High-Pressure Liquid Chromatography Detection of the Antithyroid Compound 5-Vinyloxazolidine-2-thione in Milk

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A method is described for the analysis in milk of low levels of the goitrogen 5-vinyloxazolidine-2-thione (OZT), a hydrolytic product of a glucosinolate present in some rapeseed meal used in animal feed. Milk at pH 8.5 was treated with hydrogen sulfide prior to extraction with diethyl ether. The residue obtained from the ether extract was reextracted with hexane–water (pH 7) and recovered from the aqueous phase (pH 8.5) using diethyl ether. The OZT in the ether residue was separated by high-pressure liquid chromatography on a Lichrosorb Si60 (5 μm) column in isooctane containing 10% isopropyl alcohol and 0.1% ammonium hydroxide. The absorption was measured at 254 nm. Less than 2 μg of OZT per liter of milk can be detected on a relatively small sample (50 mL). OZT was not detected in winter milk from two dairies in Manitoba, Saskatchewan, and Alberta and three dairies in Ontario.

Antithyroid activity has been attributed to thiocyanate and 5-vinyloxazolidine-2-thione (OZT), which are hydrolytic products of glucosinolates present in rapeseed and other members of the family Cruciferae. Since rapeseed meal is used as animal feed in Canada and Europe, the transfer of OZT to milk consumed by humans has been the subject of several investigations (Arstila et al., 1969; Hoppe et al., 1971; Krusius et al., 1966; Virtanen, 1961; Virtanen et al., 1958, 1959, 1963). The detection of low levels of antithyroid substances is of particular importance since milk is consumed in large quantities by infants and children.

Previous methods of determination involve the extraction from milk, separation by paper chromatography, and measurement by ultraviolet spectrophotometry. The reported detection of 5–10 μg of OZT per liter was achieved by the extraction of 300 mL of milk with ethyl acetate, removal of neutral lipids, reextraction with chloroform, and isolation of OZT by two-dimensional paper chromatography before spectrophotometry (Kreula and Kiesvaara, 1959). More recently similar extraction procedures requiring 200 mL of milk have been reported (Madjeski, 1973).

Recently we investigated the GLC characteristics and response of four different detectors to OZT and its heptafluorobutyryl derivative (McLeod et al., 1978). It was possible to detect 0.29 ng of the derivative by electron capture, 5 ng by the Coulson electrolytic conductivity detector (CECD) in the halogen mode, 15 ng by the Melpar flame emission for sulfur at 394 nm, and 35 ng by CECD in the nitrogen mode. Application of the derivatization technique was demonstrated using milk extracts cleaned up by high-pressure liquid chromatography (LC) and spiked with OZT at 2 ppm.

A brief reference has been made to the use of LC for the separation of OZT on Porasil 3M column in isooctane–ethanol (Report Government Chemist, 1975), but no details were given. After the completion of this work another LC method for the determination of OZT in milk was reported in Sweden (Josefsson and Åkerstrom, 1978). In this paper we present the LC detection and measurement of low levels of OZT in milk. Pasteurized milk from three dairies in Ontario and six western dairies were analyzed using this method.

MATERIALS AND METHODS

Preparation of 5-Vinyloxazolidine-2-thione (OZT). OZT was prepared from rapeseed meal extract generously provided by Dr. J. Jones, Food Research Institute, Ottawa. This was a freeze-dried warm water extract of the dehulled *Brassica napus* cv. Target meal. The extract (1 g) was hydrolyzed for 18 h with thioglucosidase (30 mg) in 0.02 M citrate–phosphate buffer (5.0 mL) at pH 7.0. The hydrolysate was extracted with diethyl ether. After removal of the ether under reduced pressure, the residue was dissolved in water by gentle warming, extracted with hexane to remove neutral lipids, and then reextracted with diethyl ether. The ether residue was applied to silica gel G plates activated at 110 °C for 30 min and then developed in chloroform containing 1% ethanol. The R_f value was determined by chromatography of OZT on a separate plate and staining with iodine vapor. After removal of the neutral lipids with hexane, the OZT was isolated by thin-layer chromatography on activated silica gel G in chloroform containing 1% ethanol. The OZT was eluted from the gel with absolute ethanol and reextracted with diethyl ether. After removal of the ether the residue was stored at −20 °C under nitrogen.

The identity of the isolated OZT was established by mass and nuclear magnetic resonance spectroscopy. The prepared OZT and that obtained from Lancaster Synthesis Ltd, England, had identical spectra. The infrared absorption spectrum of our preparation of OZT is shown in Figure 1.

OZT was quantitated by measuring the optical density at a wavelength of 248 nm (correcting for background absorption) and applying the molar absorbance of 15 800 (Appelqvist and Josefsson, 1967) as determined on an OZT preparation kindly supplied by Dr. M. E. Daxenbichler.

Preparation of Milk Samples. For the purpose of establishing a suitable extraction procedure, milk was obtained from the Department of Agriculture to ensure that the cows' diet, and subsequently the milk, would be reasonably uniform and free of glucosinolates and their hydrolytic products. The milk was heated at 70 °C in a water bath for 30 min and then cooled to room temperature before the addition of OZT in aqueous solution. OZT was added to 50.0 mL of milk to produce concentrations of 0, 2, 4, 7, and 113 ng per milliliter. These were stored at −20 °C.

Extraction of OZT from Milk. The procedure for the extraction is outlined in Table I. Prior to the extraction the pH of the milk was adjusted to 8.5 with NaOH (2 N) and hydrogen sulfide was bubbled through the samples at a moderate rate for 5 min (Arstila et al., 1969). The first two extractions with anhydrous diethyl ether required careful manipulation to avoid emulsion formation. The transfer of the residue, obtained from the first set of ether extracts, was facilitated by the use of 1% ammonia and

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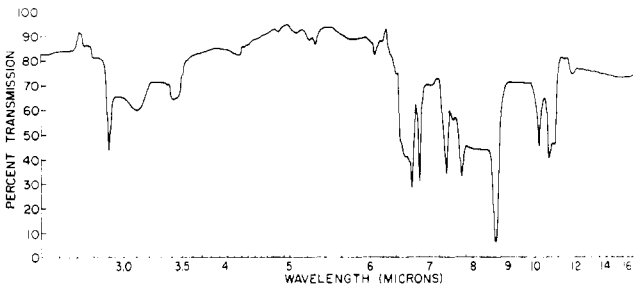

Figure 1. Infrared spectrum of OZT isolated from a hydrolyzed extract of *Brassica napus* cv. Target. A solution of OZT in redistilled chloroform was analyzed using a double-beam Beckman infrared spectrophotometer, Model 4230.

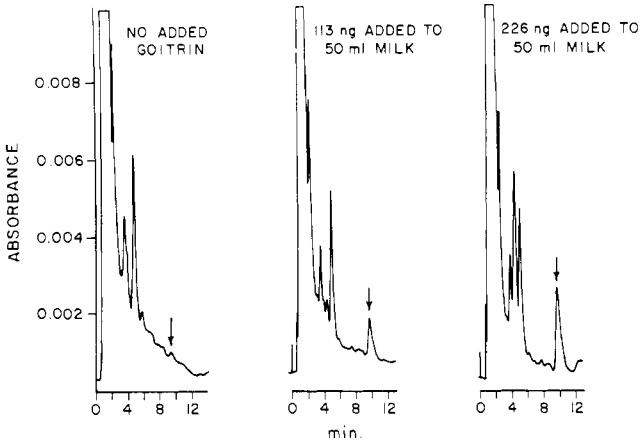

Figure 2. LC chromatograms of milk (50.0 mL) containing added OZT, extracted as described in the text, and dissolved in 0.2 mL of isooctane containing 10% isopropyl alcohol. Detection at 254 nm. Mobile phase: isooctane, isopropyl alcohol, ammonium hydroxide (90:10:0.1).

Table I. Extraction Procedure for the Analysis of OZT in Milk

milk, 50.0 mL
pH adjusted to 8.5 with 2 N NaOH
H_2S bubbled through sample for 5 min
extracted with diethyl ether[a] (130 mL × 4)
ether-phase residue transferred with ammonium hydroxide
1% (3 mL × 3) neutralized to pH 6.7-7.5
extracted with hexane (3 mL × 4)
pH of aqueous phase adjusted to 8.5.
aqueous-phase extracted with diethyl ether (5 mL × 4)
ether-phase residue dissolved in isooctane containing 10% isopropyl alcohol (0.2 mL) and 30 μL applied to LC column

[a] MillinKrodt, anhydrous, containing 1-2 ppm butylated hydroxytoluene.

gentle warming on a water bath at 40 °C. To avoid losses in the hexane, the pH was adjusted to 6.5–7.5 with concentrated HCl (50 μL) (Kreula and Kiesvaara, 1959). Prior to the final extraction with diethyl ether, the hexane was removed by evaporation under a stream of nitrogen gas and the pH adjusted to 8.5 with 2 N NaOH (2 drops). The residue obtained from the final set of ether extracts was dissolved in an appropriate volume of isooctane containing 10% isopropyl alcohol, applied to the LC column, and chromatographed as described in the next section for purified OZT. The chromatograms are presented in Figure 2.

High-Pressure Liquid Chromatography. The apparatus consisted of a Waters Model 6000A pump with a 254-nm fixed wavelength detector and a U6K loop injector. A 250 × 3.2 mm stainless steel column packed with Li-

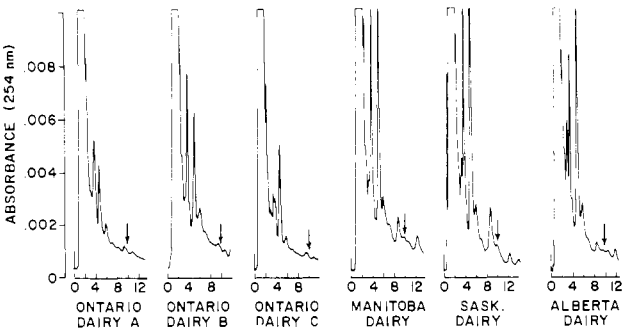

Figure 3. LC chromatograms of milk (50.0 mL) extracted and analyzed as in Figure 2.

Table II. Recovery of OZT from Fortified Milk

OZT (ng) added to 50.0 mL of milk	recov., %[a]	no. of estimations
0		3
113	76 ± 9	3
226	62 ± 3	4
339	64 ± 8	4
5640	81 ± 6	3

[a] Data are expressed as mean ± SE.

chrosorb Si60 (5 μm) was used at ambient temperature. Commercially available analytical grade solvents were used. The column was eluted with isooctane containing 10% isopropyl alcohol and 0.1% ammonium hydroxide.

An accurately determined quantity of purified OZT, prepared as described, was dissolved in isooctane containing 10% isopropyl alcohol. Thirty microliters was applied to the column. The flow rate was 1.5 mL/min.

Analysis of Milk Samples. Pasteurized milk was obtained from two dairies in Alberta, Saskatchewan, and Manitoba, as well as three different dairies in Ontario. These samples were analyzed in duplicate as described. The LC chromatograms are presented in Figure 3. These chromatograms were obtained from the application of 30 μL of the ether phase residue dissolved in 200 μL of isooctane containing 10% isopropyl alcohol and chromatographed under the same conditions as the purified OZT.

RESULTS

The infrared spectrum of the isolated OZT (Figure 1) was similar to those of Astwood (1949) and Lanzani et al. (1971). Nuclear magnetic resonance and mass spectrometry confirmed that the material was similar to a commercial sample. The preparation of OZT dissolved in diethyl ether showed an absorption maximum at 248 nm.

The purified OZT, applied to the LC column and chromatographed at a flow rate of 1.5 mL/min, had a retention time of 9.5 min. A peak of 1 cm (Δ OD = 0.0004) was obtained after injection of 4.9 ± 0.2 ng.

The LC chromatograms in Figure 2 were obtained from 50.0 mL of milk containing 0, 113, 226 ng of added OZT. The peak at the retention time of OZT was sharp and well separated from the large amount of material eluted at the beginning of the chromatogram. The identity of the peak was confirmed by spiking the milk extract with a known amount of OZT.

Recoveries of five levels of OZT added to 50.0 mL of milk are presented in Table II. The recoveries ranged from 62% for the low level addition to 81% for the addition of 5.64 μg. The maximum standard error was 9%.

The LC chromatograms from the residues of milk obtained from six western and three eastern dairies are presented in Figure 3. There was no evidence of a peak

at the retention time of OZT. This was confirmed by spiking the extract with a known quantity of OZT. A peak appeared after 8.5 min in the milks from six western dairies, but this peak was not detected in milk from three dairies in Ontario.

DISCUSSION

The level of OZT in milk in Finland was reported to be as high as 35–100 μg/L of milk (Arstila et al., 1969). Formerly levels of OZT less than 100 μg/L were considered to be insignificant because this amount does not inhibit radioiodine uptake (Virtanen et al., 1963). Rats dosed orally with 500 ng of OZT daily for 2 weeks showed significant thyroid enlargement but radioiodine uptake was not affected (Krusius and Peltola, 1966). The lowest single dose required to inhibit radioiodine uptake was found to be 500 μg which is 1000 times greater than the oral daily dose required to produce enlargement of the rat thyroid. A single oral dose of 25 mg of OZT inhibited radioiodine uptake in adult human (70 kg) (Langer et al., 1971). Extrapolation from the results obtained for rats indicates that thyroid enlargement is possible at an OZT level of 2 μg/L of milk for an infant of 6 kg ingesting 1 L of milk per day.

With the extraction procedure described and the use of LC, relatively small volumes (50 mL) of milk can be analyzed to detect levels of OZT as low as 1–2 μg/L.

Analysis of pasteurized milk samples from six western and three eastern dairies indicated that the winter milk supply from these dairies did not contain OZT in amounts greater than 2 μg/L. An unknown compound with a retention time, close, but not identical with that of OZT, was detected in milk from six western dairies.

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Extraction of Free and Bound Carboxylic Acid Residues from Field Soils Treated with the Herbicides Benzoylprop-ethyl, Diclofop-methyl, and Flamprop-methyl

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The breakdown of the herbicidal esters benzoylprop-ethyl, diclofop-methyl, and flamprop-methyl was studied in small field plots at three locations in Saskatchewan after two separate growing seasons. Following extraction of soil samples with aqueous acidic acetonitrile, the parent esters and acid hydrolysis products were determined gas chromatographically. Residues of benzoylprop-ethyl and flamprop-methyl, together with their corresponding acids, were recovered from treated soils at all sites both years, whereas no significant amounts of diclofop-methyl (or acid) were observed in any sampled soils. Following extraction with the acidic acetonitrile solvent, the soil residua were reextracted with cold aqueous sodium hydroxide or hot aqueous triethanolamine solutions to release any benzoylprop, diclofop, or flamprop acids held on the soil in a bound form. From these alkaline digestion studies and experiments conducted to compare the extractions of the three herbicidal acids from fortified soils using cold sodium hydroxide and hot triethanolamine as extractants, it was concluded that any such bound residues could only be present in negligible quantities.

The herbicidal esters benzoylprop-ethyl [1, R = Cl, R′ = C_2H_5; ethyl (±)-2-[*N*-(3,4-dichlorophenyl)benzamido]-propionate], diclofop-methyl [2, R = CH_3; methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propionate], and flamprop-methyl [1, R = F, R′ = CH_3; methyl (±)-2-[*N*-(3-chloro-4-fluorophenyl)benzamido]propionate] are used at rates of approximately 1 kg/ha for the control of wild oats (*Avena* sp.) and other annual grasses in a variety of crops.

Although these herbicides are applied as postemergence treatments, some of the spray can come into contact with the soil where they undergo hydrolysis to their respective carboxylic acids (Beynon et al., 1974; Roberts, 1977; Smith, 1977). Initially these acids can be recovered almost quantitatively from treated soils using acidic polar solvent mixtures (Wright and Mathews, 1976; Smith, 1976, 1977). Using ^{14}C-labeled herbicides it has been shown that with

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