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Determination and Qualitative Confirmation of Melengestrol Acetate Residues in Beef Fat by Electron Capture Gas Chromatography and Gas Chromatographic/Chemical Ionization Mass Spectrometry

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A method for the detection of melengestrol acetate in beef fat is presented. Crude extracts are purified by preparation thin-layer chromatographic techniques. Residues of melengestrol acetate in the range 1–100 ppm are quantitated. Mass spectral confirmation is possible without additional cleanup of the sample extract. Test results obtained on Canadian beef heifers for 1982–1984 are presented.

Current Canadian regulations (Agriculture Canada (No. 46), 1984) allow the use of melengestrol acetate, MGA (17-hydroxy-6-methyl-16-methylenepregna-4,6-diene- 3,20-dione acetate), at a rate of 0.40 mg/animal per day. It is administered as a feed additive and restricted to intact heifers 181 kg and over. A withdrawal period of 48 h prior to slaughter is required.

At present a zero tolerance exists for MGA residues in foods (Campbell, 1978). With current sensitive analytical techniques, detectable residues may occur in spite of proper withdrawal (Ryan and Dupont, 1975).

Various residue detection techniqueshave been developed with spectrophotometric (Krzeminski et al., 1968), gas chromatographic with flame ionization (Duggan, 1968, 1969), and gas chromatographic with electron capture (Krzeminski and Cox, 1973; Krzeminski et al., 1976; Ryan and Dupont, 1975) detection principles. Only the method of Ryan and Dupont has the required sensitivity to detect MGA residues at the low levels that might occur in animal tissues (Krzeminski et al., 1981). The methods employing electron capture detection suffer from quantitation problems due to the severe effect of coextractants on the gas chromatographic base line.

In this paper, a relatively simple cleanup technique is described, which allows quantitation of MGA residues in the range 1–100 μg/kg in animal fat. Direct injection into a gas chromatograph/mass spectrometer without further cleanup was possible, thus allowing unambiguous identification of the residue.

This method was used in a 2-year survey of MGA residue levels in the fat tissue of Canadian slaughter heifers. During 1982–1984 671 samples were collected from Canadian packing plants for analysis. In 1982–1983, MGA levels ranged from less than 2 to 20.4 μg/kg with a mean of 3.4 μg/kg. In 1983–1984, MGA levels ranged from less than 2 to 28.7 μg/kg, with a mean of 2.8 μg/kg.

EXPERIMENTAL SECTION

Materials. Melengestrol acetate (minimum 99% purity) was provided by Upjohn Co., Kalamazoo, MI. Precoated silica gel thin-layer chromatography plates with preabsorbant spotting area (Whatman LK5F) were purchased from Terochem Laboratories Ltd., Edmonton, Alberta, Canada T6E 3A4. All solvents used were glass-distilled residue grade.

Apparatus. The gas chromatograph was a Hewlett-Packard 5880 equipped with a ^{63}Ni high-temperature electron capture detector. The mass spectrometer was a Finnigan 4021 capable of positive/negative multiple ion monitoring and operating in both electron impact and chemical ionization modes.

Standard Solutions. Stock solutions A and B were prepared as previously described (Krzeminski et al., 1976) except that solution B used acetone instead of methanol. A 5-mL portion of solution B was diluted to 200.0 mL with acetone (solution C, 2.5 μg/mL) and prepared daily as needed. Portions of 1, 2, 3, and 4 mL each of solution C were diluted to 10.0 mL

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with acetone. Respectively, these are solutions D (0.25 μg/mL), E (0.50 μg/mL), F (0.75 μg/mL), and G (1.0 μg/mL) and represent tissue equivalents of 5, 10, 15, and 20 μg/kg MGA.

Sampling Scheme. Samples consisting of 100 g of fat from slaughtered heifers were collected at packing plants across Canada according to a random survey plan from April 1, 1982, to March 31, 1984. The tissues were packaged in plastic bags, frozen, and shipped to the analytical laboratory. The samples were stored at −18 °C until analysis. Samples received in a thawed state were discarded.

Extraction. The frozen sample was grated and immediately extracted as described by Krzeminski et al. (1976) with the following exceptions. The residue from the round-bottom flask was quantitatively transferred with three rinses of 3 mL of acetone into a 10-mL sample vial. The acetone was evaporated in a 50 °C water bath, under a stream of dry nitrogen, to a volume of approximately 0.5 mL. A blank sample spiked to contain 10 μg/kg MGA was analyzed with every run to determine recovery values.

Thin-Layer Chromatography. Thin-layer chromatography was used for sample cleanup. A 20 cm × 20 cm plate was divided into nine vertical channels by scraping the silica layer with a metal probe. The channels had the following widths (as measured from the left edge): 0.6 cm, 1 cm, 0.6 cm, 7.5 cm, 0.6 cm, 7.5 cm, 0.6 cm, 1 cm, 0.6 cm. The TLC plate was then placed on a heating strip (surface temperature 60 °C), which heated the preadsorbent spotting zone. The two 1-cm channels were spotted with 20 μL of MGA solution A (1000 μg/mL). Two sample extracts were streaked onto the two 7.5-cm-wide channels. The plate was then placed into a developing tank containing hexane, in such a fashion that the upper 2 cm protruded past the lid, which was slid open slightly, allowing evaporation of solvent from this point. This continuous development was allowed to proceed for 16–18 h. The plate was removed from the tank, allowed to dry for 20 min, and then immediately developed to a height of 12 cm in a tank saturated with a freshly prepared solution of 1.5% methanol in benzene. A UV source at 254 nm was used to visualize the MGA standard zones. Sections of silica gel (1.5-cm-wide bands) were scraped from the sample channels at the R_f corresponding to the MGA standard. The silica gel was drawn, with the aid of a vacuum, into a Pasteur pipet containing a silanized glass wool plug. Each sample was eluted with 2 × 2 mL of acetone into a 5-mL sample vial. The solvent was evaporated just to dryness under a stream of dry nitrogen, and the residue was redissolved in 0.50 mL of acetone.

Gas–Liquid Chromatography. Operating conditions: 0.6 m × 2 mm (i.d.) borosilicate glass column packed with 3% OV-3 on Chromosorb WHP, 80/100 mesh; detector temperature, 320 °C; injection port temperature, 250 °C; oven temperature, 230 °C; carrier gas, argon–methane (5%), 50 mL/min flow. The GC settings were adjusted so that a 2-μL injection of 0.10 μg/mL solution gave a peak height of 1.5–2.5 cm. The column was conditioned by injecting 3 μL of a previously analyzed high-fat-content sample extract. Duplicate injections of the working solutions and single injections of the samples were made. A standard curve of peak height vs concentration in the range 5–20 μg/kg (tissue equivalency) was prepared, and the peak heights of the samples were calculated from this standard curve.

The MGA had a retention time of approximately 6 min under these conditions.

Gas Chromatography/Mass Spectrometry. Operating conditions: 5.5 m × 0.2 mm (i.d.) wall-coated OV-1 fused silica capillary column; carrier gas, helium, head pressure, 8 psi; injection port temperature, 270 °C; source temperature 200 °C; manifold temperature 120 °C; oven temperature initially 50 °C for 1.5 min, rate 20 °C/min, final temperature 275 °C for 5 min; electron multiplier, 1.2 keV. For the analyses, isobutane was used as CI reagent gas. The capillary column was threaded through the transfer line directly into the source. The extracts from the GC quantitation and 0.5 mL of the 0.40 μg/mL working solutions were evaporated to dryness. Approximately 25 μL hexane was added to redissolve the residues. A cold-column injection technique was used to deliver 2 μL of the standard and each sample solution. Integration times were set to 100 ms/amu for each monitored mass. The monitored masses were as follows: positive ions, m/z 397$^+$ and 337$^+$; negative ions, m/z 396$^-$ and 337$^-$.

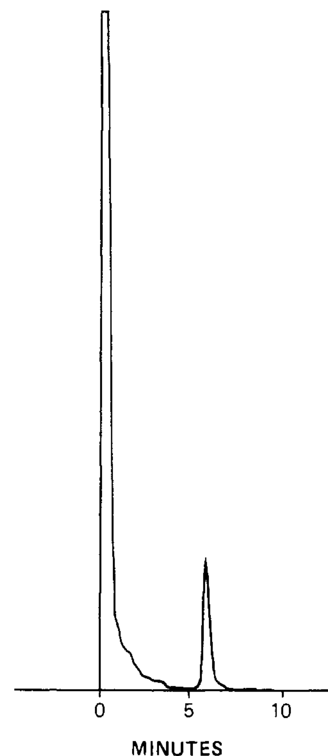

Figure 1. Typical GC tracing of beef fat extract containing 5 μg/kg MGA residue.

RESULTS AND DISCUSSION

Initially prechanneled TLC plates were used (19 channels per plate), with the sample being spotted on two or three adjacent channels, allowing five to six samples to be run per plate. However, depending on the nature of the sample, its condition upon receipt, and its age, these plates did not always have sufficient capacity to handle coextracted materials. By channeling plates ourselves, we were able to produce a channel large enough to accommodate high levels of coextractants. After grinding, adipose tissues must be immediately covered with hexane and extracted. Freezer storage, after grinding, caused a large increase in the amount of coextractant (up to 0.5 g), which overloaded the TLC plate and resulted in losses of MGA.

The continuous development in hexane removed most of the interfering material to the top of the plate and in addition concentrated the MGA as a narrow band at the interface of the silica gel and the preabsorbant material. As a result, it was possible to achieve reproducible and reliable R_f values for the MGA. For example, the R_f for MGA was an identical 0.47 in the standard and the overspiked extract after development in 1.5% methanol/benzene. The MGA band in the standard had a width of about 6 mm. The MGA zone in the sample was taken to include this plus an additional 5 mm above and below the visualized zones.

Figure 1 shows a typical GLC tracing obtained from a 5 μg/kg MGA residue in beef fat. While earlier electron capture detection methods for MGA residues suffered from poor detection limits (Krzeminski and Cox, 1973) and poor resolution of the MGA peak from the trailing solvent peaks (Ryan and Dupont, 1975), the present method does not suffer from these shortcomings. At a level of 2 μg/kg, the signal/noise exceeded 6.5 and the recorder had returned to base line before the appearance of the MGA peak.

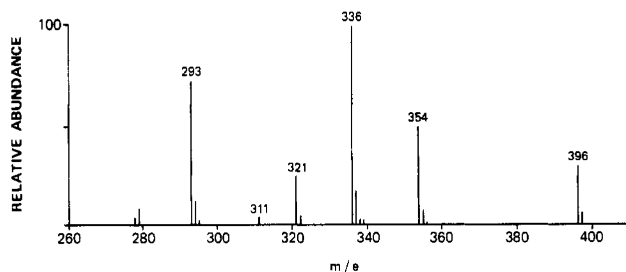

Figure 2. Electron impact mass spectrum of MGA.

Table I. Ion Abundances

	chemical ionization gas	
major ion (m/z)	methane	isobutane
425^+	1	
397^+	10	100
396^-	100	58
338^+	4	30
337^+	5	90
337^-	40	80
336^-	-	60

Problems were initially encountered with the lack of repeatability of peak heights from replicate injections. One remedy was to "condition" the column by injecting a sample several times in succession (Krzeminski et al., 1976) or with the injection of a solution of beef fat in hexane (Ryan and Dupont, 1975). Even upon conditioning the column in this manner, we found it necessary to intersperse standard solution injections between sample injections to obtain good repeatability for manual injections. Quantitation was based on a comparison of the peak height in the sample against the standard curve of peak height vs concentration, which is determined from the injection of the working solutions. A linear response from 5 to 20 μg/kg resulted. Samples with levels in excess of 20 μg/kg were diluted such that the resulting peak height would fall within the established linear range, when reinjected. Calculations were corrected for the dilution factor.

The electron impact mass spectrum of MGA, although producing all characteristic fragments (Figure 2), was not useful for the confirmation of low-level (<10 μg/kg) samples. Variation in the relative abundance of the molecular ion (m/z 396) (0–30%) and the other characteristic ions (m/z 354, 337, 336, 321, 311) in the presence of matrix materials precluded the use of this technique for confirmation. In addition, the abundances of these ions were very low, apparently due to the equally low ionization efficiency for MGA. Positive and negative chemical ionization with methane and isobutane produced the ions listed in Table I. With multiple ion monitoring, samples containing 1 μg/kg could be confirmed by mass spectrometry.

Confirmation was achieved using isobutane reagent gas and monitoring the ions at m/z 397^+, 337^+, 396^-, and 337^-. Presence of MGA was confirmed if all monitored masses appeared at the correct retention time (±2%) and the ratios of the ions $397^+/396^-$, $337^+/397^+$, and $337^-/396^-$ were within ±20% of their relative abundances in the standard.

Table II. MGA Residue Levels in Canadian Beef Heifers

level,[a] μg/kg	no. samples (%)
<2.0	391 (58.3)
2.0–5.0	139 (20.7)
5.1–10.0	110 (16.4)
10.0–15.0	16 (2.4)
15.1–20.0	8 (1.2)
20.1–25.0	4 (0.6)
>25.1	3 (0.4)

[a] 1982–1983 range <2 to 20.4 μg/kg, mean 3.4 μg/kg; 1983–1984 range <2 to 28.7 μg/kg, mean 2.8 μg/kg.

Although these mass ratios varied with source temperature and ionization gas pressure, there was little fluctuation due to matrix and other uncontrolled factors.

Optimum performance was achieved with the instrument tuned so that (1) the most abundant peak was m/z 397^+, (2) m/z 337^+ and 337^- were both at least 80% of m/z 397^+, and (3) m/z 396^- was at least 50% of m/z 397^+.

A source temperature of 200 °C and source pressure of 0.32 Torr met these conditions.

Table II shows the frequency distribution of the levels of MGA residues found in Canadian beef heifers during the 1982–1984 testing period. Only 4.6% of all samples had residues of MGA in excess of 10.0 μg/kg of fat. This program was deemphasized after 2 years since no samples in excess of the administrative action limit of 30 μg/kg were detected.

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