol. When starting system, change mobile phase to HPLC grade water, and wash reservoirs and associated tubing with water before adding reaction solutions.

System Suitability Test

See Chapter 6, HPLC, for further information about evaluating HPLC systems.

- Prepare mixed standard solution containing 1 µg/mL each aldicarb sulfonate, aldicarb sulfone, carbofuran, and carbaryl.
- Chromatograph solution, using HPLC system operation described above. Retention times will be about 6.5, 8, 20, and 21 min, respectively.

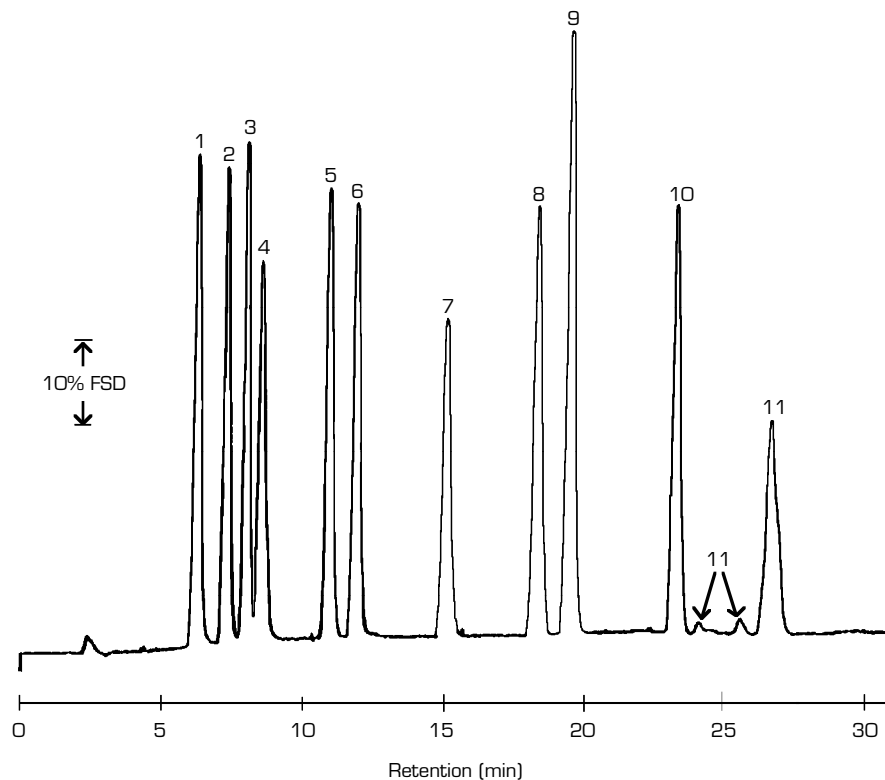
- Baseline resolution should be achieved between aldicarb sulfoxide and aldicarb sulfone and between carbaryl and carbofuran.

Directions

See Figure 401-d for typical chromatogram of carbamate pesticides and metabolites.

- Filter methanol extract from C1 r-b flask through filtration device.
- Collect filtrate in 10 mL centrifuge tube or other suitable container. About 4.5 mL filtrate will be collected. Exact volume of filtrate collected is not critical because sample concentration (g sample/mL methanol) is known.
- If solution requires dilution, pipet aliquot into another container and dilute to volume, as needed.
- Inject 10 μ L methanol solution into HPLC system.
- Tentatively identify residue peaks on basis of retention times. Measure peak area or height and determine residue amount by comparison to peak area or height obtained from known amount of appropriate reference standard(s). To ensure valid measurement of residue amount, sizes of peaks from sample residue and reference standard should match within $\pm 25\%$. Chromatograph reference standard(s) immediately after sample.

Figure 401-d
HPLC Chromatogram of Carbamates and Metabolites



Chromatographed at conditions described in DL1, with post-column derivatization. 1) aldicarb sulfoxide; 2) aldicarb sulfone; 3) oxamyl; 4) methomyl; 5) 3-hydroxycarbofuran; 6) methiocarb sulfoxide; 7) aldicarb; 8) carbofuran; 9) carbaryl; 10) methiocarb; 11) bufencarb.

ALTERNATIVE:

DL2 HPLC, FLUORESCENCE DETECTION

**Reference**

Krause, R.T. (1983) *J. Chromatogr.* **255**, 497-510

Principles

Residues in methanol solution are separated on a C-8 reverse phase HPLC column using an acetonitrile/water gradient mobile phase. Naturally fluorescent residues eluting from the column are detected and measured by a fluorescence detector.

Directions

- Set up and operate an HPLC system in the same manner as DL1, except:
 - operate hydrolysis chamber at ambient temperature.
 - set detector excitation and emission wavelengths at 288 and 330 nm, respectively.
 - turn off pumps or nitrogen flow that add sodium hydroxide and OPA-MERC reaction solutions to mobile phase.
- Perform determination as in DL1.

CONFIRMATION



Confirm tentative identification of naturally fluorescent residues by using DL2. See Table 401-b for list of chemicals for which this confirmatory step is appropriate. Use excitation and emission wavelengths that are optimum for residue being confirmed.

Confirm N-methylcarbamates that include phenolic structure by injecting final extract into HPLC with post-column hydrolysis-electrochemical detection method described in Krause, R.T. (1988) *J. Chromatogr.* **442**, 333-343. This method is based on selective detection of phenolic group of insecticides, rather than carbamate moiety. Intact carbamates are separated by reverse phase HPLC using gradient acetonitrile/water mobile phase as described above. Eluted carbamates are hydrolyzed in-line with dilute sodium hydroxide at 100° C, and resulting phenols are detected with coulometric electrochemical detector. Technique has been tested with six carbamates (bufencarb, carbaryl, carbofuran, 3-hydroxycarbofuran, isoprocarb, and methiocarb) and four crops (apples, cabbage, grapes, and tomatoes).

402: METHOD FOR ACIDS AND PHENOLS

BASIC REFERENCE

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

GENERAL PRINCIPLES

Acidic and phenolic residues are extracted from commodity acidified with sulfuric acid by various techniques dictated by the type of commodity. The extract is cleaned up by gel permeation chromatography (GPC). Residues in the concentrated extract are methylated by ion pair alkylation and further cleaned up by Florisil column chromatography. The resulting methyl esters are determined by GLC. Certain residues can be determined only by element-selective GLC detectors.

APPLICABILITY

Consult Guide to PAM I for additional information pertinent to the appropriate application of multiresidue methodology.

Method is applicable to a wide variety of fatty and nonfatty foods; several different extraction steps are available for the different food types. Cleanup by GPC is most practical when a large number of analyses are being performed. Method was originally designed for chlorophenoxy acids, but has been found applicable to a variety of acidic and phenolic residues. See Table 402-a, following method description, for pesticides and metabolites tested through the steps of this method.

REFERENCE STANDARDS

Use reference standards of methyl esters/ethers of acids or phenols, if available. Otherwise, use standards of the acids/phenols methylated through C1a and cleaned up through C1b. Prepare stock solutions in acetone.

STEPS OF THE METHOD

Extraction (E)

- | | | |
|-----------|-------------|---|
| E1 | (p. 402-3) | Extraction with solvents from acidified, denatured products |
| E2 | (p. 402-7) | Extraction with acidified methylene chloride |
| E3 | (p. 402-9) | Extraction with acidified methanol |
| E4 | (p. 402-10) | Extraction with acidified water/methanol |
| E5 | (p. 402-11) | Extraction with acidified methanol |
| E6 | (p. 402-13) | Dissolution in methylene chloride/hexane |
| E7 | (p. 402-15) | Extraction with acidified methylene chloride |

Recommended Use

- | |
|---|
| animal tissues, dairy products, fats, and shortenings |
| fruits, vegetables other than legumes, and beverages |
| legumes |
| grains and cereals |
| sugar and high sugar processed foods |
| vegetable oils |
| water |



Cleanup and methylation (C)

- | | | | |
|-----------|-------------|---|--------------|
| C1 | (p. 402-17) | GPC cleanup, methylation, and Florisil column cleanup | all products |
|-----------|-------------|---|--------------|



**Determination (D)**

DG1 (p. 302-25) GLC, 100% methyl siloxane column, 200°, EC detector

DG3 (p. 302-29) GLC, 100% methyl siloxane column, 200°, EICD-X

DG4 (p. 302-31) GLC, 100% methyl siloxane column, 200°, EICD-N

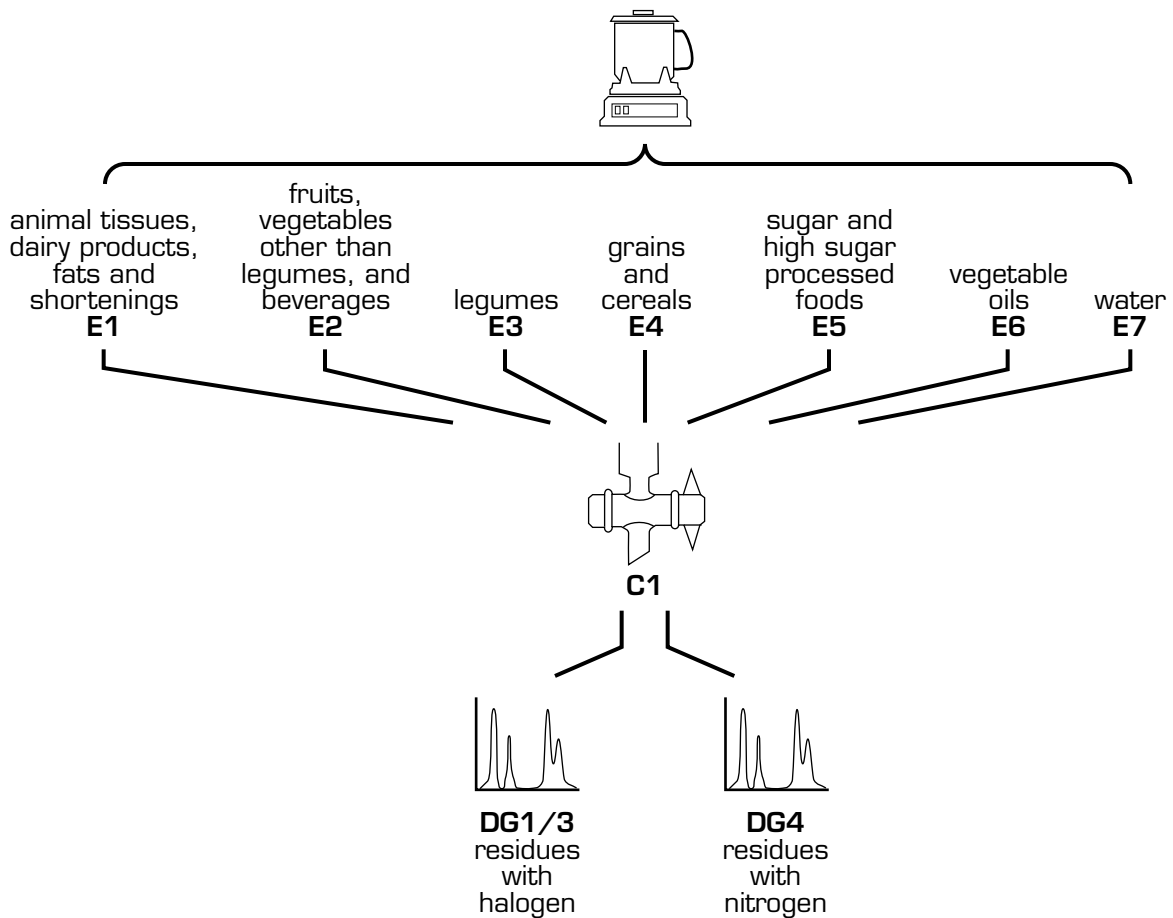
Recommended Use

halogenated acids and phenols

halogenated acids and phenols

acids and phenols containing nitrogen

Figure 402-a
Method for Acids and Phenols

**VALIDATION**

The following combination has undergone validation in a single laboratory, over many years, with repeated recoveries performed in conjunction with FDA's Total Diet Study; these are recommended for use:

E1 + C1 + DG1

Validation report:

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713. Where slight differences occur between the method description in Section 402 and in the Hopper *et al.* 1992 publication, this section reflects standard operating procedure in the Total Diet Study.

E1 EXTRACTION WITH SOLVENTS FROM ACIDIFIED, DENATURED PRODUCTS**Reference**

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

Principles

Fat and residues are dissolved in ethyl ether and petroleum ether after the fatty product has been denatured with oxalate and alcohol and acidified with sulfuric acid. Ether extract is washed with large quantities of water to remove co-extractives.

Apparatus

blender, high speed; explosion-proof Waring Blendor, 1 qt jar

centrifuge, explosion-proof, to hold 500 mL bottles

centrifuge bottle, glass, 500 mL. Use glass stopper or cover rubber stopper with aluminum foil to avoid contamination.

delivery tube apparatus (Figure 402-b), fabricated in laboratory

Kuderna-Danish concentrator (K-D), 500 mL, with Snyder column, plain receiving flask

separatory funnel (separator), 250 mL and 1 L

Reagents

boiling chips, 20-30 mesh carborundum

ethyl ether, distilled from all-glass apparatus, with 2% ethanol as preservative, see Section 204 for peroxide test

methanol, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

petroleum ether, distilled from all-glass apparatus

sodium (or potassium) oxalate, reagent grade

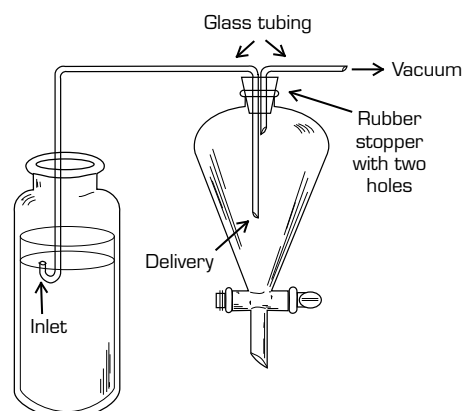
sodium chloride aqueous solution, saturated

10% sulfuric acid, reagent grade

1+1 (v/v) ethyl ether/petroleum ether

50% (v/v) methylene chloride/hexane

Figure 402-b
Delivery Tube Apparatus



Glass tube, inserted in one hole of two-hole rubber stopper, is used to draw upper solvent layer from centrifuge bottle into separatory funnel. Siphon tube is straight or bent in U-shape and inlet end placed at interface of two phases in centrifuge bottle. Second hole in stopper is fitted with another glass tube. Vacuum drawn through second tube causes upper phase from centrifuge bottle to transfer into separator.

(Corrigan, E. (Nov. 1963) (FDA) Bureau By-Lines 5, 20; Sawyer, L.D., and Baca, J.R. (May 1978) LIB 2188, FDA, Rockville, MD.)

Directions

For each batch of samples analyzed, fortify commodity with chlorophenoxy acid herbicide(s) and pentachlorophenol and analyze it with others to verify adequacy of recoveries.

Animal Tissues

- Weigh 50 g sample into blender jar.
- Add 50 mL distilled water, 100 mL methanol, 10 mL 10% sulfuric acid, and about 2 g sodium or potassium oxalate.
- Blend at high speed 2 min and transfer to centrifuge bottle with aid of powder funnel. Rinse blender jar and funnel with 50 mL ethyl ether and add to bottle.

Dairy Products

- Grind cheese and other solid products before analysis. Weigh appropriate amount into centrifuge bottle: 100 g milk or other relatively low fat commodity, 25-50 g cheese or other high fat commodity.
- Add 100 mL methanol, 10 mL 10% sulfuric acid, and about 2 g sodium or potassium oxalate; mix. Add 50 mL ethyl ether.

Fats and Shortenings

- Weigh 25 g sample into centrifuge bottle.
- Add 50 mL distilled water, 100 mL methanol, 10 mL 10% sulfuric acid, and about 2 g sodium or potassium oxalate; mix. Add 50 mL ethyl ether.

All

- Shake centrifuge bottle vigorously 1 min; then add 50 mL petroleum ether and shake vigorously 1 min.
- Centrifuge about 5 min at 1500 rpm. Transfer top (solvent) layer, with delivery tube apparatus, into 1 L separator containing 500-600 mL water, 30 mL saturated sodium chloride solution, and 10 mL 10% sulfuric acid.
- Re-extract aqueous residue in centrifuge bottle twice, shaking vigorously 1 min with 50 mL (1+1) ethyl ether/petroleum ether. After each extraction, centrifuge and transfer solvent layer into same 1 L separator.
- Mix combined extracts and water in separator thoroughly but cautiously to prevent emulsion formation. Drain and discard water.
- Rewash (gently) solvent layer twice with 100 mL water, 10 mL 10% sulfuric acid and 30 mL saturated sodium chloride solution; discard wash solution each time. If emulsions form, add additional 5 mL saturated sodium chloride solution to wash.
- After final wash is discarded, transfer ether layer to 250 mL separator. Let stand ≥ 30 min. Drain and discard any water and emulsion from separator.
- Transfer ether to K-D with plain receiving flask, add boiling chips, and evaporate solvent.
- Cool, add 50 mL methylene chloride to extracted fat, and mix. Add boiling chips and evaporate on steam bath until level in receiving flask does not change and there is still methylene chloride in Snyder column traps. Cool.
- Use approximate fat content of commodity, as listed in Section 201, to determine what dilution is required to achieve concentration of ≤ 0.16 g fat/mL. Quantitatively transfer fat from receiving flask to glass-stoppered (g-s) graduate of appropriate volume. Use 50% methylene chloride/hexane for rinsing during transfer and then dilute solution to predetermined volume with that solvent mixture.

- Transfer aliquot of solution to tared vessel; evaporate to dryness at steam bath temperature under current of dry air. Weigh and record weight of fat extracted. If necessary, adjust remaining solution volume so that solution contains ≤ 0.16 g fat/mL.
- Clean up extract by C1; equivalent weight of whole product cleaned up is:

$$\frac{\text{original sample weight}}{\text{mL final extract}} \times \text{mL loaded onto GPC}$$

E2 EXTRACTION WITH ACIDIFIED METHYLENE CHLORIDE**Reference**

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

Principle

Residues are extracted from fruits and vegetables with methylene chloride after acidification.

Apparatus

blender, high speed; explosion-proof Waring Blendor, 1 qt jar

centrifuge, explosion-proof, to hold 500 mL bottles

centrifuge bottle, glass, 500 mL. Use glass stopper or cover rubber stopper with aluminum foil to avoid contamination.

delivery tube apparatus, see Figure 402-a in E1; apparatus with straight tube inlet (rather than U-shape) is preferred

graduated cylinder (graduate), 250 mL

Kuderna-Danish concentrator (K-D), 500 mL, with Snyder column, 10 mL graduated receiving flask

separatory funnel (separator), 250 mL

Reagents

boiling chips, 20-30 mesh carborundum

glass wool, Pyrex

hexane, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

10% sulfuric acid, reagent grade

Directions

For each batch of samples analyzed, fortify commodity with chlorophenoxy acid herbicide(s) and pentachlorophenol and analyze it with others to verify adequacy of recoveries.

- Weigh 100 g sample (chopped fruits or vegetables other than legumes or beverages) into blender jar. Add 10 mL 10% sulfuric acid and 250 mL methylene chloride.
- Blend 2 min at high speed and pour into centrifuge bottle with aid of powder funnel.
- Centrifuge 5 min at 1500 rpm. Transfer top (water) layer, with delivery tube apparatus, into 1 L separator, then discard.
- Carefully decant methylene chloride (leaving cake in centrifuge bottle) through funnel containing glass wool plug into separator. Let stand ≥ 30 min. Drain and discard any water and emulsion from separator.
- Transfer methylene chloride to graduate and record volume.

- Transfer measured volume of methylene chloride to K-D with 10 mL receiving flask. Add boiling chips and concentrate to 5 mL on steam bath. Dilute extract to 10 mL with hexane and mix.
- Clean up extract by C1; equivalent weight of product cleaned up is:

$$100 \text{ g} \times \frac{\text{mL methylene chloride recovered}}{250 \text{ mL}} \times \frac{\text{mL loaded onto GPC}}{10 \text{ mL}}$$

E3 EXTRACTION WITH ACIDIFIED METHANOL**Reference**

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

Principle

Residues are extracted from legumes with methanol after acidification and partitioned into methylene chloride.

Apparatus

blender, high speed; explosion-proof Waring Blendor, 1 qt jar

centrifuge, explosion-proof, to hold 500 mL bottles

centrifuge bottle, glass, 500 mL. Use glass stopper or cover rubber stopper with aluminum foil to avoid contamination.

graduated cylinder (graduate), 100 and 250 mL

Kuderna-Danish concentrator (K-D), 500 mL, with Snyder column, 10 mL graduated receiving flask

separatory funnel (separator), 2 L

Reagents

boiling chips, 20-30 mesh carborundum

glass wool, Pyrex

hexane, distilled from all-glass apparatus

methanol, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

sodium chloride aqueous solution, saturated

10% sulfuric acid, reagent grade

Directions

For each batch of samples analyzed, fortify commodity with chlorophenoxy acid herbicide(s) and pentachlorophenol and analyze it with others to verify adequacy of recoveries.

- Weigh 100 g chopped legume vegetable into blender jar. Add 10 mL 10% sulfuric acid and 200 mL methanol.
- Blend mixture in blender jar 2 min at high speed and pour into centrifuge bottle with aid of powder funnel.
- Centrifuge 5 min at 1500 rpm and carefully pour top layer through funnel containing glass wool plug into 250 mL graduate. Measure volume re-covered if <250 mL or take 250 mL.
- Pour measured extract into 2 L separator and add 100 mL methylene chloride. Shake separator 30 sec, then add 30 mL saturated sodium chloride solution, 10 mL 10% sulfuric acid, and 650 mL water.
- Shake 30 sec and allow emulsion to settle ≥ 30 min. Drain and discard any water and emulsion from separator.

- (If emulsion does not break, drain emulsion into centrifuge bottle and centrifuge at 1500 rpm 5 min. Siphon off and discard water layer. Pour methylene chloride through funnel containing glass wool plug into 250 mL separator. Let stand ≥ 30 min to ensure complete separation of any remaining water. Drain and discard any water and emulsion from separator.)
- Transfer methylene chloride layer to 100 mL graduate and record volume.
- Transfer measured volume of methylene chloride to K-D with 10 mL receiving flask. Add boiling chips and concentrate extract on steam bath to about 5 mL. Dilute to 10 mL with hexane and mix.
- Clean up extract by C1; equivalent weight of legumes cleaned up is:

$$100 \text{ g} \times \frac{\text{mL extract recovered after centrifugation}}{200 + 10 + (100 \text{ g} \times \% \text{ moisture})} \times \frac{\text{mL methylene chloride recovered}}{100 \text{ mL}} \times \frac{\text{mL loaded onto GPC}}{10 \text{ mL}}$$

ALTERNATIVE:**E4 EXTRACTION WITH ACIDIFIED WATER/METHANOL****Principle**

Water/methanol replaces methanol for extraction from grains and cereal products to accommodate their low moisture.

Additional Reagents

30% (v/v) water/methanol

Directions

- Weigh 50 g ground grain or cereal product into blender jar. Add 10 mL 10% sulfuric acid and 340 mL 30% water/methanol.
- Continue as in E3, "Blend mixture in blender jar 2 min. . ."
- Equivalent weight of grains or cereal products cleaned up is:

$$50 \text{ g} \times \frac{250 \text{ mL}}{340 \text{ mL} + 10 \text{ mL}} \times \frac{\text{mL methylene chloride recovered}}{100 \text{ mL}} \times \frac{\text{mL loaded onto GPC}}{10 \text{ mL}}$$

E5 EXTRACTION WITH ACIDIFIED METHANOL

**Reference**

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

Principles

Residues are extracted from sugar or high sugar processed foods with aqueous methanol after acidification; residues are then transferred to methylene chloride by partitioning.

Apparatus

blender, high speed; explosion-proof Waring Blendor, 1 qt jar

centrifuge, explosion-proof, to hold 500 mL bottles

centrifuge bottle, glass, 500 mL. Use glass stopper or cover rubber stopper with aluminum foil to avoid contamination.

graduated cylinder (graduate), 250 mL

Kuderna-Danish concentrator (K-D), 500 mL, with Snyder column, 10 mL graduated receiving flask

separatory funnel (separator), 250 mL and 2 L

Reagents

boiling chips, 20-30 mesh carborundum

glass wool, Pyrex

methanol, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

sodium chloride aqueous solution, saturated

10% sulfuric acid, reagent grade

Directions

For each batch of samples analyzed, fortify commodity with chlorophenoxy acid herbicide(s) and pentachlorophenol and analyze it with others to verify adequacy of recoveries.

- Weigh 50 g sugar or high sugar processed food into blender jar. Add 10 mL 10% sulfuric acid, 100 mL water, and 200 mL methanol.
- Blend 2 min at high speed. Pour mixture through powder funnel containing glass wool plug into 2 L separator.
- Add 250 mL methylene chloride and shake 30 sec. Add 700 mL water, 10 mL 10% sulfuric acid, and 35 mL saturated sodium chloride solution. Shake separator 1 min and let emulsion settle.
- Drain remaining emulsion and methylene chloride into 500 mL centrifuge bottle and centrifuge 5 min at 1500 rpm. Siphon off and discard water layer.
- Pour methylene chloride layer through funnel containing glass wool plug into 250 mL separator. Let stand ≥ 30 min to ensure complete separation of any remaining water. Drain and discard any water and emulsion from separator.

- Transfer methylene chloride layer to 250 mL graduate and record volume.
- Transfer measured volume of methylene chloride to K-D fitted with 10 mL receiving flask. Add boiling chips and concentrate extract on steam bath to about 5 mL. Dilute to 10 mL with hexane and mix.
- Clean up extract by C1; equivalent weight of product cleaned up is:

$$50 \text{ g} \times \frac{\text{mL methylene chloride recovered}}{250 \text{ mL}} \times \frac{\text{mL loaded onto GPC}}{10 \text{ mL}}$$

E6 DISSOLUTION IN METHYLENE CHLORIDE/HEXANE**Reference**

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

Principle

No actual extraction of residues is done for vegetable oils; instead, they are dissolved in solvent for subsequent cleanup on GPC.

Reagents

hexane, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

50% (v/v) methylene chloride/hexane

Directions

For each batch of samples analyzed, fortify commodity with chlorophenoxy acid herbicide(s) and pentachlorophenol and analyze it with others to verify adequacy of recoveries.

- Weigh 16 g pure vegetable oil into 100 mL volumetric flask. Dilute to volume with 50% methylene chloride/hexane (0.16 g/mL).
- Clean up oil solution by C1; equivalent weight of product cleaned up is:

$$\frac{16 \text{ g}}{100 \text{ mL}} \times \text{mL loaded onto GPC}$$

E7 EXTRACTION WITH ACIDIFIED METHYLENE CHLORIDE**Reference**

Hopper, M.L. *et al.* (1992) *J. AOAC Int.* **75**, 707-713

Principle

Residues in water are extracted with methylene chloride after acidification.

Apparatus

Kuderna-Danish concentrator (K-D), 500 mL, with Snyder column, two-ball micro Snyder column, 10 mL graduated receiving flask

separatory funnel (separator), 1 L

Reagents

acetone, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

10% sulfuric acid, reagent grade

Directions

For each batch of samples analyzed, fortify commodity with chlorophenoxy acid herbicide(s) and pentachlorophenol and analyze it with others to verify adequacy of recoveries.

- Weigh 500 g water, transfer to 1 L separator. Add 10 mL 10% sulfuric acid and 60 mL methylene chloride. Shake vigorously 1 min.
- Allow layers to separate and drain methylene chloride layer into K-D with 10 mL receiving flask. Repeat extraction with two 60 mL portions methylene chloride. Combine all extracts.
- Add boiling chips and evaporate to near dryness on steam bath. Add 50 mL acetone and evaporate to about 3 mL.
- No GPC cleanup is necessary. Proceed to methylation, C1b. Entire solution (therefore, entire weight of original product) is methylated.

C1 GPC CLEANUP, METHYLATION, AND FLORISIL COLUMN CLEANUP**C1a GPC CLEANUP****Reference**

Hopper, M.L. (1982) *J. Agric. Food Chem.* **30**, 1038-1041

Principles

Co-extractives in the extract are separated from residues on GPC by molecular size exclusion; larger molecules (fats, *etc.*) elute first and are discarded. Advance calibration of the GPC column dictates the optimum amount of eluate to discard in order to remove most large molecule co-extractives and recover as much residue as possible. Once calibrated, the column can be used repeatedly.

Apparatus

filtration device for solutions, 10 mL syringe with Luer-Lok tip, fitted with either (a) 13 mm diameter Swinny stainless steel filter holder and 13 mm diameter filters, 5.0 μm LS-type, or (b) disposable membrane filters, 25 mm diameter, 5 μm Teflon membrane, encased in polypropylene. (Pre-assembled devices that do not require a syringe are also available.)

GPC apparatus; automated equipment optional but recommended. GPC apparatus must include:

- 1) sample introduction valve
- 2) pump, low pressure, suitable for use with organic solvents, capable of 5 mL/min flow
- 3) sample loading loop, 1/16" Teflon tubing coiled in cylindrical form, about 13 mL capacity
- 4) pulse dampener, about 6' of 1/8" copper tubing coiled and closed at one end, installed between pump and sample introduction valve with a connecting tee. Pulse dampener is needed only when pump is not pulseless.

GPC column, glass, 25 mm id \times 300 or 500 mm with organic solvent plunger kit

GPC syringe, 10 mL syringe with Luer-Lok tip, with Millipore Swinny stainless steel adapter, Millipore 5.0 μm LS-type filter

Kuderna-Danish concentrator (K-D), 500 mL, with Snyder column, two-ball micro Snyder column, 5 and 10 mL volumetric or graduated receiving flasks

Reagents

Bio-Beads SX-3 resin, 200-400 mesh (Bio-Rad Laboratories, Richmond, CA; pretested resin is available from ABC Laboratories)

hexane, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

eluant: 1+1 (v/v) methylene chloride/hexane

Preparation of GPC column

- Weigh 35 g Bio-Beads SX-3 into 400 mL beaker.
- Add 150 mL 50% methylene chloride/hexane.
- Stir beads with glass or steel rod until all beads have swelled and lumps are no longer present.
- Pour slurry into GPC column with aid of stirring rod.
- Hold column in upright position with plunger tightened about 25 mm from bottom end of usable length of column, ignoring threaded ends.
- Add slurry to column continuously so beads never become completely settled until all beads have been added.
- Place other plunger in column after beads have settled and liquid has drained completely.
- Compress each plunger an equal distance from its respective end until bed length is about 200 mm.
- Connect column to GPC solvent delivery system, and pump solvent from bottom to top of column until all air is expelled.
- Adjust flow rate of system to 5 mL/min and check column pressure. Adjust operating pressure for column to 8-11 psig by moving plunger(s).
- Allow GPC system to equilibrate by pumping solvent through it.
- Re-adjust flow rate to 5 mL/min if it has changed.

Calibration of GPC column

Elution of Fat

- Melt butter and decant butterfat and solids through fluted filter paper into suitable container; do not include water layer. Heat funnel if necessary to facilitate filtration.
- Weigh 4 g warm, filtered butterfat into 25 mL g-s graduate; dilute to 25 mL with 50% methylene chloride/hexane; mix until fat is dissolved (0.16 g fat/mL).
- Filter fat solution through filtration device and load 5 mL onto GPC column.
- Elute with 50% methylene chloride/hexane.
- Collect column effluent in tared beakers in 10 mL increments from 0 to 100 mL.
- Evaporate solvent from each beaker, cool, and weigh to calculate amount of fat eluted in each 10 mL increment. (For manual GPC, collect 10 mL fractions in separate graduates and transfer to tared beakers for evaporation and calculation of fat.)
- 95% of fat should elute in first 60 mL. If >5% of fat appears in 60-70 mL fraction or later, reject column and prepare new one by repacking with original batch of beads. Visual evaluation of yellow band of fat as it passes through column usually shows tailing or streaking when column is inadequate. Use new batch of beads if second column is still inadequate.

Elution of Pesticides

- Prepare mixed standard solution containing 1.2 µg ethion/mL, 0.4 µg diazinon/mL, 0.4 µg heptachlor epoxide/mL, 0.2 µg dicloran/mL, and 0.6 µg dieldrin/mL in 50% methylene chloride/hexane.
- Place 5 mL aliquot standard solution in K-D with 10 mL receiving flask; add 50 mL hexane and 2-3 boiling chips; concentrate to 10 mL. This removes methylene chloride prior to determinative step.
- Filter mixed standard solution through filtration device and load 5 mL onto GPC column.
- Elute with 50% methylene chloride/hexane.
- Collect 10 mL fractions from 0 through 160 mL.
- Transfer each fraction to K-D with 10 mL receiving flask and add 50 mL hexane and 2-3 boiling chips; concentrate to 10 mL.
- Calculate recoveries by comparison to mixed standard solution that has also undergone concentration to 10 mL. Use determinative steps DG1 and DG2.
- Column is normal if diazinon and ethion start to elute in either 50-60 mL or 60-70 mL fraction, and dicloran starts to elute in 90-100 mL fraction.
- Determine what volume should be discarded (usually first 60 mL) and what should be collected (usually 60-160 mL fraction) by examining fat and mixed standard elution profiles developed above. Use these calibrated fraction volumes in subsequent calibration steps and in sample cleanup.

Elution of Pesticides from Fat

- Weigh 1.6 g warm, filtered butterfat into tared 10 mL g-s graduate, add 5 mL mixed standard solution from pesticide elution test above, dilute to volume with 50% methylene chloride/hexane, and mix until fat is dissolved.
- Filter fortified fat solution through filtration device and load 5 mL onto GPC column.
- Elute with 160 mL 50% methylene chloride/hexane.
- Discard and collect respective volumes determined during calibration.
- Transfer collected fraction to K-D with 10 mL receiving flask and concentrate as in pesticide elution test.
- Calculate recoveries as in pesticide elution test.
- For normal column, ≥80% diazinon, parathion, and ethion, and ≥95% of organochlorine pesticides are recovered.

Elution of Herbicides

- Prepare mixed standard solution of 0.1 µg 2,4,5-T/mL and 0.05 µg pentachlorophenol/mL in 50% methylene chloride/hexane by diluting acetone stock solutions.
- Load 5 mL onto GPC column and elute with 160 mL 50% methylene chloride/hexane.
- Discard and collect respective volumes determined above.

- Transfer collected fraction to K-D with 5 mL receiving flask.
- Add 2-3 boiling chips and concentrate.
- Cool, add 50 mL acetone and fresh boiling chips, and reconcentrate to about 3 mL.
- Cool solution and methylate as in C1b.
- Clean up methylated solution on Florisil as in C1c and concentrate to 5 mL.
- Calculate amount of herbicide in concentrated extract using determinative step DG1 and reference standards of pentachlorophenol methyl ether and 2,4,5-T methyl ester. Convert amount of methylated chemicals to amount of corresponding acid/phenol by multiplying respective values by 0.95, which represents ratio of molecular weights of acid/phenol to methylated chemical for both 2,4,5-T and pentachlorophenol (255.49/269.52 and 266.35/280.37, respectively). Calculate percentage recovered by comparing converted values to amounts added (0.5 µg 2,4,5-T and 0.25 µg pentachlorophenol).
- Gel column is acceptable if $\geq 80\%$ of added 2,4,5-T and pentachlorophenol are recovered.

Directions

- Use GPC column prepared and calibrated as described above. Column can be used repeatedly.
- Centrifuge cloudy solutions of extract from E1-E6 before loading them onto GPC. Filter all solutions through filtration device before GPC.
- Load filtered sample extract onto GPC column in 5 mL loops. About 0.8 g fat or 15-50 g equivalent weight of nonfatty product can be loaded in each loop. Use original sample weight, aliquots taken during extraction, volume of final extract, and loading loop size to calculate amount being loaded.
- If targeted limit of quantitation is lower than can be achieved with this size aliquot, load additional aliquots of extract in separate 5 mL loops and combine concentrated eluates from each after GPC cleanup.
- Elute column with 160 mL 50% methylene chloride/hexane. Discard volume previously calibrated and collect remainder in beaker or graduate. Transfer collected eluate to K-D fitted with 10 mL receiving flask. Rinse collection vessel with several mL acetone and add to concentrator.
- Add 2-3 boiling chips and concentrate to about 3 mL. Cool, add 50 mL acetone and fresh boiling chips, and reconcentrate to about 1 mL. Use micro-Snyder column to reach final volume if necessary. Methylate cleaned up extract by procedure in C1b.

C1b METHYLATION

**Reference**

Hopper, M.L. (1987) *J. Agric. Food Chem.* **35**, 265-269

Principles

Acids and phenols in cleaned up extract solution are ionized by an alkali (tetrabutylammonium hydroxide) and methylated with methyl iodide to their respective esters and ethers.

Apparatus

graduated cylinder (graduate), 10 mL, g-s
microliter syringes: 25, 50, or 100 μ L, for adding reagents
water bath, capable of maintaining 40° C

Reagents

acetone, distilled from all-glass apparatus
hexane, distilled from all-glass apparatus
methyl iodide, certified grade
tetrabutylammonium hydroxide (TBAH) titrant, 1.0 M in methanol

Cautions

Note the following cautions that must be observed during methylation:

Use well ventilated hood and protective gloves when adding reagents for methylation.

With each batch of samples, also methylate aliquot of same mixed standard solution of chlorophenoxy acids and pentachlorophenol used for fortification in above recovery tests. Clean up methylated standard on Florisil column.

Determine completeness of methylation by calculations using primary standards of chlorophenoxy acid methyl esters and pentachlorophenyl methyl ether (*i.e.*, not esters/ether generated by this methylation step). If methylation reaction appears incomplete by test, prepare fresh mixed standard solution of acids and pentachlorophenol (these solutions have been found susceptible to degradation).

Methylate fresh mixed standard and old mixed standard, and clean up both by Florisil chromatography. Determine degree of methylation in both old and new standards as above.

The comparison between degrees of methylation will indicate whether problem is caused by standard or by methylation step. If low recoveries are not repeated with new standards, problem can be assumed to be with old standards. If recoveries of old and new standards are both poor (*i.e.*, consistently <40% or >120%), methylation step is at fault.

Presence of water may prevent complete methylation.

Directions

- Dilute concentrated extract from GPC, C1a, to 3 mL with acetone.
- Add 80 μ L 1.0 M TBAH/methanol and 40 μ L methyl iodide. Immediately stopper tube.
- Mix, then place stoppered tube in 40° C water bath for 1.5 hr, with water level of bath above fluid level in tube.
- Remove tube from water bath and attach to 250 mL K-D. Add 50 mL hexane and boiling chips. Evaporate to about 1 mL (avoiding dryness).
- Dilute to appropriate volume with hexane, add 2 mL distilled water, and shake stoppered tube. Discard water.
- Clean up methylated extract on Florisil, C1c.

**C1c FLORISIL COLUMN CLEANUP****Reference**

Griffitt, K.R. *et al.* (Feb. 1983) "Miniaturized Florisil Cleanup of Chlorophenoxy Acid Herbicides and Pentachlorophenol in Total Diet Samples," LIB 2695, FDA, Rockville, MD

Principle

Solution of methylated residues is cleaned up by adsorption chromatography on Florisil column.

Apparatus

chromatographic column, 10 mm id \times 300 mm, Teflon stopcock, coarse porosity fritted disc

Kuderna-Danish concentrator (K-D), 125 or 250 mL, with Snyder column, graduated or volumetric receiving flask

Reagents

acetonitrile, distilled from all-glass apparatus; see Section 204 for distillation directions

boiling chips, carborundum, 20 mesh, or other suitable boiling chips

ethyl ether, distilled from all-glass apparatus, with 2% ethanol as preservative; see Section 204 for peroxide test

Florisil, PR grade; see Section 204 for handling and testing directions and calculation of lauric acid (LA) value

hexane, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

sodium sulfate, anhydrous, granular, reagent grade; see Section 204 for handling directions

eluants: 1—20% methylene chloride/hexane (v/v). Dilute 200 mL methylene chloride with hexane. Allow mixture to reach room temperature, and adjust volume to 1 L with hexane.

2—50% methylene chloride/0.35% acetonitrile/49.65% hexane (v/v/v). Pipet 3.5 mL acetonitrile into 500 mL methylene chloride and dilute with hexane. Allow mixture to reach room temperature and adjust to 1 L with hexane.

Florisil Check

For each batch of Florisil, verify that weight calculated from LA value is suitable by this test.

- Place activated Florisil (weight = $110/\text{LA value} \times 4 \text{ g}$) in chromatographic column; add about 2 cm sodium sulfate.
- Add 5 mL mixed standard solution of 0.1 μg 2,4,5-T methyl ester/mL, 0.05 μg pentachlorophenyl methyl ether/mL, and 0.2 μg picloram methyl ester/mL.
- Elute as directed below, concentrate eluates to 5 mL, and examine by DG1.
- Calculate recoveries by comparison to original mixed standard solution.
- Elute column further with about 100 mL ethyl ether and collect in 10 mL fractions.
- Concentrate each fraction to 5 mL, and examine each by DG1.
- Determine volume of ethyl ether needed to elute picloram methyl ester from column and record for each batch of Florisil.
- Pentachlorophenyl methyl ether should elute in eluate 1; 2,4,5-T methyl ester (and methyl esters of other chlorophenoxy acids) should elute in eluate 2. Picloram methyl ester should elute in about 100 mL ethyl ether.
- If test chemicals do not elute as expected, test adjusted weights of Florisil (similar to tests described in Section 204) or use different batch of Florisil.

Directions

- Add appropriate weight of Florisil (determined above) to 10 mm id \times 300 mm column; add about 2 cm sodium sulfate.
- Prewash column with 15 mL hexane. Do not allow column to go to dryness.
- Place K-D fitted with appropriate receiving flask under column.
- Quantitatively transfer methylated extract from C1b to column. Rinse flask with hexane and add to column. Extract and rinse volume together should be ≤ 15 mL.
- With stopcock completely open, elute column with 35 mL eluant 1.
- Change K-Ds and elute column with 60 mL eluant 2.
- To analyze for picloram, change K-Ds and elute column with appropriate volume of ethyl ether determined above.
- Add boiling chips to K-Ds and concentrate each eluate to suitable definite volume. When volume < 5 mL is needed, use two-ball micro-Snyder or micro-Vigreux column during evaporation.

- Add 50 mL hexane to K-D containing Eluate 2 and reconcentrate to remove final traces of acetonitrile. Add 50 mL hexane to K-D containing ethyl ether eluate and reconcentrate.

DETERMINATION

Determine methylated residues in concentrated solution from C1 with determinative steps DG1, DG3, and DG4 (see Section 302).

Chlorinated residues are determined using DG1 or DG3; residues containing nitrogen may be determined using DG4.

Inject volume of concentrated extract equivalent to the following weights, based on whole product:

	<i>Products $\geq 20\%$ fat</i>	<i>Products $< 20\%$ fat</i>
Eluate 1	≤ 5 mg	≤ 10 mg
Eluate 2	≤ 10 mg	≤ 20 mg
Ethyl ether	≤ 10 mg	≤ 20 mg

CONFIRMATION

Confirm tentatively identified residues according to the principles discussed in Section 103. Review PESTDATA (Appendix I) to find GLC systems applicable to residue; rechromatograph on other systems if available.

403: METHOD FOR PHENYLUREA HERBICIDES

BASIC REFERENCE

Luchtefeld, R.G. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 740-745

GENERAL PRINCIPLES

Phenylurea herbicide residues are extracted with methanol. The extract is cleaned up by partitioning and column chromatography on a Florisil column. Residues are selectively determined with an HPLC system consisting of a reverse phase (RP) column, post-column photodegradation and derivatization, and detection of the resultant derivative by fluorescence detection.

APPLICABILITY

Consult Guide to PAM I for additional information pertinent to the appropriate application of multiresidue methodology.

Method is applicable to residues of phenylurea herbicides in nonfatty foods. See Table 403-a, following method description, for pesticides tested through the method.

Limits of detection and quantitation for selected compounds are also included in Table 403-a; method is applicable to determination of at least 14 phenylurea herbicides at concentrations of 0.05, 0.5, and 1.0 ppm in 14 nonfatty food products.

REFERENCE STANDARDS

Prepare stock solutions (1 mg/mL) of each standard in HPLC grade isopropyl alcohol. Combine appropriate amounts of stock standard solutions and further dilute with (1+1) acetonitrile/HPLC grade water for working standards. Use standards at concentration of 1.25 µg/mL for HPLC determination. Keep stock solutions refrigerated prior to use and prepare working standards weekly.

STEPS OF THE METHOD

Extraction (E)

E1 (p. 403-3) Extraction with methanol

Recommended Use

nonfatty foods



Cleanup (C)

C1 (p. 403-5) Liquid-liquid partitioning and Florisil column cleanup

nonfatty foods



Determination (D)

DL3 (p. 403-7) HPLC, post-column photolysis and derivatization, fluorescence detection

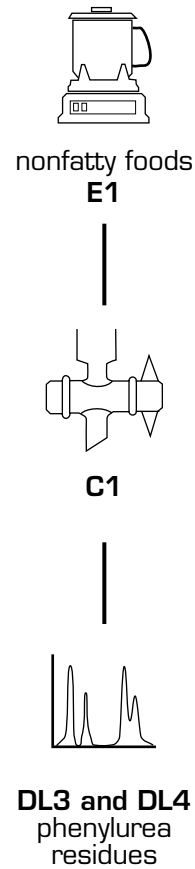
phenylureas



DL4 (p. 403-10) HPLC, different mobile phase

alternative to DL3,
confirmation of
identity

Figure 403-a
Method for Phenylureas



VALIDATION

The following combination has undergone interlaboratory validation and is recommended for use:

E1 + C1 + DL3

Validation report:

Luchtefeld, R.G. (May 1989) "Validation of a Multiresidue Procedure for Determining Phenylurea Herbicides," LIB 3309, FDA, Rockville, MD

E1 EXTRACTION WITH METHANOL

**Reference**

Luchtefeld, R.G. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 740-745

Principles

Residues of phenylurea herbicides are extracted from nonfatty foods with methanol, and the methanol solution is separated from food solids by centrifugation.

Apparatus

centrifuge, explosion-proof, to hold 500 mL bottles

centrifuge bottle, glass, 500 mL. Use glass stopper or cover rubber stopper with aluminum foil to avoid contamination.

funnel, glass

graduated cylinder (graduate), 250 mL

homogenizer, Polytron Model PT 10-35, with PT 35K generator containing knives, head equipped with metal (not Teflon) bushing

Reagents

glass wool, Pyrex

methanol, distilled from all-glass apparatus

Directions

- Weigh 50 g sample into centrifuge bottle. Add 100 mL methanol and homogenize mixture 1.5 min using Polytron with speed set at 8.
- Centrifuge homogenate 5 min at 1500 rpm and decant 80-100 mL through funnel fitted with glass wool plug into 250 mL graduate.
- Add 100 mL methanol to material in centrifuge bottle and homogenize 1.0 min with speed set at 8.
- Centrifuge homogenate and decant as much liquid as possible through funnel into same 250 mL graduate.
- Record volume of combined methanol extract.

C1 LIQUID-LIQUID PARTITIONING AND FLORISIL COLUMN CLEANUP**Reference**

Luchtefeld, R.G. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 740-745

Principles

Co-extractives are removed by adding sodium chloride to the methanol extract and partitioning with hexane, which is discarded. Residues are partitioned from methanol into methylene chloride. Concentrated methylene chloride extract is cleaned up on a Florisil column, and residues are eluted with acetone/methylene chloride.

Apparatus

chromatographic column, 10 mm id × 300 mm, Teflon stopcock, coarse porosity fritted disc

chromatographic column, 25 mm id, plain

Kuderna-Danish concentrators (K-D), 250 and 500 mL, with Snyder column and 5 mL graduated receiving flasks

separatory funnel (separator), 500 mL and 1 L

Reagents

acetone, distilled from all-glass apparatus

acetonitrile, HPLC grade

boiling chips, 20-30 mesh carborundum

Florisil, PR grade; see Section 204 for handling and testing directions

hexane, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

sodium chloride, reagent grade

sodium chloride aqueous solution, saturated

sodium sulfate, anhydrous, granular, reagent grade; see Section 204 for handling directions

eluant: 20% (v/v) acetone/methylene chloride

1+1 (v/v) acetonitrile/HPLC grade water

Directions*Partitioning*

- Transfer entire methanol extract (E1) to 500 mL separator.
- Add 30 mL saturated sodium chloride solution and 50 mL hexane, and shake mixture 30 sec. Let layers separate.
- Transfer lower aqueous layer to 1 L separator containing 500 mL water and 30 mL saturated sodium chloride solution. Discard hexane.
- Add 200 mL methylene chloride to the 1 L separator and shake 1 min.
- Drain methylene chloride through 25 mm × 50 mm column of sodium sulfate into 500 mL K-D fitted with 5 mL receiving flask.

- Re-extract aqueous phase with two 100 mL portions methylene chloride. Drain each extract through sodium sulfate into K-D.
- Rinse sodium sulfate with 50 mL methylene chloride; add rinse to K-D.
- Add boiling chip to K-D and evaporate combined extracts and rinse to about 3 mL for transfer to Florisil column.

Florisil Cleanup

- Place 4 g activated Florisil in 10 mm id chromatographic column; add 1 cm sodium sulfate. Wash column with 30 mL methylene chloride and discard wash.
- When methylene chloride reaches top of sodium sulfate, place 250 mL K-D with 5 mL graduated receiving flask under column. Transfer concentrated extract to column. Rinse container with about 3 mL methylene chloride and add rinse to column.
- When extract reaches top of sodium sulfate, rinse column with two 3 mL portions methylene chloride; allow each rinse to reach top of sodium sulfate.
- When final rinse reaches top of sodium sulfate, elute column with 50 mL 20% acetone/methylene chloride.
- Concentrate solution on steam bath to about 3 mL. Remove receiving flask and evaporate to dryness, using stream of nitrogen and water bath at 40° C.
- Pipet 2 mL (1+1) acetonitrile/HPLC grade water into receiving flask.
- Calculate sample equivalent in final cleaned up extract according to following formula:

$$\frac{\text{g sample}}{\text{mL extract}} = \frac{\text{mL methanol filtrate collected}}{200 \text{ mL} + (50 \text{ g} \times \% \text{ water in sample}) - 5} \times \frac{50 \text{ g}}{2 \text{ mL final extract}}$$

*DL3 HPLC, POST-COLUMN PHOTOLYSIS AND DERIVATIZATION,
FLUORESCENCE DETECTION*



Reference

Luchtefeld, R.G. (1985) *J. Chromatogr. Sci.* **23**, 516-520

Principles

Residues in acetonitrile/water solution are separated on a C-18 RP HPLC column using methanol/water gradient mobile phase. Residues eluting from the column are photolyzed in-line to primary amines by exposure to UV light; the flow's passage through a sleeve of Teflon tubing increases exposure to UV light. The amines are reacted in-line with o-phthalaldehyde and 2-mercaptoethanol to form fluorophores that are measured by a fluorescence detector. The determinative step is very selective for phenylureas.

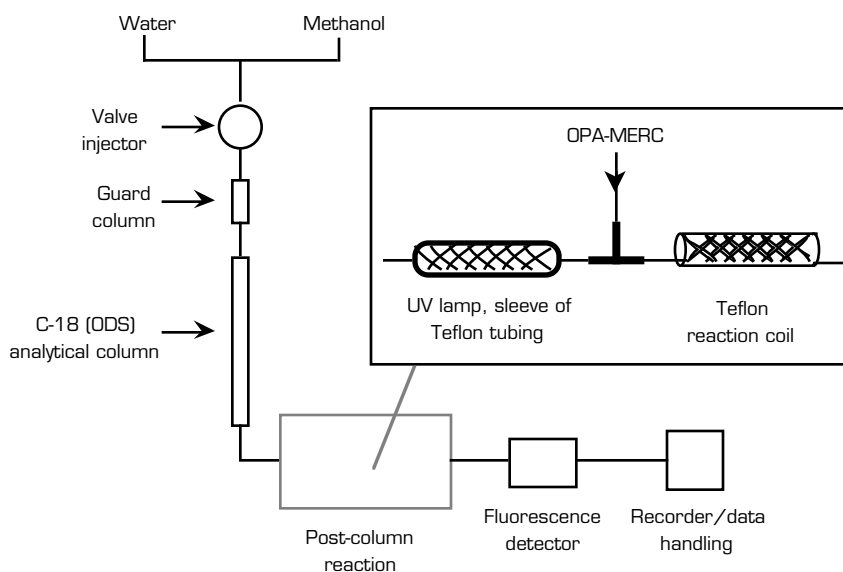
Apparatus

filtration device for solutions, 10 mL syringe with Luer-Lok tip, fitted with either (a) 13 mm diameter Swinny stainless steel filter holder and 13 mm diameter filters, 5.0 μm LS-type, or (b) disposable membrane filters, 13 mm diameter, 0.22 μm nylon membrane, encased in polypropylene. (Preassembled devices that do not require a syringe are also available.)

HPLC apparatus (Figure 403-b) must meet system suitability test below. Complete system consists of:

- 1) mobile phase delivery system: dual pump gradient system
- 2) injector with 40 μL valve loop injector
- 3) guard column, direct connect cartridge system containing prepacked C-18 cartridge (Alltech Associates, Deerfield, IL 60015, Cat. No. 28013)
- 4) column oven or heater, to maintain analytical column at constant temperature

Figure 403-b
HPLC System for Determination of Phenylurea Herbicides



- 5) analytical column, 25 cm × 4.6 mm id, containing spherical particles with monomeric bonded layer of octadecylsilane (ODS, C-18); *e.g.*, Econosphere C-18, Alltech Associates
- 6) post-column photolysis and derivatization unit as shown in telescoped portion of Figure 403-a. Unit is assembled from:
 - a) UV lamp for photodegradation, 17 cm × 9 mm od (Model 80-1178-01) with power supply (Model 90-0001-01), BHK Inc., Pomona, CA
 - b) Teflon sleeve for photodegradation lamp, 10' × 0.5 mm id delay coil (Cat. No. 5-9206, Supelco, Inc., Supelco Park, Bellefonte, PA). Place coiled Teflon tubing over lamp and connect one end to mixing tee and other end to column.
 - c) low flow-rate pump for OPA-MERC solution (Model 396-31, LDC/Milton Roy, Riviera Beach, FL). Connect cone-shaped coil of 13' × 1/8" od stainless steel tubing as pulse dampener between pump and 0.5 mm id Teflon tubing, which is connected to tee.
 - d) mixing tee, stainless steel, 0.25 mm bore, 1/16" standard fittings (No. ZT1C, Valco Instruments, Inc., Houston, TX)
 - e) reaction coil, 10' × 0.5 mm id delay coil (Supelco; Cat. No. 5-9206), with one end connected to mixing tee and other end to detector
- 7) fluorescence detector

Reagents

acetic acid, glacial, reagent grade

2-mercaptoethanol (MERC), reagent grade. Prepare stock solution by diluting 10 mL MERC to 100 mL with methanol.

methanol, HPLC grade. Before use, degas in glass bottles by helium sparging or other suitable method.

monobasic potassium phosphate, certified ACS grade. Prepare 0.002 M solution by dissolving 0.27 g in 1 L HPLC grade water.

o-phthalaldehyde (OPA), reagent grade. Prepare stock solution by dissolving 300 mg OPA in 100 mL methanol.

sodium borate buffer solution. Dissolve 19.1 g ACS grade sodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) in approximately 500 mL HPLC grade water. Dilute to 1 L with HPLC grade water and adjust pH to 10.5 with sodium hydroxide, using pH meter. Filter through filtration device.

water, HPLC grade, commercial product or prepared from water purification equipment that produces distilled, de-ionized water. For HPLC, degas water as described for acetonitrile. Water must be adequately purified to prevent plugging HPLC column and extraneous peaks in chromatograms. All water used in HPLC procedure must be HPLC grade. ("Water" that does not specify HPLC grade means distilled water.)

OPA-MERC solution. Transfer about 250 mL degassed sodium borate buffer solution to 500 mL volumetric flask. Add 25 mL OPA stock solution and 5 mL MERC stock solution. Dilute to volume with borate buffer solution with mixing.

System Operation

- Operate column overnight with 0.3 mL/min (90+10) 0.002 M monobasic potassium phosphate/methanol mobile phase, using single or dual pump system. Change mobile phase to 10% methanol/HPLC grade water and operate 30 min at 1.0 mL/min. If chromatogram baselines begin to drift after several days of use, repeat this procedure.
- Maintain analytical column at 35° C. Equilibrate system with 40% methanol/HPLC grade water at 1.0 mL/min, with 0.2 mL/min OPA-MERC solution added through mixing tee. Allow detector to stabilize after starting OPA-MERC solution flow and turning on UV photodegradation lamp.
- After injecting sample or standard, begin 30 min linear gradient from 40% methanol/HPLC grade water to 80% methanol/HPLC grade water. Operate detector at excitation wavelength of 340 nm and emission wavelength of 455 nm. Adjust detector sensitivity to obtain 50% recorder or integrator deflection when 40 µL 1.0 µg/mL diuron solution is injected. Diuron elutes in approximately 24 min under these conditions.
- Flush pump and tubing used for addition of OPA-MERC solution daily after use by pumping 3% glacial acetic acid in water through system at 1.0 mL/min for about 20 min.
- After each day's use, rinse HPLC column 20 min with 80% methanol/HPLC grade water. Column may be stored in this mobile phase.

System Suitability Test

See Chapter 6, HPLC, for further information about evaluating HPLC systems.

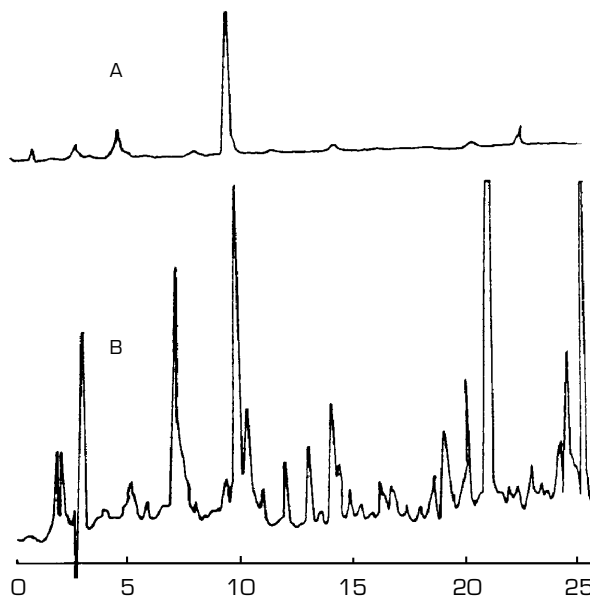
- Prepare solution containing 1 µg/mL each of metobromuron and diuron.
- Chromatograph solution three times. Retention times are approximately 22 and 24 min for metobromuron and diuron, respectively.
- Determine relative standard deviation (RSD) (standard deviation/mean) of peak heights measured in three chromatograms; also determine resolution between the two peaks, according to formulas in Figure 602-a.
- RSD is <3% and resolution will be ≥ 1.5 on adequate system.

Directions

Figure 403-c compares chromatogram from this system to one from system using UV detection at 245 nm.

- Filter both acetonitrile/water sample extract, C1, and reference standard solution(s) through filtration device; inject 40 µL filtrate into HPLC system.
- Compare detector responses (peak height is preferred) to sample extract and to reference standard using same chromatographic conditions.
- Use (1+1) acetonitrile/HPLC grade water to make further dilutions of sample extract, as necessary to make peak heights of sample and standard match closely.

Figure 403-c
HPLC Chromatograms of Carrot Extract



(A) HPLC determinative step, Section 403 DL3; (B) Detection by UV detector at 245 nm. Large peak in chromatogram A is caused by interference from carrot sample.

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ALTERNATIVE:



DL4 HPLC, DIFFERENT MOBILE PHASE

Reference

Luchtefeld, R.G. (1985) *J. Assoc. Off. Anal. Chem.* **70**, 740-745

Principles

HPLC chromatographic pattern for phenylureas is changed by using a gradient mobile phase of acetonitrile/water instead of methanol/water. Differences provide useful confirmatory evidence of residue identity.

Additional Reagent

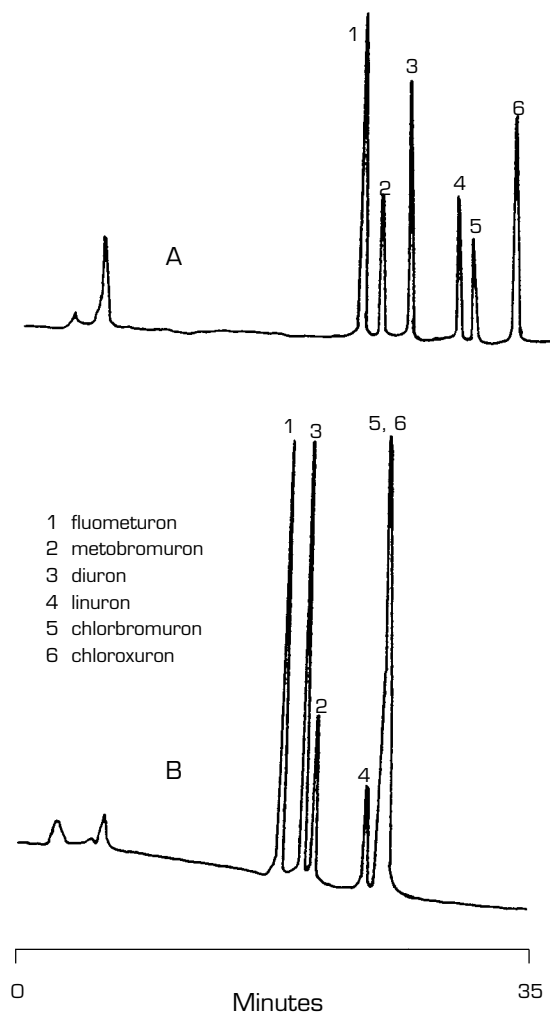
acetonitrile, HPLC grade. Before use, degas in glass bottles by helium sparging or other suitable method.

Directions

Figure 403-d demonstrates different chromatographic patterns produced by DL3 and DL4. Table 403-a lists retention times (relative to diuron) for 14 phenylurea herbicides on the two systems.

- Set up and operate HPLC system as in DL3, except use 30 min linear gradient from 30% acetonitrile/HPLC grade water to 80% acetonitrile/HPLC grade water as mobile phase. All other operations remain the same.

Figure 403-d
Chromatographic Pattern Comparison



Chromatography of six phenylurea herbicides on Econosphere ODS column, 25 cm × 4.6 mm id with two different mobile phases. [A] linear gradient from 40-80% methanol/water in 30 min; 1 mL/min flow rate. [B] linear gradient from 30-80% acetonitrile/water in 30 min; 1 mL/min flow rate.

[Reprinted with permission of Association of Official Analytical Chemists, from *J. Assoc. Off. Anal. Chem.* (1987) **70**, 740-745, Figure 5, page 743.]

CONFIRMATION



Confirm tentatively identified residues according to the principles discussed in Section 103. Rechromatograph on whichever HPLC system, DL3 or DL4, was not used in original determination.

404: METHOD FOR BENZIMIDAZOLES

BASIC REFERENCE

Gilvydis, D.M., and Walters, S.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 753-761

GENERAL PRINCIPLES

Residues are extracted with methanol and partitioned into methylene chloride after initial acidification and again after subsequent alkalization of the extract. Residues are separated and quantitated by reverse phase (RP) ion pair HPLC with both UV and fluorescence detectors.

APPLICABILITY

Consult Guide to PAM I to find additional information pertinent to appropriate application of multiresidue methodology.

Method is applicable to fruits and vegetables and determines residues of allophanate, MBC (resulting from use of benomyl, carbendazim, or thiophanate-methyl), thia-bendazole, and thiophanate-methyl. UV detector responds to all residues to which the method is applicable, but fluorescence detector responds only to MBC and thiabendazole. Alternative determinative step permits examination for residues in commodities from which interfering materials are co-extracted. Method variations applicable to coffee beans are designed for determination of MBC only.

Method limit of quantitation is 0.1 ppm for each residue. UV detection is nonselective and prone to crop interferences, so limit of quantitation may be affected by the particular commodity. Limit of quantitation for thiabendazole can be increased to 0.01 ppm by use of a fluorescence detector at conditions of maximum absorbance for the compound.

See Table 404-a, following method description, for results of recovery tests.

REFERENCE STANDARDS

Obtain reference standards from repository or commercial sources.

Prepare stock solutions (25 µg/mL) in acetone of each of following: allophanate, MBC, thiabendazole, and thiophanate-methyl. (Do not use benomyl as reference standard, because overnight standing is required to ensure complete decomposition to MBC.) Solutions are stable in acetone and in HPLC mobile phase.

Prepare mixed standard solution by combining 1 mL of each stock standard solution, evaporate to dryness with gentle heat (30-40° C) under stream of nitrogen, dissolve residue in 4.0 mL methanol, add 6.0 mL HPLC ion pairing solution, and mix to give final concentration of 2.5 µg/mL of each standard.

STEPS OF THE METHOD

Choose from these method options:

Extraction (E)

- | | |
|---------------|--|
| E1 (p. 404-5) | extraction with methanol, transfer to methylene chloride |
| E2 (p. 404-7) | extraction with methanol, removal of oil with hexane, transfer to methylene chloride |

Recommended Use

- | |
|-----------------------|
| fruits and vegetables |
| green coffee beans |



E3 (p. 404-8) extraction with methanol, removal of oil with methylene chloride, transfer to methylene chloride roasted coffee beans

Cleanup: There are no traditional cleanup steps in this method.



Determination (D)

DL5 (p. 404-9) HPLC, 4.1 mM ion pairing reagent in mobile phase, UV and fluorescence detection

DL6 (p. 404-12) HPLC, 32.7 mM ion pairing reagent in mobile phase, UV and fluorescence detection

DL7 (p. 404-14) HPLC, 4.1 mM ion pairing reagent in mobile phase, changes in detector settings

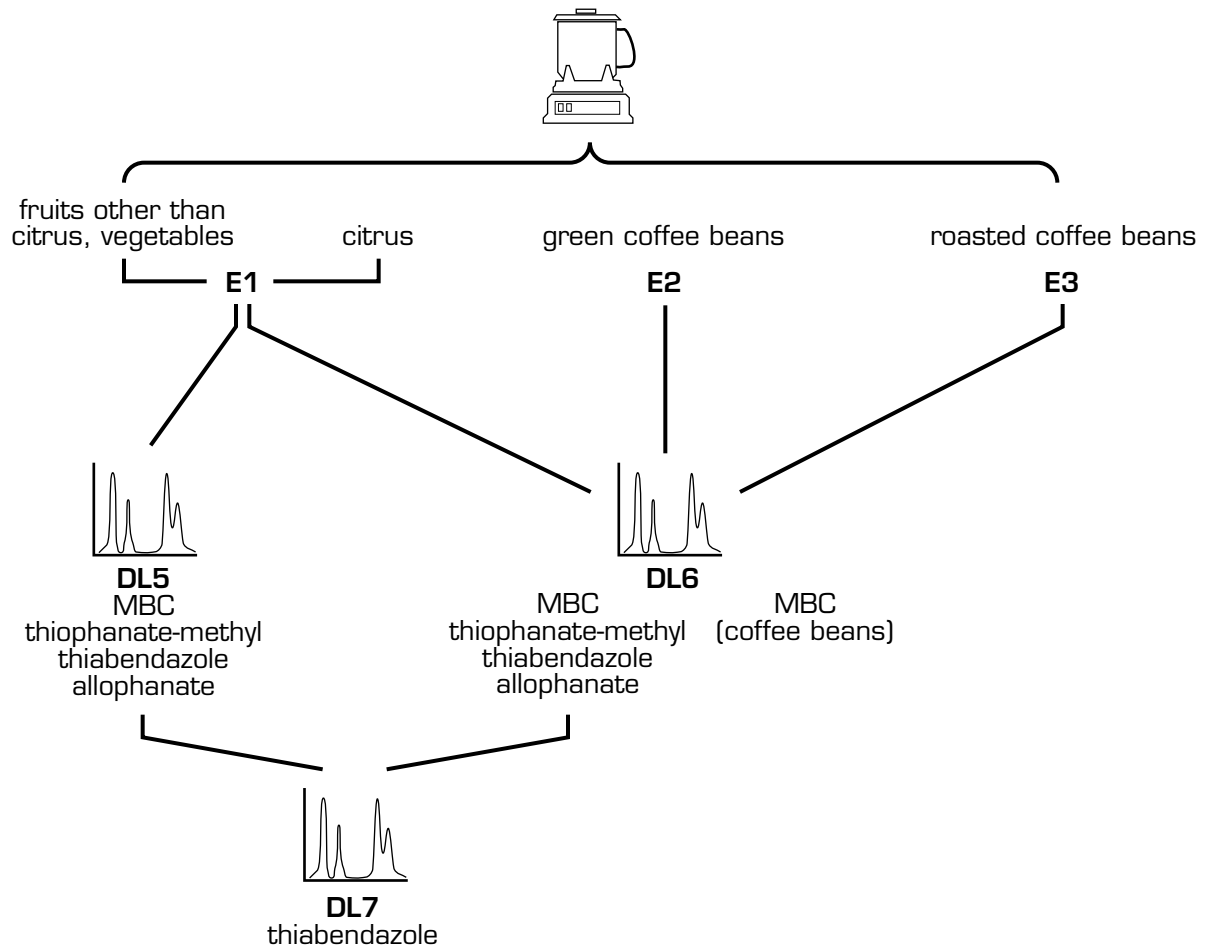
Recommended Use

fruits and vegetables

citrus, coffee beans (green and roasted)

thiabendazole

**Figure 404-a
Method for Benzimidazoles**



VALIDATION

Several combinations of method options are possible. The following combinations have undergone interlaboratory validation and are recommended for use:

E1 + DL5

Validation report:

Gilvydis, D.M. (July 1990) Quarterly Report on methods research, FDA internal communication

E2 + DL6

Validation report:

Jacobs, R.M., and Yess, N.J. (1993) *Food Addit. and Contam.* **10**, 575-577

E3 + DL5

Validation report:

Roy, R.R. (1993) FDA private communication of results (applied to many different commodities) from Interagency Agreement No. FDA 224-90-2479, work performed by USDA National Monitoring and Residue Analysis Laboratory, Gulfport, MS

E1 EXTRACTION WITH METHANOL, TRANSFER TO METHYLENE CHLORIDE**Reference**

Gilvydis, D.M., and Walters, S.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 753-761

Principle

Residues are extracted with methanol and partitioned into methylene chloride after initial acidification and again after subsequent alkalization of the extract.

Apparatus

Buchner funnel (Buchner), porcelain, 12 cm diameter
evaporator, vacuum rotary
filter paper, sharkskin, to fit Buchner funnel
flask, round-bottom (r-b), 1 L
separatory funnel (separator), 500 mL
shaker, mechanical, Burrell wrist action
vacuum filtration flask, 1 L

Reagents

All solvents must be suitable for liquid chromatography and spectrophotometry and must be filtered through 0.2 μm filter.

hydrochloric acid, reagent grade, 1 M

methanol, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

sodium chloride, 1% aqueous solution

sodium hydroxide, 5 M and 1 M solutions

sodium sulfate, anhydrous, granular, reagent grade; see Section 204 for handling directions

Directions

- Weigh 50 g finely chopped sample into 500 mL glass-stoppered (g-s) Erlenmeyer flask. Add 100 mL methanol and shake 10 min on mechanical shaker. To analyze bananas (whole or pulp only), shake vigorously by hand prior to mechanical shaking to disperse mass.
- Filter contents with suction through Buchner fitted with filter paper into vacuum filtration flask. Rinse Erlenmeyer and filter cake with additional 50 mL methanol.
- Transfer filtrate to 500 mL separator, and add 10 mL 1 M hydrochloric acid and 100 mL 1% sodium chloride solution; for low moisture crops, *e.g.*, wheat grain, use 150 mL 1% sodium chloride solution. Mix and allow to cool.
- Extract aqueous filtrate with two 100 mL portions methylene chloride, shaking vigorously 1 min each time.

- Dry methylene chloride layers through column of about 70 g sodium sulfate, collecting in r-b flask. Save aqueous methanol layer in separator for later extractions.
- Rinse sodium sulfate with about 50 mL methylene chloride and collect in r-b flask.
- Evaporate methylene chloride in r-b flask just to dryness using vacuum rotary evaporator with $\leq 30^{\circ}$ C water bath.
- Dissolve material in r-b flask in 4 mL methanol. This extract contains residues of allophanate and thiophanate-methyl and some MBC and thia-bendazole.
- Determine residues in this extract with DL5, except use DL6 for citrus. Avoid delay in determination to minimize loss of thiophanate-methyl by degradation.
- Drain aqueous methanol phase from separator into beaker. Rinse separator with two 10 mL portions water, and add rinsings to beaker.
- Adjust pH of solution in beaker to 7.5-8 with 5 M and 1 M sodium hydroxide solutions and 1 M hydrochloric acid, as necessary. Do not allow solution to become strongly alkaline during adjustment.
- Return solution to separator and extract with two 100 mL portions methylene chloride, shaking vigorously 1 min each time.
- Dry methylene chloride layers through column of about 70 g sodium sulfate, collecting in r-b flask. (Sodium sulfate column used to dry previous methylene chloride layer, above, may be reused.) Rinse sodium sulfate with about 50 mL methylene chloride and collect in r-b flask.
- Evaporate combined methylene chloride extracts to dryness in vacuum rotary evaporator. Dissolve material in r-b flask with 4 mL methanol. Most MBC and thiabendazole are recovered in this extract.
- Determine residues with DL5, except use DL6 for citrus.

*E2 EXTRACTION WITH METHANOL, REMOVAL OF OIL WITH HEXANE,
TRANSFER TO METHYLENE CHLORIDE*



Reference

Gilvydis, D.M., and Walters, S.M. (Aug. 1989) "Modification of LIB 3217 for Carbendazim (MBC) in Green and Roasted Coffee Beans," LIB 3353, FDA, Rockville, MD

Principles

Residues are extracted from green coffee beans with methanol and the filtrate is acidified. Oils are removed from acidified extract by partitioning into hexane, which is discarded. Aqueous methanol extract is then made alkaline, and residues are extracted into methylene chloride by partitioning. Only MBC is targeted by this analysis, so extraction from acidified filtrate (done in E1) is not performed.

Apparatus

Buchner funnel (Buchner), porcelain, 12 cm diameter
evaporator, vacuum rotary
filter paper, sharkskin, to fit Buchner funnel
flask, round-bottom (r-b), 1 L
separatory funnel (separator), 500 mL
shaker, mechanical, Burrell wrist action
vacuum filtration flask, 1 L

Reagents

All solvents must be suitable for liquid chromatography and spectrophotometry and must be filtered through 0.2 μm filter.

hexane, distilled from all-glass apparatus

hexane, methanol-saturated

hydrochloric acid, reagent grade, 1 M

methanol, distilled from all-glass apparatus

methylene chloride, distilled from all-glass apparatus

sodium chloride, 1% aqueous solution

sodium hydroxide, 5 M and 1 M solutions

sodium sulfate, anhydrous, granular, reagent grade; see Section 204 for handling directions

Directions

- Grind coffee beans, using centrifugal mill, to pass 20-mesh screen.
- Weigh 50 g prepared sample into 500 mL g-s Erlenmeyer flask. Add 100 mL methanol and shake 10 min on mechanical shaker.
- Filter contents with suction through filter paper in Buchner and rinse with additional 50 mL methanol. Transfer filtrate to 500 mL separator.

- Add to filtered extract in separator 10 mL 1 M hydrochloric acid and 150 mL 1% sodium chloride solution. Mix and allow to cool.
- Extract acidified methanol once with 100 mL methanol-saturated hexane. Drain lower aqueous layer into beaker and discard hexane.
- Adjust pH of solution in beaker to 7.5-8 with 5 M and 1 M sodium hydroxide solutions and 1 M hydrochloric acid, as necessary. Do not allow solution to become strongly alkaline during adjustment.
- Return solution to separator and extract with two 100 mL portions methylene chloride, shaking vigorously 1 min each time.
- Dry methylene chloride layers through column of about 70 g sodium sulfate, collecting in r-b flask. Rinse sodium sulfate with about 50 mL methylene chloride and collect in r-b flask.
- Evaporate combined methylene chloride extracts to dryness in vacuum rotary evaporator. Dissolve material in r-b flask with 4 mL methanol. Residues of MBC will be in solution. Determine with DL6.

ALTERNATIVE:



E3 EXTRACTION WITH METHANOL, REMOVAL OF OIL WITH METHYLENE CHLORIDE, TRANSFER TO METHYLENE CHLORIDE

Reference

Gilydis, D.M., and Walters, S.M. (Aug. 1989) "Modification of LIB 3217 for Carbenidazim (MBC) in Green and Roasted Coffee Beans," LIB 3353, FDA, Rockville, MD

Principle

Roasted coffee beans contain more oil than green coffee beans, so methylene chloride, rather than hexane, is used to remove oil.

Directions

- Extract as in E2, except after acidification of filtered extract and cooling of mixture:
- Extract acidified methanol once with 100 mL methylene chloride, shaking vigorously 1 min. Drain lower aqueous layer into beaker and discard methylene chloride.
- Continue, as in E2, by making solution alkaline.

DL5 HPLC, ION PAIR MOBILE PHASE, UV AND FLUORESCENCE DETECTOR**Reference**

Gilvydis, D.M., and Walters, S.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 753-761

Principles

Benzimidazole residues are chromatographed on RP HPLC system. MBC and thia-bendazole are ionized in acidic mobile phase and paired with negatively charged counter-ions from sodium decanesulfonate to control system selectivity and separate analytes from interfering sample co-extractives. Thiophanate-methyl and allophanate, neutral at the conditions in the column, are unaffected by mobile phase modifications and chromatograph according to RP principles.

UV and fluorescence detectors each respond to MBC and thia-bendazole; UV detector also responds to thiophanate-methyl and allophanate.

Apparatus

Filtration device for ion pairing solution: stainless steel glass filter holder, 300 mL capacity, fitted with hydrophilic membrane of pH range 2-10, 0.45 μm pore size, 47 mm diameter. Use with vacuum filtration flask, 1 L.

Filtration device for sample solutions, 10 mL syringe with Luer-Lok tip, fitted with nylon 13 mm diameter disposable filter unit, 0.45 μm pore size.

HPLC system must meet system suitability test below. Complete system consists of:

- 1) mobile phase delivery system, constant volume isocratic pump
- 2) injector, automatic sample injection module, preferably with loop volume $\geq 25 \mu\text{L}$
- 3) analytical column, 25 cm \times 4.6 mm id, containing highly end-capped 5 μm C-18 bonded silica suitable for chromatography of basic compounds
- 4) guard column, compatible with analytical column, packed with same or comparable C-18 bonded silica
- 5) column oven, with configuration that accommodates guard and analytical columns
- 6) variable wavelength UV detector or photodiode array detector, equipped with flow cell of about 8 μL
- 7) fluorescence detector, dual monochromator, equipped with 5 μL flow cell
- 8) recorder, strip chart recorder or computing integrator compatible with each detector

Reagents

1-decanesulfonate, sodium salt, 98% pure

ion pairing solution, 4.1 mM 1-decanesulfonate, sodium salt. Pipet 7.0 mL phosphoric acid into 200 mL HPLC grade water; dissolve 1.0 g 1-decanesulfonate, sodium salt in this mixture. Pipet 10.0 mL triethylamine into solution and dilute to 1 L with HPLC grade water. Filter through $<1 \mu\text{m}$ porosity membrane. (pH of solution should be about 2.4.)

methanol, distilled from all-glass apparatus

mobile phase, prepared by mixing *manually* 65 parts ion pairing solution with 35 parts methanol. (Do not mix mobile phase using pump, because exothermic reaction caused by mixing generates bubbles that prevent stable pump operation.) Before use, degas by sparging with helium while stirring solvent with magnetic stirrer.

phosphoric acid, 85%

triethylamine, 99% pure

water, HPLC grade, commercial product or prepared from water purification system that produces distilled, de-ionized water. Resistivity of water must be >12 megohms-cm.

System Operation

- Connect fluorescence detector in tandem with (following) UV detector.
- Set column oven temperature at 40° C and equilibrate system with mobile phase at flow rate of 1.5 mL/min for ≥30 min or until constant retention times are achieved for all four analytes.
- Rinse system with 50% methanol/water when not using for extended period (*e.g.*, ≥24 hr). When not in use for shorter periods, maintain slow flow (0.1-0.2 mL/min) of mobile phase to prevent salt deposition.
- To rid column of highly retained sample components as necessary, rinse column with methanol after rinsing with 50% methanol/water; then rinse with 50% methanol/water again before introducing mobile phase. Mobile phase must be rinsed from column with aqueous solvent before adding methanol and *vice versa* to avoid salt precipitation and possible clogging of system.
- Operate UV detector initially at absorbance wavelength of 250 nm; immediately following elution of allophanate, reset wavelength to 280 nm and rezero baseline for determination of thiophanate-methyl, MBC, and thiabendazole. Adjust detector and/or recorder sensitivity so that 40-70% full scale deflection (FSD) is obtained for 62.5 ng of each standard (25 µL mixed standard solution).
- Operate fluorescence detector at excitation wavelength of 280 nm (20 nm slit width) and emission wavelength of 310 nm (10 nm slit width). Adjust detector, attenuator, and/or recorder sensitivity so that 60-80% FSD is obtained for 62.5 ng MBC (25 µL mixed standard solution); at these conditions, 62.5 ng thiabendazole will cause 40-50% FSD.
- Alternatively, use wavelength-programmable fluorescence detector and wavelength-programmable or diode array UV detector for concurrent determinations of residues at optimum wavelength settings for each.

System Suitability Test

See Chapter 6, HPLC, for further information about evaluating HPLC systems.

- Prepare mixed standard solution (2.5 ng/µL for each compound) as directed above. Prepare additional dilutions to produce mixed standard solutions of 4 ng/µL and 0.4 ng/µL.
- Allow HPLC system to equilibrate at conditions described in System Operation.

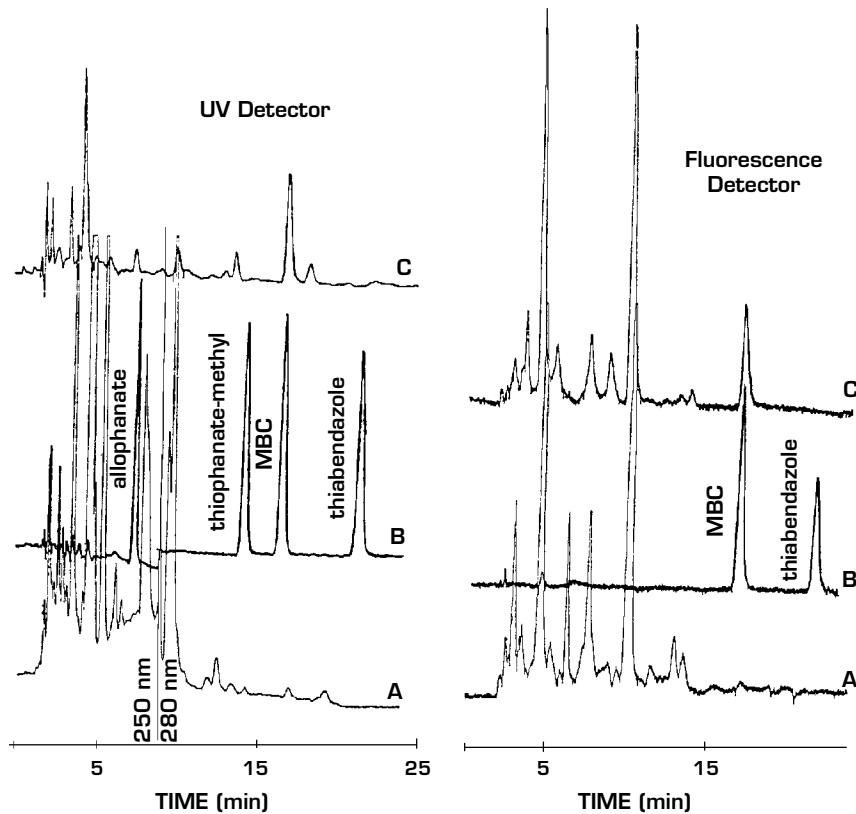
- Inject at least three 25 μL portions 2.5 ng/ μL mixed standard solution. Determine following parameters:
 - 1) retention time and peak height for each peak; relative standard deviations (RSD) for repetitive retention times and peak height measurements
 - 2) column efficiency (N) for thiabendazole peak
 - 3) asymmetry factor (As) for thiabendazole peak
- Inject each of three different concentrations of mixed standard solutions (10-100 ng/25 μL injection). Plot peak height vs. amount injected.
- HPLC systems adequate for analysis for benzimidazoles will meet following minimum criteria: retention times of about 8, 15, 18, and 23 min for allophanate, thiophanate-methyl, MBC, and thiabendazole, respectively (retention times may vary among columns but should remain constant for particular column); RSD <0.5% for retention times and <3% for peak heights of individual peaks in three consecutive chromatograms; N>12,000 and As <1.3 for thiabendazole peak.
- Examine systems not meeting these criteria for problems, using various troubleshooting sections of Chapter 6. Correct problems uncovered by troubleshooting until system meets criteria defined above.
- System will typically respond linearly to 10-100 ng of each compound, but linear range may vary among systems. Perform quantitative analyses only within calculated linear range of system as determined above. Dilute sample extracts as needed to permit injection of analyte level within linear range. Adjust amounts injected so that peak heights of analyte and reference standard do not differ >25% from one another.

Directions

See Figure 404-b for typical chromatograms produced by HPLC system.

- To extract from E1-E3 (dissolved in 4.0 mL methanol), add 6.0 mL ion pairing solution; mix. Residue *must* be dissolved in methanol prior to adding ion pairing solution.
- Filter through 0.45 μm porosity membrane; filter will plug as solution is applied, so filter only volume needed for HPLC determination, about 1 mL.
- Inject 25 μL sample solution and chromatograph as described in System Operation.
- Compare chromatographic response (peak retention times, heights, and/or areas) with that of standard solution and calculate residue amount.
- If further dilutions are necessary, use mixture of 4:6 methanol:ion pairing solution as diluent.
- To convert calculated MBC (MW 191.2) to equivalent benomyl (MW 290.4), multiply by 1.52.
- To convert calculated MBC to equivalent thiophanate-methyl (MW 342.4), multiply by 1.79.
- Peaks of 50% FSD at conditions established for screening analysis are equivalent to about 0.5 ppm each of thiophanate-methyl, allophanate, and thiabendazole; MBC peak of 50% FSD at these conditions represents about 0.3 ppm.

Figure 404-b
Chromatograms of Benzimidazole Compounds



Chromatograms of: (A) peach extract partitioned from the acidic phase of 404 E1, (B) standard solution, (C) peach extract partitioned from basic phase of 404 E1. HPLC operation as directed in DL5. Sample contains 0.14 ppm field-incurred MBC.

ALTERNATIVES:



DL6 HPLC, CONCENTRATED ION PAIR MOBILE PHASE, UV AND FLUORESCENCE DETECTOR

Reference

Gilydis, D.M., and Walters, S.M. (Aug. 1989) "Modification of LIB 3217 for Carbendazim (MBC) in Green and Roasted Coffee Beans," LIB 3353, FDA, Rockville, MD

Principles

Concentration of ion pairing reagent is increased eight times to increase k' values of analytes and improve separation from early eluting co-extractives.

Additional Reagents

ion pairing solution, 32.7 mM 1-decanesulfonate, sodium salt. Pipet 7.0 mL phosphoric acid into 200 mL HPLC grade water; dissolve 8.0 g 1-decanesulfonate, sodium salt in this mixture. Pipet 10.0 mL triethylamine into solution and dilute to 1 L with HPLC grade water. Filter through $<1 \mu\text{m}$ porosity membrane. (pH of solution should be about 2.4.)

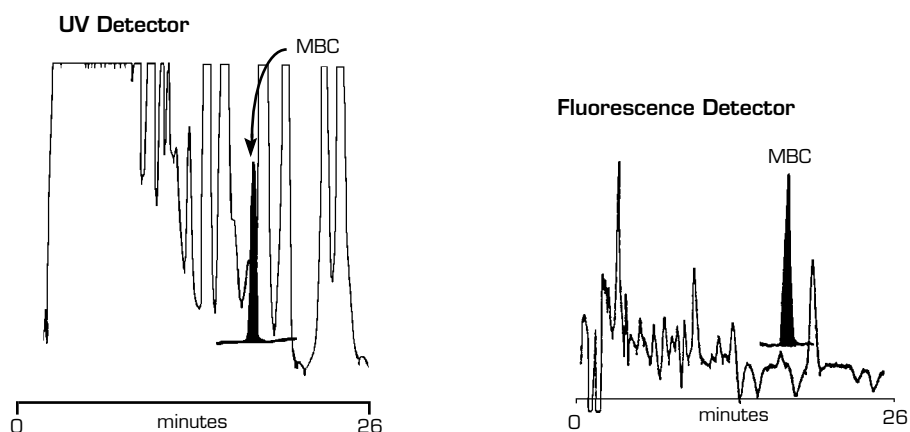
mobile phase, prepared by mixing *manually* 63 parts ion pairing solution with 37 parts methanol. Do not mix using pump. Before use, degas by sparging with helium while stirring solvent with magnetic stirrer.

Directions

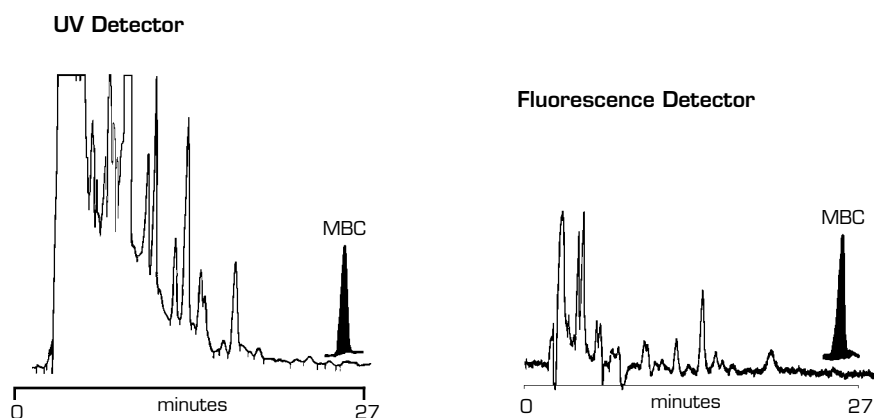
See Figure 404-c for depiction of improvements provided by higher concentration of ion pairing solution in mobile phase.

- Perform HPLC determination as in DL5, except use ion pairing solution and mobile phase described here. At these conditions, MBC will elute in about 26 min.

Figure 404-c
Effect of Ion Pairing Solution Concentration on Chromatography of MBC



HPLC operated as directed in DL5, 4.1 mM 1-decanesulfonate, sodium salt



HPLC operated as directed in DL6, 32.7 mM 1-decanesulfonate, sodium salt

Chromatograms of extract from green coffee beans, extracted according to 404 E2, and chromatographed at conditions directed in DL5 and DL6. Superimposed chromatograms of MBC represent 0.3 ppm.

**DL7 HPLC, ION PAIR MOBILE PHASE, CHANGES IN DETECTOR SETTINGS****Reference**

Gilvydis, D.M., and Walters, S.M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 753-761

Principle

Changes in detector settings provide increased sensitivity to thiabendazole.

Directions

- Perform HPLC determination as in DL5, except adjust wavelength of UV detector absorbance to 305 nm and adjust excitation and emission wavelengths of fluorescence detector to 305 and 345 nm, respectively.
- Dilute sample extract containing thiabendazole and rechromatograph. UV detector response to thiabendazole at this setting will be about twice that obtained at 280 nm, and fluorescence detector response to thiabendazole will be about 10 times that obtained at 280 and 310 nm.

**CONFIRMATION**

See Section 103 for additional information about confirmation.

Confirm initial findings of residues by rechromatographing with alternative systems described here, as appropriate to residue(s) found.