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Received for review April 27, 1989. Revised manuscript received August 29, 1989. Accepted September 25, 1989.

Determination of Bis(2-ethylhexyl) Phthalate in Cow's Milk and Infant Formula by High-Performance Liquid Chromatography

John A. Giust,† C. Thomas Seipelt, Brian K. Anderson, David A. Deis,* and John D. Hinders

Ross Laboratories, 625 Cleveland Avenue, Columbus, Ohio 43215

A method for the rapid extraction and analysis of milk or infant formula for bis(2-ethylhexyl) phthalate (DEHP) by high-performance liquid chromatography (HPLC) is reported. The method eliminates separatory funnel extraction and does not require a residue cleanup by gel permeation chromatography (GPC). Samples are solvent-extracted after being mixed with 10% deactivated silica. The extract is concentrated by Kuderna–Danish evaporation and the resulting residue analyzed directly by HPLC with UV detection. A method detection limit of 100 ppb was calculated. The overall time required for a complete determination is improved over methods using separatory funnel extraction and GC analysis by approximately 3-fold. Spike recovery of DEHP from infant formula and four different milk types (raw, whole, low-fat, and skim milk) ranged from 65% to 80% recovery at the 10 mg/L spike level.

Plasticizers are nonvolatile solvents compounded with plastic resins to increase their workability, flexibility, or extensibility. Bis(2-ethylhexyl) phthalate (DEHP) is the most common plasticizer, with an estimated annual production rate of 2 billion pounds worldwide (National Research Council, 1986).

DEHP has been found in soils, human and animal tissues, air, foods, and marine life (Peakall, 1975). Two studies have estimated the total daily human consumption of DEHP from all sources of exposure at 5.8 mg in the United States (U.S. Department of Health and Human Services, 1985) and 2.1 mg in Japan (Nakamura et al., 1979). Due to the apparent ubiquity of DEHP in the environment, the effects of human exposure to DEHP have been extensively researched during the past two decades (Department of Health, Education and Welfare, 1972; National Toxicology Program, 1981; Thomas et al., 1978).

Foods become contaminated with DEHP by contacting containers, wrappings, and food-processing equipment made of plastic or using plastic parts containing DEHP. For example, various types of plastic tubing are commonly used for conveying milk, and PVC food wrap is widely used in the United Kingdom (Castle et al., 1987). Since a plasticizer is not permanently bound to the plastic resin, it will migrate into foods containing lipophilic materials such as fats and oils. Because of this, the Food and Drug Administration (FDA) limits the DEHP content in materials contacting food to 3% (w/w) (*Code of Federal Regulations*, 1987).

Analytical methods commonly used for determining DEHP in milk or infant formula have been residue methods based upon a separatory funnel extraction and gas chromatographic analysis with flame ionization, electron capture, or mass detectors (Cocchieri, 1986; Thuren, 1986; Ferrario et al., 1985; Kamps et al., 1985; Petitjean-Jacquet and Vergnaud , 1983; Suzuki et al., 1979; Giam et al., 1975; Anderson and Lam, 1979). These analytical methods have excellent sensitivity; however, they often have very tedious extraction procedures. Also, milk extracts generally require a rigorous cleanup step, usually by gel permeation chromatography (GPC), to remove coextracted fats and oils before GC analysis.

In some instances, impurities can be intolerable since they limit the size of injections and require long, high-temperature oven hold times to completely desorb the chromatographic column. In these cases, a second cleanup step, either column chromatography or additional GPC, has been used (Burns et al., 1981). This laboratory initially employed a pesticide method developed by Kamps et al. (1985). The method consisted of a separatory funnel extraction, centrifugation of the resulting emulsion, and GPC cleanup of the residue, followed by GC/MS analysis. The method was effective, but very laborious. To overcome this disadvantage, a new method was devel-

† Present address: Hewlett-Packard, 675 Brooksedge Blvd., Westerville, OH 43081.

0021-8561/90/1438-0415$02.50/0

oped to circumvent many of the time-consuming steps. In the newly developed method, the sample is extracted after adsorption onto silica gel, eliminating any emulsion problem, and HPLC with UV detection is used to eliminate residue cleanup requirements.

MATERIALS AND METHODS

Chemicals and Reagents. Pesticide-grade acetonitrile, methylene chloride, and methanol were obtained from the Burdick and Jackson Division of the Baxter Healthcare Corp. Deactivated silica (10%) was prepared by adding 10 g of deionized organic-free water to 90 g of 60/200-mesh silica (SilicAR Grade 62 special) obtained from Mallinckrodt Chemical Co. The bis(2-ethylhexyl) phthalate standard was obtained from the Aldrich Chemical Co. Dilutions of the reference standard were prepared in methanol.

Apparatus. DEHP analyses were performed on a Varian 5500 liquid chromatograph equipped with variable-wavelength UV detector and computing integrator. The analytical column was a 4 mm × 30 cm Varian MCH5 C18 column. A column heater set at 40 °C was used but is not required.

Analytical Procedure. *Special Precautions.* Glassware must be scrupulously cleaned. Detergent washings followed by pesticide-grade methanol rinses will sufficiently clean the glassware. Cleaned glassware should be sealed or capped with aluminum foil and stored in a clean environment.

Additionally, because of the ubiquity of plastics, extreme care must be taken to prevent inadvertent sample contamination. As an example, the plastic coating on popular types of laboratory clamps easily contaminates a sample when any solvent dripping down the sides of glassware contacts plastic and falls into the collection flask.

Poor-quality solvents can also be a source of interferences. If the quality of the solvent is unknown, it is advisable to determine purity by analyzing a 1-L sample of the solvent that has been evaporated to a few milliliters.

Sample Extraction. Milk samples are prepared without dilution. Mix any concentrated infant formula with reagent water to ready-to-use proportions, and place 25 mL of the sample in a 600-mL beaker. Slowly add to the sample enough 10% deactivated silica gel to form a wet slurry. Mix well using a stainless steel spatula. Continue adding silica a few grams at a time with thorough mixing until the mixture is dry and free-flowing and does not cling or stick to the sides of the beaker. The amount of silica gel used per sample will be very close to a 4:3 weight ratio.

Place a plug of glass wool into the neck of an addition funnel, and position it to drain into a 500-mL Kuderna–Danish (K–D) evaporator flask fitted with a 10-mL concentrator tube. Pour the sample/silica gel mixture into the addition funnel, and extract the mixture by adding 300 mL of 99% methylene chloride/1% methanol extracting solvent to the funnel in one addition.

The solvent addition should be made quickly and forcefully enough to disturb the silica bed. This mixing action will help ensure that the bed is completely wetted by the solvent. Any dry areas persisting in the bed should be mixed into the slurry with the mixing spatula.

The solvent flow rate through the bed in not critical. However, the flow should be rapid enough to allow the funnel to drain completely in approximately 15–20 min.

After the extracting solvent has drained, pass an additional 50 mL of the extracting solvent through the mixture twice without disturbing the bed. When the last 50-mL addition has drained completely, the addition funnel is removed from atop the K–D flask and the silica gel mixture discarded. To the K–D flask containing the collected solvent, add 10 mL of methanol and a glass or PTFE boiling chip and attach a three-ball Snyder column.

Extract Concentration. Prewet the Snyder column with a few milliliters of methylene chloride, and evaporate off the extracting solvent by placing the assembly in a water bath set to 80–85 °C. During evaporation, add 10 mL of methanol through the Snyder column when the solvent volume has been reduced to approximately 50 mL. Do not allow the solvent volume to drop significantly below 50 mL before the 10-mL methanol addition. Continue evaporation until the solvent volume is approximately 2–3 mL. Remove the K–D apparatus from the water bath, and allow it to drain and cool for at least 10 min.

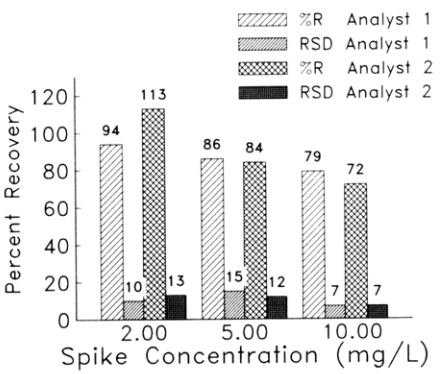

Figure 1. Bar graph of percent recovery (% R) and relative standard deviation (RSD) DEHP spike concentration using a typical milk based infant formula. The performance study involved two analysts performing triplicate analyses at three different spike levels.

Adjust the final residue volume to 5 mL with methanol, and transfer to a screw-top vial for storage. In some cases, a small, immiscible layer of fat may be present after the residues have been adjusted to their final volumes. In these cases, the mixture is shaken vigorously, the layers are allowed to settle, and only the top methanol layer is analyzed.

High-Performance Liquid Chromatography. A 20-μL portion of the final extract is injected. A column flow rate of 1.25 mL/min is used with a mobile phase of 90% acetonitrile/10% water. The mobile phase is switched to 100% methanol after 16 min to clean the column. After 14 min the mobile phase is switched back to 90% acetonitrile/10% methanol for a 20-min reequilibration before the next analysis. The detector wavelength is set at 225 nm. The detector response is recorded from triplicate injections of the calibration standards. The average peak height or area for each standard is used to calculate a calibration curve or calibration response factor (CRF). If the ratio of response to the amount of DEHP injected (CRF) is consistent over the working range (<10% RSD), linearity through the origin is assumed and the average ratio or response factor is used in place of a calibration curve.

RESULTS AND DISCUSSION

Figure 1 illustrates the results of a method performance study using a milk-based infant formula spiked with DEHP. Two analysts performed parallel experiments consisting of triplicate sample analyses at three different spiking concentrations of 2, 5, and 10 mg/L. Spike recoveries ranged from 94–113% at 2 mg/L to 75–85% at the higher concentrations.

The accuracy and precision indicated in Figure 1 compared very well to the method described by Kamps et al. (1985). The overall speed of analysis, however, was greatly improved. As an example, a later study involving the analysis of 87 milk samples was accomplished in about 40 working hours, including instrumental analysis. The same study using the more extensive GPC cleanup and GC/MS analysis would have required 120 working hours.

From Figure 1, a trend of lower recovery at the higher DEHP spike concentrations can be noted. Initially, the linearity of the detector was suspected. However, subsequent checks of the detector response demonstrated linearity 10 times higher than that of the highest residue concentration. This would indicate that some aspect of extraction or concentration is responsible for the recovery trend. The exact cause for the observed trend between analyte concentration and recovery is not known. However, the low recovery has been observed to correspond

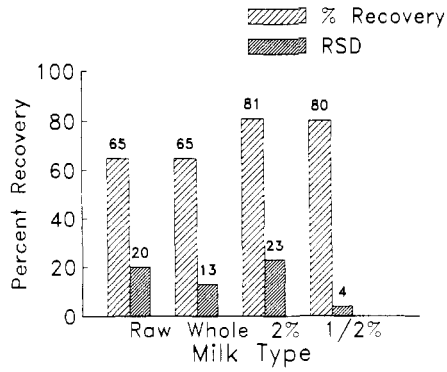

Figure 2. Bar graph of percent recovery and relative standard deviation (RSD) vs DEHP spike concentration in unprocessed milk (raw) and commercially available milk with 3.5% milk fat (whole), low-fat or 2% milk fat (2%), and skim or $^1/_2$% milk fat (1/2%). The performance study consisted of triplicate analyses of the four milk types at a single spike concentration of 10 mg/L.

with higher total solids and higher fat matrices. Therefore, overloading of the silica is suspected.

A method detection limit for infant formula samples of 0.1 mg/L was calculated using methods described in Appendix B of the *Federal Register*, Oct 26, 1984. The detection limit study was performed with a DEHP spike concentration of 0.3 mg/L with a total of eight replicate analyses. The calculated detection limit produces a detector response of at least 3 times the normal signal to noise ratio for a sample at 0.3 mg/L.

The calculated detection limit for this method is approximately 10 times greater than the detection limit achievable by the method developed by Kamps et al. The higher detection limit is primarily due to the smaller concentration factor (5×), as well as instrument sensitivity (30 ng of DEHP on-column). A larger sample size could be used to decrease the limit of detection; however, the 25-mL sample size represents the optimum that the method can easily handle without the use of GPC cleanup.

Figure 2 illustrates the results of a method performance study on unprocessed cow's milk (raw), whole processed milk (whole), low-fat milk (2% fat), and skim milk ($^1/_2$% fat). With the exception of the unprocessed milk sample, the sampling included plastic and wax paper containers as well as different commercial brands. The study consisted of triplicate analyses of the four milk types at a single spike concentration of 10 mg/L.

The average spike recovery at 10 mg/L compared very well with recovery from infant formula for spikes at the same concentration. The 73% (RSD 15%) and 76% (RSD 7%) recoveries for cow's milk and infant formula, respectively, indicate that the method is applicable to either matrix. The data in Figure 2 also indicate that analyte recovery may be related to the fat content of the milk sample.

A formal determination of a method detection limit for cow's milk was not included in the above performance study. Due to the similarities in matrices, the method detection limit of 100 μg/L determined for infant formula samples was assumed to be sufficiently accurate for the needs of this laboratory. No detectable amount of DEHP was found in any of the milk samples used in the study.

Figure 3 is a typical chromatogram for the HPLC mobile-phase system that was found to be satisfactory for the analysis of the sample residues. Figure 3 represents a 0.8 mg/L DEHP-spiked sample analyzed with 87% acetonitrile/13% water at 225 nm and 1.25 mL/min. The

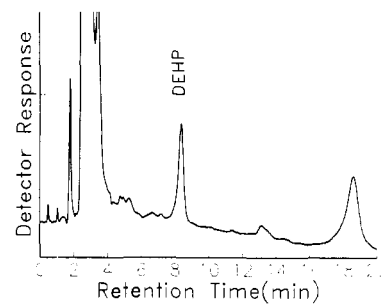

Figure 3. Typical chromatogram formula residue from a sample spiked to 800 ppb DEHP. Analytical conditions: mobile phase of 87% acetonitrile and 13% water at an analytical wavelength of 225 nm and a column flow of 1.25 mL/min.

chromatogram demonstrates that these conditions have adequate resolution and sensitivity. Additional resolution of the DEHP peak from background components can be gained by increasing the polarity of the mobile phase. However, any gain in resolution is accompanied by a decrease in sensitivity. Also, the 225-nm analytical wavelength requires a 15–20-min reequilibration period after the column has been washed postrun with 100% methanol. This postrun wash to remove late-eluting interferences still requires significantly less time than either GPC or open column cleanup prior to analysis.

Initial method development work was performed with a methanol/water mobile phase and analytical wavelength of 240 nm. Later development work investigated the potential of using shorter analytical wavelengths and a more UV-transparent solvent (acetonitrile) for greater sensitivity. The mobile-phase conditions and analytical wavelength of Figure 3 were finally chosen as the optimum conditions for the method.

During our development of the method, diatomaceous earth was investigated as an alternative adsorbent and was found to be inferior to 10% deactivated silica gel. When diatomaceous earth was used as the adsorbent, obtaining a dry, free-flowing mixture of adsorbent and sample was very difficult. Analyses of infant formula samples that substituted diatomaceous earth for 10% deactivated silica gel had a calculated detection limit of 157 μg/L.

The described method provides a rapid and accurate technique for determining the concentration of DEHP in milk or infant formula. Typical analyte recovery of 70–100% is comparable to more complicated residue techniques. The greatest utility of the method is in analyzing milk matrices that severely emulsify during solvent extraction and that have residues containing high boiling point components. In addition, the speed of the method is improved over more traditional methods based on separatory funnel extraction and gas chromatographic analysis.

ACKNOWLEDGMENT

We thank Lawrence Smith for providing unprocessed milk samples as gifts from the Ohio Agricultural Research and Development Center, Wooster, OH.

Registry No. DEHP, 117-81-7.

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Received for review January 25, 1989. Accepted August 4, 1989.

Preparation and Purification of Malonaldehyde Sodium Salt

André Lacombe,† Selim Kermasha,* Frederik R. van de Voort, and Bernice L. Mills†

Department of Food Science and Agricultural Chemistry, Macdonald College of McGill University, 21,111 Lakeshore Road, Ste Anne de Bellevue, Québec, Canada H9X 1C0

Malonaldehyde is not commercially available and is generally prepared when required from 1,1,3,3-tetramethoxypropane (TMP). Although sodium malonaldehyde (NaMA) is potentially a stable product that could be preprepared to meet research needs, present procedures either are tedious or have been shown to be inadequate in terms of purity. NaMA was prepared by adding 1 N HCl to TMP, stored at 4 °C for 24 h, and neutralized to pH 8.0 and the moisture removed under vacuum at <40 °C. The dry end products were selectively solubilized in anhydrous alcohol and filtered twice through neutral charcoal to remove any color compounds, the ethanol was removed under vacuum, and the residue was lyophilized. Analysis of the lyophilisate by HPLC produced a single peak, shown to be free of methanol and higher polymeric forms by infrared analysis, and its purity was determined to be >98% by NMR. The procedure developed is an improvement over previous methods, has an overall recovery of about 60%, and provides a product of sufficient purity for mutagenicity testing and as a standard for the thiobarbituric acid test (TBA) commonly used for assessing the autoxidation of fats and oils.

Malonaldehyde has come under increasing scrutiny by the health profession as a potential mutagenic agent (Basu and Marnett, 1983) that has ramifications in relation to the ingestion of oxidized lipids. The effects of malonaldehyde are difficult to study because it cannot be obtained commercially, is generally unstable in solution, and has to be prepared as required, limiting its ready availability as a standard material for mutagenicity studies or more general work such as standardizing the thiobarbituric acid test commonly used to evaluate the degree of autoxidation of fats and oils.

Malonaldehyde was first described by Claisen (1903) as a three-carbon dialdehyde resulting from the acid hydrolysis of β-ethoxyacrolein diethyl acetal. In 1941, Hüttel developed a simple procedure for producing malonaldehyde but found it to be hygroscopic, generally unstable, but could be stabilized in the form its sodium salt. The subsequent recognition that malonaldehyde was a

† Present address: Department of Nutrition, University of Montréal, P.O. Box 6128 Station A, Montréal, Québec, Canada H3C 3J7.

0021-8561/90/1438-0418$02.50/0