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CHROMATOGRAPHIC ANALYSIS OF HORMONE RESIDUES IN FOOD

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CONTENTS

1. INTRODUCTION

The use of hormone drugs has become an indispensable tool for the efficient production of animal food. This practice has resulted in the need for simple, reliable methodology to monitor for the levels, distribution, and metabolism of hormones in food from animal sources. Chemical, physical and chromatographic techniques are admirably suited to fulfil this requirement but, so far, no general review has been published on methods in this field.

There are several classes of hormone drugs, of which the estrogens were the first to be used in animals. In the 1940s, it was observed that diethylstilbestrol in

several forms had an effect on the growth of some animal species. This fact resulted in both addition of diethylstilbestrol to animal feed and implantation of the animals. The effect of this strong estrogen on cattle was anabolic such that muscle tissue was accumulated more quickly and less feed was used per pound of weight gained. Other classes of hormone drugs are the progestogens and the androgens, which are given to food animals to provide easier management in their development whether it be for synchronization of breeding, suppression of ovulation and estrus, or weight addition. The corticosteroids are another class of hormone drugs which are legally used in animal production. These steroids are employed in the treatment of inflammation, particularly from mastitis, and also in the treatment of bovine ketosis. In addition to these compounds, antithyroid hormones have been used to add weight to food animals, several hormonal polypeptides are known and, more recently, prostaglandins have been hailed as a new class of animal drugs. Because of this widespread agricultural custom, residues of these products can appear in food either from tolerance allowances, abuse, or misuse, and these and their metabolites can be further altered by handling procedures, processing, storing, and cooking.

From the presence of drug residues in food, the hazard to the consumer is varied. It can be a direct toxic effect by ingestion of the drug, an allergenic reaction to the drug, possible effects from tumorigenic compounds, or alteration of drug sensitization of both animal and man to microorganisms. In consequence, withdrawal periods and regulations are often specified to ensure that the levels or tolerances of drugs in food are at acceptable levels. The toxicology and use of animal hormones have been recently summarized[1].

Scope

The types of hormone drugs which are considered are those used in food producing animals such as poultry, cattle, and swine, and are listed in Table 1 under several classes. Many other products are used in dogs, cats, and horses, but these are unlikely to enter the human food chain. As a consequence, the samples considered are the meat products of muscle, fat, liver, and kidney, the dairy products cheese and milk, the poultry product eggs, and cereal animal feeds. All other sources are of secondary interest. However, techniques have been developed for the measurement of hormone drugs in tissues from experimental animals, *e.g.*, rats and mice, and these

TABLE 1

HORMONES IN FOOD PRODUCING ANIMALS

Class	*Examples*
Estrogens	diethylstilbestrol, hexestrol, dienestrol diacetate, zeranol, trenbolone acetate, estradiol
Androgens	testosterone
Progestogens	chlormadinone acetate, fluorogestone acetate, medroxyprogesterone acetate, melengestrol acetate, progesterone
Corticosteroids	cortisone, cortisol (hydrocortisone), prednisone, prednisolone, methylprednisolone, betamethasone, dexamethasone, flumethasone, fluoroprednisolone, triamcinolone
Others	prostaglandins, polypeptides (oxytocin), antithyroid hormones

have been included here when no other procedures for tissues from food animals were available from the literature. It is also explicit that the review considers only those methods which are directly applicable to tissues, human foods, and animal foods (feeds) and excludes methodology in such biological materials as plasma, blood, and urine. For instance, there is a wealth of procedures for measuring progesterone and testosterone, but few of these possess extraction or separation steps to measure the drugs in tissue. The review is also oriented to those procedures which have a high degree of specificity, since the problems involved in measuring a hormone drug in a tissue of unknown history are much greater than measuring the same drug in a controlled experimental protocol. The hormones are discussed under their general effects as estrogens, androgens, progestogens, corticosteroids, and others, even though these are not necessarily the effects for which they have primarily been intended, *e.g.*, zeranol is a weak estrogen but its primary effect is anabolic on the tissues of growing animals.

The general types of methods for measuring hormones in any matrix can be roughly classified into: (i) chemical-physical methods, (ii) bioassay techniques including microbiological inhibition, and (iii) competitive binding procedures such as radioimmunoassay (RIA). All of these types can employ chromatographic means for both extraction, separation, and purification to various degree but it is with chemical-physical detection techniques that chromatographic means are most highly developed. Hence, this review will deal mainly with this type of measurement and describe less thoroughly those detection techniques of the other two classes which include some chromatographic cleanup.

Up to the present, methods for the analysis of hormone drugs have been summarized using bioassays[2,3] and examples of methods, by no means comprehensive, for steroidal hormones have been reviewed in blood and urine[4] and by gas–liquid chromatography (GLC) alone[5]. The regulatory control of some hormone residues in foods by chemical analysis has been briefly described[6]. However, much of the material on hormones in food is of recent appearance and that which has been reported is scattered throughout the literature. Thus, it was deemed advisable to summarize the material on the measurement of hormone drugs in foods from animal sources. Particular emphasis has been placed on chromatographic procedures in order to give the analyst better access to the capabilities and shortcomings in this field. The literature has been covered from 1957 to 1974 systematically plus many references of more recent vintage.

2. ESTROGENS

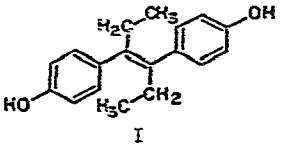
The estrogenic compounds under consideration are almost all phenolic and their structures are shown in Figs. 1 and 2. The classical way for measuring the estrogen content of tissue or food has been the bioassay technique of Umberger *et al.*[7]. This is preferred to the Clauberg rabbit assay[2], as the extraction has been specifically adapted for tissue samples. In the mouse uterus test, the sample is incorporated into the diet of immature female mice and the weight change of the uterus noted. Comparison of this observation with that of controls in a dose–response curve allows a measurement, usually expressed as diethylstilbestrol units, of the estrogen content. This procedure has been found to be accurate with a low detection limit (~2 ppb*) in

* Throughout this article, the American billion (10^9) is meant.

TABLE 2

PROCEDURES FOR DIETHYLSTILBESTROL DETERMINATION IN ANIMAL FEEDS

Extraction	*Purification*	*Measurement*	*Dectection limit*	*Reference*
Benzene	NaOH on Celite column; elute with ethanol–HCl (0.005 *N*)	(a) Colour with $SbCl_5$ at 525 nm (b) Irradiate; absorbance at 410 nm	10 ppm	10
Chloroform	(a) Florisil column; elute with water (b) Partition between NaOH and chloroform	Irradiate; absorbance at 420 nm	10 ppm	11
Chloroform	Partition between NaOH and chloroform	Irradiate; absorbance at 415–420 nm	10 ppm	12–15
Chloroform	Partition between NaOH and chloroform	Disc assay; *Staphyloccus aureus*	10 ppm	16
Chloroform	Partition between NaOH and chloroform	Irradiate; absorbance at 420 nm	10 ppm	17
Chloroform	Partition between NaOH and chloroform	Acetate; GLC on SE-30 at 200°; FID	10 ppm	18
Chloroform–acetone–phosphoric acid (3:3:1)	(a) $Ba(OH)_2$ on Celite column; elute with ether (b) Partition between NaOH and chloroform	(a) Irradiate; absorbance at 415 nm (b) Confirm on TLC on silica gel; irradiate	10 ppm	19
7% ethanol in chloroform	Partition between NaOH and chloroform	Irradiate; absorbance at 415 nm	10 ppm	20
7% ethanol in chloroform	Partition between NaOAc and chloroform	Irradiate; absorbance at 418 nm	10 ppm	21
7% ethanol in chloroform	Partition between NaOH and chloroform	Silylation; GLC on OV-1 at 215°; FID	10 ppm	22
Ethanol–KOH	(a) Partition between KOH and diethyl ether (b) Alumina column (c) TLC on silica gel	Irradiate; visual colour with vanillin	0.05 ppm	23
Silylation, chloroform	—	GLC on JXR at 220°; FID	10 ppm	24
7% ethanol in chloroform	Partition between, NaOH and chloroform	Dichloroacetate, GLC on UC W-98 at 255°; ECD	2 ppm	25
Ethanol	(a) Partition between NaOH and chloroform (b) TLC on silica gel, two-dimensional	Spray vanillin in H_3PO_4; visual colour	0.2 μg	26
7% ethanol in chloroform	(a) Partition between NaOH and chloroform (b) K_3PO_4 on Celite column; elute with ether–petroleum ether (3:17)	(a) Irradiate; absorbance at 415 nm (b) Confirm TLC on silica gel; visual colour	0.55 ppm	27
7% ethanol in chloroform	(a) Partition between NaOH and chloroform (b) K_3PO_4 on Celite column; elute with ether–petroleum ether (3:17)	Irradiate; oxidise with $NaHSO_3$ Fluorescence at 385 nm	0.005 ppm	28
7% ethanol in chloroform	(a) Partition between NaOH and chloroform (b) TLC on silica gel, two-dimensional	Silylation; GLC–MS on OV-1 at *m/e* 412	0.01 ppm	29
Acetone–isoamyl alcohol, acetic acid–glycol (1:1:1:4)	Partition between NaOH and chloroform	Irradiate; oxidise with $NaHSO_3$; partition diol; fluorescence at 380 nm	0.005 ppm	30
Water	Partition between NaOAc and chloroform	Silylation; GLC on OV-1 at 210°; FID	10 ppm	31



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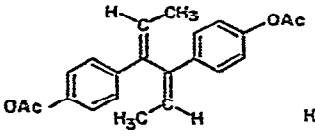

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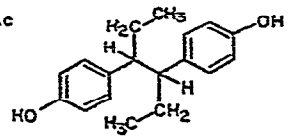


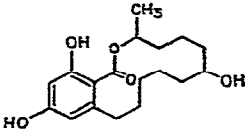
III

Fig. 1. Structural diagrams of stilbene estrogens. I = Diethylstilbestrol; II = dienestrol diacetate; III = hexestrol.

controlled experimental protocols. On the other hand, it lacks specificity, takes about ten days to perform, is not suitable for routine use and has been shown[8,9] to be strongly affected by the diet of the animal. Hence more specific tests for the estrogens have been devised.

A. Diethylstilbestrol

Due to its high potency and oral activity, diethylstilbestrol (DES) has enjoyed a wide use in animals and a more limited use in humans. Since its introduction in the early 1940s, a plethora of techniques, both chemical and biological, have evolved for its measurement in biological samples. The chemical ones are summarized in Table 2 for animal feeds and in Table 3 for animal tissues under the headings extraction, hydrolysis, purification, measurement, detection limit, and reference. The following are noteworthy general comments. Since DES is a phenol conjugated to a double bond, facile conversion of the *cis* and *trans* forms takes place through the phenol-enone. Dissolution of either pure *cis* or *trans* in almost any solvent results in a mixture of the two in a short time. This property has been studied by Winkler *et al.*[57] and White and Ludwig[58], who have isolated both forms. The active estrogenic form of DES is known to be the *trans* configuration, which has resulted in the addition of



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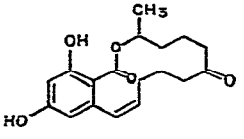

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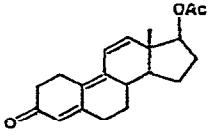

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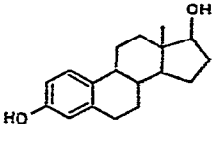


VII

Fig. 2. Structural diagrams of estrogens. IV = Zeranol (zearalanol); V = zearalenone; VI = trenbolone acetate; VII = estradiol.

TABLE 3

PROCEDURES FOR DIETHYLSTILBESTROL DETERMINATION IN ANIMAL TISSUES

Extraction	*Hydrolysis*	*Purification*	*Measurement*	*Detection limit*	*Reference*
Acetone	Phosphoric acid, pH 2, 100°	Partition between NaOH and ether	Colour with $SbCl_5$ at 540 nm	2000 ppb	32
Acetone	—	(a) Talc column (b) Methylate; extract with ether	GLC on EGAP at 200°; argon ionization detector	1000 ppb	33
Ethanol–chloroform (1:1)	—	Partition between chloroform–NaOH and ether	Irradiate; fluorescence at 410 nm	10 ppb	34
Fat by octane; liver mix on Celite, elute with ethanol	2 *N* HCl, heat	(a) Partition between chloroform and NaOH (b) Irradiate in neutral KH_2PO_4 (c) NaOH on Celite column; elute with chloroform	(a) Absorbance at 410 nm (b) Heat acid; partition between chloroform and NaOH (c) Fluorescence at 410 nm	2 ppb	35–37
Chloroform–ethanol (1:1)	4 *N* HCl, heat	(a) Partition between chloroform and NaOH, (b) TLC on silica gel; elute with hexane–ether–CH_2Cl_2 (4:3:2) (c) Irradiate; elute 2nd dimension as in (b)	Fluorescence at 366 nm; absorbance at 254 nm	1.5 ppb	38–40
Ether	Conc. phosphoric acid	(a) Partition between ether and $NaHCO_3$ (b) TLC on silica gel, two-dimensional	Irradiate; visualize by fluorescence	100 ppb	41
Ethanol	2 *N* HCl, heat	Partition between chloroform and NaOH	Silylate; GLC on OV-17 at 235°; FID	—	42
Acetonitrile–water (9:1)	5 *N* HCl, heat at 82°	Wash with hexane; partition between NaOH and benzene	GLC on OV-1 at 210°; ECD; no derivative	1 ppb	43
Ether	50% H_3PO_4, heat; or 2% NaOH heat at 82°	Partition between NaOH and ether; then NaOH and chloroform	Dichloroacetate; GLC on UC W-98 at 255°; ECD; confirm on TLC on silica gel; absorbance	10 ppb	44, 45
Ethanol	—	(a) Partition between NaOH and chloroform (b) TLC on silica gel, two-dimensional	Spray vanillin in H_3PO_4; visual colour	0.2 μg	26
Ethanol; Celite column	2 *N* HCl	(a) Partition between chloroform and NaOH	Irradiate; densitometry at 410 nm	0.5 ppb	46–48

		(b) Partition between hexane and 70% methanol (c) Silica gel column (d) TLC on silica gel			
Acetone	2 *N* HCl	(a) Partition between chloroform and NaOH–CH_2Cl_2 (b) TFA on Chromosorb W column; elute with hexane	GLC on OV-17 at 190°; ECD	2 ppb	49
Methanol for tissue; chloroform for fat	Glucuronidase (bovine liver), heat at 37°	(a) Partition between chloroform and NaOH (b) DCA	GLC on UC W-98 at 255°; ECD	1 ppb	50
Methanol	Glucuronidase, heat at 37°	(a) Partition between chloroform and NaOH–CH_2Cl_2 (b) TFA	GLC on OV-17 at 190°; ECD	5 ppb	51
Acetonitrile in triethylamine	Heptanol–triethylamine, ammonium chloride and phosphate, heat for 1–2 h	(a) Partition between NaOH and heptanol–triethylamine (1:8) (b) Partition between NaOH and diethyl ether–petroleum ether (1:4) (c) Irradiate, oxidize with $NaHSO_3$, partition diol between NaOH and chloroform	(a) Fluorescence at 380 nm (b) Confirm on TLC on silica gel; colour with $AgNO_3$	1 ppb	52
Acetonitrile–water (9:1)	2% NaOH, heat at 80°	Wash with hexane followed by benzene; partition between NaOH and benzene; silylate	GLC–MS on OV-17 at 250°; monitor at *m/e* 412	4 ppb	53
Confirmation only	–	$AgNO_3$ on Florisil column; elute with petroleum ether	GLC–MS on UC W-98 at 250°; monitor at *m/e* 488	0.2 ppb	54
Confirmation only		Hydrolyse TFA with water–methanol; form HFB with trimethylamine	GLC on OV-210 at 190°; ECD	2 ppb	55
Methanol	β-Glucuronidase	(a) Partition between NaOH and chloroform (b) DCA (c) $AgNO_3$ on Florisil column; elute with hexane–benzene (3:1)	(a) GLC on UC W-98 at 240°; ECD (b) Confirm GLC–MS; monitor at *m/e* 488, 490, 492, 494	0.5 ppb	56

antioxidants to animal feeds[59,60] to conserve the *trans* form, since it has also been shown[61] that feeding *trans*-DES gives a larger weight increase than feeding *cis*-DES. However, the reason why the *trans* form is not readily transformed into a mixture of the two in the animal has not been explained. Extraction of DES from animal feeds is best accomplished with a relatively non-polar solvent; at present, the solvent of choice for most feeds is 7% ethanol in chloroform. Extraction of DES from animal tissues is complicated by the occurrence, besides free DES, of other conjugates, a large part of which is constituted by the glucuronide. Hence, the initial dissolution is performed with more polar solvents, of which acetone, methanol, and acetonitrile all appear suitable.

At this stage, hydrolysis of the DES conjugates is usually effected to facilitate later purification steps; however, this particular step of DES measurement is the one with the greatest variation. Early work of Malpress[62] showed that the hydrolysis of DES in cow urine was difficult and was best accomplished in a purified state rather than in the crude sample. Teague and Brown[63] demonstrated that DES was partly destroyed by hydrolysis of DES glucuronide with either acid or, to a lesser extent, enzymes. They chose conditions of pH 3.5 in a phosphate buffer autoclaving at 180° as the most appropriate for hydrolysis.

In later work by Mitchell *et al.*[64] the distribution of radioactive DES in steers was studied. About 30% of the radioactivity was present in the feces as free DES and approximately 20% in the urine; about 75% of the latter was conjugated. A much smaller amount had accumulated in the tissues and 100% of this was believed to be in the free form. Total recovery of the radioactivity was about 50%. Separation of the free and glucuronide parts of DES was carried out by washing an organic phase of both, firstly with bicarbonate solution (pH 9–10) to remove the glucuronide and then with 0.1 *N* NaOH to remove the free phenols after the method of Teague and Brown[63]. Further work of Hinds *et al.*[65] corroborated the distribution and forms of DES in the feces and urine and showed that there was some DES in the liver, but it was not certain if all of this were the glucuronide. They speculated that there was a form present in tissue which was not free DES or its glucuronide and was not extracted by organic solvents. Karg *et al.*[38] believed DES to be in the free form in muscle tissue after intramuscular injection of DES diproprionate. Vogt *et al.*[40] showed a difference for residue values of DES in beef tissues as measured by biological and chemical methods. They attributed the lower chemical values to a form of DES in the tissue which was biologically active but was not freed for chemical analysis by either acid or enzymes. The screening procedure of Waldschmidt[26] for estrogens involved no enzymatic hydrolysis for meat and organ samples, implying the presence of the free form, although enzymes were used for other materials including feed. On the other hand, Umberger *et al.*[35] gave some evidence that DES was in a conjugated form in tissues, but they were unable to free all the DES with glucuronidase. Donoho *et al.*[50] arrived at much the same conclusion. This entire question was further studied in a series of papers from the United States Department of Agriculture[66–70]. Their work corroborated the distribution of DES in the feces and urine by either oral or implant administration and showed that DES could be freed from conjugates in urine samples on standing. In addition, the distribution of DES in liver tissues showed some free form with 50–100% conjugation and not all of the latter could be liberated by hydrolysis with glucuronidase-sulfatase.

In the light of the uncertain form of all the DES in food samples, the best method for hydrolysis is open to question. Many workers have used aqueous acid, either hydrochloric or phosphoric. Umberger *et al.*[35] and Coffin and Pilon[49] found better recoveries with these reagents. Basic hydrolysis has also been used with either sodium hydroxide[45,53] or organic amines[52]. The other approach to the generation of free DES is to hydrolyse with glucuronidase[50,51,56] or glucuronidase-sulfatase[26,66–70]. Petrak[4] has discussed the entire question of the hydrolysis of steroidal conjugates. In this connection, it is well known from radiolabelled drug metabolic studies that drugs in tissue, particularly liver, are covalently bound and that these are only freed with difficulty for measurement. Presently, it would appear that acid hydrolysis can give low DES recoveries by being too destructive and enzyme hydrolysis can give low recoveries by being too specific. The entire area needs further experimentation and certainly is a significant source of variation in the analysis of DES.

A characteristic property of estrogens in general and DES in particular is that they are phenols. As a result, they can be partitioned between organic and aqueous phases by the suitable choice of pH. Distribution coefficients for extraction of DES between chloroform and aqueous are shown in Table 4, taken from the work of Smiley and Schall[22]. The DES in an aqueous solution can be extracted into chloroform if the pH is lowered to the region of 9–10. Recent work shows that a value of 10.5 suffices to distribute DES into the chloroform phase with high recovery. This facile partitioning between organic and aqueous phases has resulted in a general pathway for purification; indeed, every method for measurement of DES takes advantage of this property either by liquid–liquid partitioning in a separatory funnel or by adsorption and elution from solid phases such as Celite. For feed samples, this type of separation usually gives an extract clean enough for measurement, particularly if GLC is used.

TABLE 4

DISTRIBUTION COEFFICIENTS FOR DIETHYLSTILBESTROL IN VARIOUS SYSTEMS[22]

Reproduced by permission of the publisher and the authors (I. E. Smiley and E. D. Schall) from *J. Ass. Offic. Anal. Chem.*, 52 (1969) 107.

Organic phase	*Aqueous phase*	K^*
Chloroform–ethanol (93:7)	1 N NaOH	0.02
Chloroform	10% H_2SO_4	9.35
Chloroform	1 *N* NaOH	0.06
Chloroform	Water	60

$$^* K = \frac{\text{conc. organic phase}}{\text{conc. aqueous phase}}.$$

For animal tissues, additional purification is necessary and silica gel chromatography is the adsorbant of choice. Both thin-layer chromatography (TLC) and column chromatography have been used, with the former more prevalent. In Table 5 the column and thin-layer chromatographic properties of DES in foods are summarized. In particular, European workers have employed two-dimensional TLC on silica gel to separate and purify DES from animal extracts. A semipurified extract is spotted

TABLE 5

THIN-LAYER AND COLUMN CHROMATOGRAPHY OF DIETHYLSTILBESTROL IN FOOD EXTRACTS

Type	*Adsorbant*	*Solvents*	R_F		*Reference*
			Cis	*Trans*	
TLC	Silica gel G	Acetone–chloroform (3:17)	0.4	0.6	19
TLC	Silica gel G	(1) Hexane–ether–CH_2Cl_2 (4:3:2)	0.25	0.5	39
		(2) Irradiate; repeat in 2nd dimension		0.2	38, 41
Column	Alumina	Ethanol–ether (1:1)		—	23
TLC	Silica	Petroleum ether–ether (4:6)		0.65	23
TLC	Silica gel G for DCA derivative	Hexane–ether–CH_2Cl_2 (4:3:2)		0.9	44
TLC	Silica gel G	(1) Hexane–ether–CH_2Cl_2 (4:3:2)	0.21	0.33	26
		(2) Ethyl acetate–benzene (1:3)	0.43	0.51	
TLC	Silica gel G	Acetone–diethyl ether–petroleum ether (13:8:79)	0.2	0.4	27
TLC	Silica gel G	Ether–benzene (1:4)	—	0.5	46–48
Column	Silica gel	1% ethanol in chloroform		—	46–48
TLC	Silica gel G	Chloroform–ethanol (98:2)	0.1	0.3	29
Column	Silica gel	Chloroform		—	29
Column	Chromosorb W for TFA derivative	Hexane		—	49
TLC	Silica gel for diol	Chloroform–acetonitrile (85:15)		0.3	52
Column	$AgNO_3$ on Florisil for DCA derivative	Petroleum ether–benzene (1:4)		—	54, 56

and developed in one dimension with hexane–diethyl ether–methylene chloride (4:3:2) to give two UV sensitive spots of R_F 0.25 and 0.5, corresponding to *cis*- and *trans*-DES, respectively. The sample is either developed in a second dimension with a different sol-

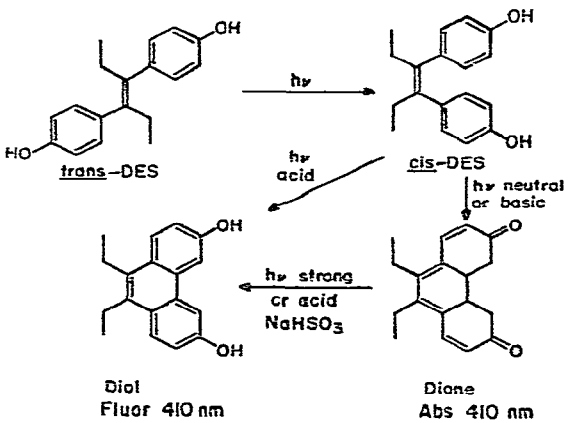


Fig. 3. UV transformations of diethylstilbestrol.

vent or irradiated to the phenanthrenedione and developed again, giving a second R_F value. Such a process results in both specificity and low detection. Aschbacker *et al.*[66] and Rumsey *et al.*[70] also used two-dimensional TLC on silica gel in several solvent systems to separate, purify, and identify [^{14}C]DES in animal metabolic studies.

The actual measurement of a purified sample containing DES requires low detection limits for the levels which are likely to be present in tissues, although this is not such a stringent requirement for animal feeds. As a result, two separate procedures have evolved, one using fluorescence and the other derivative formation and GLC. Concerning the former, it has been well established by Banes[71] and Doyle *et al.*[72] that DES is transferred to new products on irradiation. The course of the reaction is varied, as shown in Fig. 3, but it is known that *trans*-DES is converted to the *cis* isomer on irradiation. Further reaction in a buffered neutral or basic medium results in the formation of a yellow phenanthrenedione, which has an absorption maximum at about 410 nm. This property has been the basis of a validated assay for DES in feeds[73] with a sensitivity of about 10 ppm. If the irradiation of DES is carried out in an acidic medium or for a long time, an oxidation takes place, with the formation of a phenanthrenediol, which fluoresces strongly around 410 nm. This diol is also produced by oxidation of the dione in acid with sodium bisulfite. This fluorescence allows the estimation of DES in tissues in the order of 1 ppb, for which several variations have been developed[30,35,39,52].

The second procedure for measurement of DES employs GLC with either flame ionization detection (FID) or electron capture detection (ECD). The GLC characteristics of DES are summarized in Table 6, showing the variety of columns and detection systems available. With derivative formation, two peaks for DES are invariably formed from the *cis* and *trans* configuration and these are always well resolved on GLC columns. FID is commonly used for measurement of DES in animal feeds, where either the acetate[18], the trimethylsilyl (TMS) derivative[22,24,29,31], or the dichloroacetyl (DCA) derivative[35] are formed, with detection limits in the order of 2–10 ppm. For tissue samples, the derivatives which have been employed are methyl[33], TMS[42], DCA[44,45,50,56], and trifluoroacetyl (TFA)[49,51], and even no derivative formation[43]. The latter case involves direct injection of DES onto a GLC column and detection of a single peak whose origin is unknown but believed to be an on-column derivative formation to a phenanthrenedione. However, the applicability and versatility of this latter procedure have not been demonstrated. With these derivatives, the detection limit is about the same as that for fluorescence, with the ECD limit of TFA or DCA being lower than the FID limit of TMS. It would appear that, for feeds, the TMS derivative is to be preferred because of its facile formation and, that for tissues, the TFA derivative has the advantage of ease of formation and relatively low temperature of elution.

Since there are several procedures for the measurement of DES in biological samples, confirmation of one procedure can be effected by the choice of a second procedure, provided that an aliquot is available. Some of the developed procedures have included additional steps for confirmation. Heffter *et al.*[44] gave some details of the TLC of DCA derivatives and their mass spectrometry (MS), but no tissue samples were specifically analysed. For animal feeds, Jeffus and Kenner[27] reported fluorescent spots on TLC by nitration and, in tissue, a confirmation procedure was reported by Ponder[52] after TLC and nitration.

A technique which is eminently specific for confirmation is GLC–MS. This has

TABLE 6

GAS–LIQUID CHROMATOGRAPHIC ELUTION CHARACTERISTICS OF DIETHYLSTILBESTROL

AID = Argon ionization detector; FID = flame ionization detector; ECD = electron capture detector; MS = mass spectrometer.

Derivative	*Column*		*Carrier gas*	*Flow-rate (ml/min)*	*Detector*	*Column temperature (°C)*	*Retention time (min from injection)*		*Reference*
	Packing	*Dimensions, length (cm) × internal diameter (mm)*					*Cis*	*Trans*	
Methyl	13% EGAP	182 × 4	—	—	AID	200	34		33
Acetate	3% SE-30	80 × 2	helium	15	FID	200	4	6	18
Trimethylsilyl	3% OV-1	182 × 2	nitrogen	60	FID	215	5	6.5	22, 31
Trimethylsilyl	3% JXR	122 × 3	helium	60	FID	220	4	5.5	24
Dichloroacetate	5% UC W-98	122 × 3	argon–methane (9:1)	100	ECD	255	2	3	25, 50
Dichloroacetate	5% UC W-98	182 × 2	helium	32	ECD	255	3	5	44, 45
Trimethylsilyl	2% OV-17	488 × 1.7	nitrogen	40	FID	235	—	—	42
—	1.5% OV-1	182 × 2	nitrogen	60	ECD	210	—		43
Trifluoroacetate	3% OV-17	152 × 2	nitrogen	40	ECD	190	3	4	49, 51
Trimethylsilyl	2% OV-17	244 × 2	helium	16	MS	250	—	—	53
Trifluoroacetate	3% OV-210	182 × 2	argon–methane (9:1)	30	ECD	190	3.5	4.5	55
Heptafluorobutyrate	3% OV-210	182 × 2	argon–methane (9:1)		ECD	190	4.0	5.0	55
Dichloroacetate	5% UC W-98	122 × 2	nitrogen	60	ECD	240	—	4	56

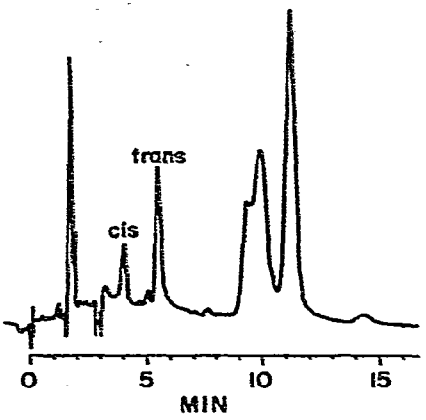


Fig. 4. Single-ion detection chromatogram of silylated liver extract containing 5 ppb DES. (Reproduced by permission of J. F. Bergmann-Verlag.)

been employed with DES in feeds by Mirocha *et al.*[29] using the methyl ether, by Day *et al.*[54] using the DCA derivative, and by Höllerer and Jahr[53] with the TMS derivative. Fig. 4 shows a GLC–MS tracing of the two DES TMS peaks taken from a liver extract of ref. 53. All of these are relevant in that they document the presence of DES in food. A chemical technique for confirmation of DES in tissues has been reported by Ryan and Pilon[55]. In this instance, the DES TFA produced by the method of Coffin and Pilon[49] was hydrolysed to free DES, which was then re-derivatized to the heptafluorobutyrate (HFB). The identity of the two peaks from both derivatives was verified by their gas–liquid chromatogram/mass spectrum. Peaks at *m/e* values of 460 and 660 occurred for the TFA and HFB, respectively, confirming the addition of two acyl groups to DES. The HFB were then eluted on GLC with ECD under different conditions to confirm the presence of DES. A typical diagram of the *cis* and *trans* peaks of both DES TFA and DES HFB is shown in Fig. 5. In this case, the injection of 0.3 ng of both on a 3% OV-210 182 cm × 2 mm I.D. glass column at 190° gives a sharp separation of all four DES peaks.

A comparison of several methods for the measurement of DES in feed and tissue has been made by Perevoshchikova *et al.*[74], who reported the UV absorbance method to be superior to the colorimetric one, but no limits of detection were given. Karg *et al.*[38] have summarized a study in cattle of methods for the detection of DES in urine, feces, and flesh. In this study, both the mouse uterus test of Umberger *et al.*[7] and the chemical procedures of Umberger *et al.*[35] and Schuller[39] were used, with preference shown for the chemical tests on account of their specificity and to the Schuller TLC method on account of its low detectability.

At this stage, there are two choices for measurement of DES in biological samples. The measurement steps are based on either fluorescence–absorbance or GLC with ECD or FID. The choice of either is subject to the judgement of the analyst and what equipment is available to him. However, it is to be noted that GLC techniques have been gradually replacing spectroscopic ones for DES due to their ease of manipulation. For tissue samples, the choice of technique for extraction and hydrolysis is uncertain, since the amount of free DES, glucuronide, and that remaining bound, has not been defined, although they merit further investigation. Purifica-

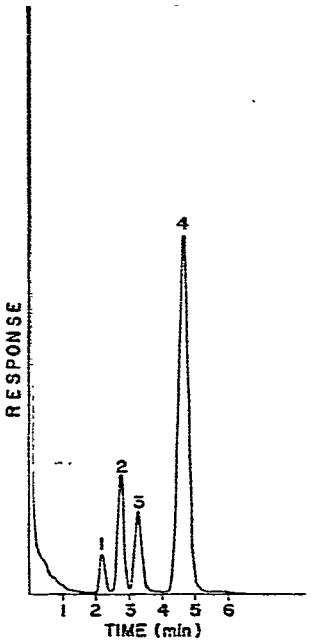


Fig. 5. GLC tracing of 0.3 ng each of DES trifluoroacetate (1,3) and DES heptafluorobutyrate (2,4) obtained on a 182-cm × 2-mm-I.D. 3% OV-210 glass column at 190° with ECD. Reproduced by permission of the publisher and the authors (D. E. Coffin and J. C. Pilon) from *J. Ass. Offic. Anal. Chem.*, 56 (1973) 852.

tion is invariably initiated with liquid–liquid partition, often followed by TLC on silica gel. Confirmation of DES in a sample is best accomplished by GLC–MS where available and by chemical means otherwise.

B. Hexestrol and dienestrol diacetate

Hexestrol and dienestrol diacetate (DDA), two phenolic synthetic estrogens, have enjoyed a more restricted use than DES in animal production and, as a result, methods for their determination are fewer. Often, they are analysed in biological samples incidental to the analysis of DES. For instance, Jeffus and Kenner[27] showed that hexestrol and dienestrol did not interfere with a method for DES in feeds.

Stuckey *et al.*[17] analysed for hexestrol in feeds by extraction with chloroform, liquid–liquid partition between chloroform and sodium hydroxide, and reversed-phase partition chromatography on a cellulose column coated with peanut oil, eluting with diethyl ether. Determination was with the blue color produced by sodium molybdophosphotungstate at 750 nm. Van Waes[23] detected both of these stilbene derivatives in feed by extraction with hot alcohol, chromatography on both an alumina column and silica thin layers, irradiation of the sample, and color formation with vanillin. A screening test for estrogens in biological extracts including feed and food was reported by Waldschmidt[26]. In this case, the estrogens were extracted with hot ethanol, enzymatically hydrolysed by a glucuronidase-aryl sulfatase, and purified by liquid partition and TLC on silica gel in two dimensions with two solvents systems. Measurement was

made by spraying with acid vanillin and observing the colour formation. A test for hexestrol alone in meat was devised by Cooper *et al.*[75]. Extraction and hydrolysis were effected simultaneously with phosphoric acid, followed by extraction into diethyl ether and purification by liquid partition with aqueous sodium hydroxide, and formation of the TFA of hexestrol. Measurement was effected by GLC on a 60 cm × 3 mm I.D. column of 2.5% Apiezon and 0.5% Epikote 1001 at 160° with ECD. In contrast to DES, hexestrol yields a single derivative and hence a single peak on GLC or TLC. The detection limit of the procedure was 0.1 ppm, which is somewhat inadequate for a hormone residue technique.

The only publication dealing solely with DDA measurement is that of Hill *et al.*[76]. DDA was purified in feeds by extraction with diethyl ether, hydrolysis with sodium hydroxide to dienestrol, and liquid partition between chloroform and aqueous sodium hydroxide. Measurement was done spectrophotometrically at 610 nm after condensation in acid with anisaldehyde with a detection limit of about 10–20 ppm.

More recently, Höllerer and Jahr[53] reported a screening method for estrogens in bovine liver with combined GLC–MS. Liver was extracted with acetonitrile–water (9:1), conjugates hydrolysed with sodium hydroxide, the extract cleaned up by liquid partition and the TMS derivative formed with bis(trimethylsilyl)trifluoroacetamide (BSA). For resolution and detection of the TMS derivatives, a 2% OV-17 244 cm × 2 mm column was used at 250° and single-ion monitoring of the molecular ion peak showed the presence of hexestrol at *m/e* 414 and dienestrol at *m/e* 410 amongst other estrogens. Detectability was 4 ppb for dienestrol but only 40 ppb for hexestrol; specificity, of course, was high.

C. Zeranol

Zeranol, a semisynthetic estrogen, used in ruminant production for increased growth, is a resorcylic acid lactone, and is also known as zearalanol. Zeranol is obtained by hydrogenation of zearalenone, which in turn is a mycotoxin produced by several *Fusarium* species growing on grain crops in particular corn, *Zea mays*. There are many methods[77] available for the measurement of zearalenone in feed crops making use of TLC and high-pressure liquid chromatography (HPLC), which take advantage of the natural fluorescence of zearalenone. Zeranol, however, does not fluoresce strongly but has been measured in meat by both GLC and UV absorbance after derivatization. In 1968, the Commercial Solvents Corp. published their method[78] for determination of this hormone in beef tissues. The latter were treated with methanol, conjugates were hydrolysed with hot hydrochloric acid and the residue purified by successive liquid partitions with chloroform, sodium hydroxide and diethyl ether. After silylation, the derivative was separated and eluted on a 3% SE-52 182 cm × 4 mm I.D. column at 275° with FID. The detection limit was in the order of 20 ppb. This procedure was somewhat long and difficulties were encountered with silicone deposits on the flame towers.

For confirmation, zeranol and zearalenone, as their TMS derivatives, were resolved from each other on a 3% OV-17 182 cm × 4 mm I.D. column but the separations were small, necessitating standards of each for assurance[79]. The technique of GLC–MS was also successfully used for confirmation where a peak at *m/e* 538 appeared for the zeranol TMS corresponding to three positions silylated on the mole-

cule. Additionally, zeranol was methylated with Methelutex® by formation direct on the column and this second derivative eluted at a lower temperature (245° *vs.* 270°) than the TMS derivative on either OV-17 or SE-52 (ref. 79).

A second procedure has been published by Waldschmidt[26] as a screening test for estrogens including zeranol in biological samples. After extraction of feed or tissue with ethanol, enzymatic hydrolysis, purification by partition and TLC on silica in two dimensions, the estrogens were sprayed with vanillin in H_3PO_4 and the colour and R_F values noted.

Recently, Ingerowski *et al.*[80] reported their method for the estimation of zeranol in cattle tissues by colour formation and TLC. After extraction of the sample with methanol, conjugates were hydrolysed with glucuronidase-sulfatase and the hormone was extracted into ethyl acetate. The evaporated extract was partitioned between acetonitrile and hexane, the evaporated acetonitrile residue purified on an alumina column eluting with tetrahydrofuran (THF), and the eluant partitioned between sodium hydroxide and chloroform. Further cleanup was effected by TLC in two dimensions using silica gel eluting, firstly, with chloroform–acetone–THF (94:4:2.5) and then with ethyl acetate–diethyl ether–hexane (1:1:1). The plate was then sprayed with Fast Blue Salt B in base and the dark violet spot and the R_F values were noted. This long procedure detected 10 ppb zeranol and quantitatively measured at 520 nm about 400 ppb. It provided an alternative technique to the GLC determination for measuring zeranol in food.

D. Trenbolone acetate

Trenbolone acetate (TA), a steroidal hormone, which is estra-4,9,11-trien-17β-ol-3-one, 17-acetate, is used on an experimental basis in Europe as an anabolic agent in cattle production. At this writing, there have been no approvals of its use, nor has there been any published work on its chromatographic measurement in animal tissues but TA and its alcohol have been measured by RIA[81]. Recent publications by Pottier *et al.*[82] and Grandadam *et al.*[83] gave data on the distribution of radiolabelled TA in ruminants. Lean tissues were extracted with ethyl acetate and fat with chloroform and were then applied to a silica gel column and eluted with ethyl acetate. The residue from either was deposited on silica thin layers, developed with benzene to remove lipids, and then with ethyl acetate–chloroform (4:1). R_F values of the 17-acetate, 17-alcohol and 17-ketone (trendione) were 0.6, 0.4, and 0.5, respectively. Spots were identified by the fluorescence at 366 nm and by radioactive counting.

An interesting and sensitive chromatographic technique for TA and TA alcohol has lately been reported by Oehrle *et al.*[84]. Both compounds were eluted and separated on silica gel thin layers with chloroform–ethyl acetate (2:1) as the developing solvent (R_F values of 0.68 and 0.34, respectively). The eluted zones were then dissolved in 75% 6 *M* HCl in methanol and excited at 365 nm. A strong reversible fluorescence was producted at 498 nm, enabling 1 ng to be detected. As this property was not exhibited by other common hormones, specificity was good. Such a technique could readily be applied to the measurement of this hormone in foods.

E. Estradiol

Estradiol is the first naturally occurring or endogenous hormone to be considered. Semisynthetic analogues such as the 3-benzoate and the 17-palmitate are also utilized in food production for specific purposes. Since estradiol is naturally present, all tissues and biological samples have this hormone as a constituent. The actual levels present are usually very low (less than 1 ppb) but show wide variation, depending on the reproductive condition of the animal. Hence, the measurement of added estradiol in animal tissues must contend with a baseline of existing estrogen whose level cannot always be specified.

A vast array of chemical techniques have been developed and continue to be developed for the determination of this important compound in blood (serum and plasma), urine, and drug formulations. Often the measurement portion of these procedures detects small amounts to qualify as residue procedures but, almost invariably, the extraction and purification techniques are insufficient to isolate estradiol from tissue samples per se. Some examples, by no means all inclusive, of reviews on chromatographic measurement of estrogens are given in the works of Petrak[4], Wotiz and Chattoraj[5], and Preedy[85].

Radiolabelled compounds are often used to estimate recovery percentages when dealing with small amounts and to give a high degree of specificity, particularly in controlled research experiments. Other techniques, besides chemical (chromatographic), have been developed for this compound. Noteworthy amongst these are the competitive binding techniques including radioimmunoassay and the bioassay techniques of Umberger *et al.*[7] utilizing the weight change of the mouse uterus from diets containing estrogenic substances. In the former case, application has only been to blood or urine and not as yet successfully to tissue, while in the latter case detection limits are good but specificity is low. Nevertheless, there exist a few procedures, somewhat specialized, for estradiol in tissues.

Ittrich[86] has carried out a comprehensive study of the fluorometric determination of estrogens including estradiol in biological samples such as blood, urine, and milk. A diluted sample was first hydrolysed with HCl to free any conjugates and the estrogens extracted into aqueous sodium hydroxide. After two liquid partition steps between diethyl ether, NaOH and benzene, the residue was adsorbed on a 4% water-deactivated alumina column and defatted by washing with carbon tetrachloride. The estrogens, estrone, estradiol, and estratriol, in that order, were then eluted in various concentration of ethanol in benzene. For determination, the evaporated column fractions were heated with aqueous acid and hydroquinone to give fluorescent products (Kolber reaction). The latter were then extracted into acetylene tetrabromide with *p*-nitrophenol and the fluorescence measured at 546 nm. The entire procedure was evaluated for ruggedness, accuracy, and precision and detected 0.1 ng estradiol per millilitre of milk.

Double isotope labelling has been used by Mondain-Monval-Gerondeau *et al.*[87] to detect several hormones including estradiol in milk. After the addition of tritium as the indicator isotope for estradiol, the estrogens were extracted into diethyl ether, the residue defatted by freezing, and the phenols derivatized with [^{35}S]pipsyl chloride. By chromatography on silica thin layers eluting with hexane–ethyl acetate (1:1), the estradiol ester was separated from estrone and estratriol esters and the radio-

activity was counted. The limit of measurement was less than 0.1 ppb in whole milk and values were given for the estrogen content of milk from various sources. This specialized procedure was eloquently specific but, being long and involving rather expensive reagents, was not suitable for routine use.

Two additional thin-layer techniques have been developed for estradiol. The first[26] of these in meat consisted of extraction with alcohol, enzymatic hydrolysis, liquid partition, and two-dimensional TLC on silica gel. Detection was by visualization after colour formation with vanillin–phosphoric acid. The other[88], in milk, involved extraction with chloroform, TLC on silica gel in two solvent systems, and UV detection by the absorption at 254 nm. The method was rapid but there is a need to increase the detection limit to measure levels of less than 1 ppm.

The Munich school[89] studied the distribution and residues of estradiol in calves from intramuscular implantation. After extraction of tissues with ethanol, hydrolysis in hot base and partition between aqueous base and diethyl ether and benzene, the estradiol in the extract was methylated to the dimethyl compound. This was followed by further partition and adsorption on a 9–10% water-deactivated alumina column eluting with benzene in petroleum ether. The estradiol was then determined in the residue by the fluorescence at 560 nm after addition of hydroquinone. Recovery of estradiol was less than 50% with a limit of detection of 2 ppb. The procedure had been adopted from the earlier work of Brown[90], Brown *et al.*[91], and Ittrich[86].

Recently combined GLC–MS techniques have been applied to tissue extracts containing steroidal hormones. Millington *et al.*[92] determined estradiol in human tissue by extraction into acetone, defatting with petroleum ether and formation of the TMS ether. After separation on a 2 m × 2 mm I.D. column of 3% OV-225 and 1% OV-210 at 255°, detection was effected by high-resolution MS with single-ion monitoring of the molecular ion peak at *m/e* 416.257. The procedure made no correction for non-quantitative recoveries but could specifically detect in the order of 1 ng estradiol per gram of tissue. Höllerer and Jahr[53] used a similar procedure with low-resolution MS to show the presence of estrogens in animal livers. Their removal of estrogen from liver was carried out with acetonitrile–water (9:1), followed by alkaline hydrolysis, liquid parition, and formation of the TMS analogue. After separation of TMS-estrogens on a 2% OV-17 244 cm × 2 mm I.D. column at 250°, detection was with a low-resolution MS monitoring at *m/e* 416 for estradiol with a limit of measurement of 4 ppb. The method was successfully applied to calf and chicken livers, where values between 4 and 15 ppb were recorded.

F. Screening techniques for estrogens

All screening techniques for the estrogens considered have been described under the individual compounds. However, the methods of Waldschmidt[26], Höllerer and Jahr[53], and Mondain-Monval-Gerondeau *et al.*[87] are adaptable for measuring several estrogens at once. Valette and Ferrando[93] gave R_F values of several estrogens on silica thin layers after eluting with hexane–ethyl acetate (3:1) and detecting by UV and spray reagents. Detection limits were given for several standards but no meat samples were specifically analysed. Godglück and Siewart[94] have described, in a general way, the methods available for estrogens.

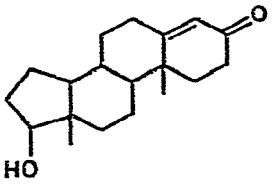


Fig. 6. Structural diagram of testosterone.

3. ANDROGENS

The single compound used in animal production from this class of hormones is testosterone, the structure of which is shown in Fig. 6. Androgen activity is usually measured by a bioassay[2], using the weight change in the comb of chickens on application of a suitable tissue extract, and detects to about 0.1 ppm.

Testosterone

Testosterone, as the 17-proprionate, is used in heifer production in combination with estradiol-3-benzoate for increase in weight gain. The remarks made about estradiol concerning its endogenous nature, the number of techniques for blood and urine, and the use of radiolabelling and competitive binding assay also apply to testosterone. Examples of reviews on its measurement can be found in Wotiz and Chattoraj[5], Petrak[4], and Dorfman[2]. Again, specific residue methods for the measurement of testosterone in tissues or food are few and scattered, but include the following.

Struck *et al.*[95] determined testosterone in testicular tissue by methanol extraction, chromatography on both a silica column (eluting with ethyl acetate) and Kieselgel thin layers in two dimensions, developing with mixtures of chloroform and acetone, and detection by UV absorption with reflectance. Radiolabelled testosterone was used to monitor recovery with limits of detection in the order of 20–50 ppb, *i.e.*, 0.2–0.5 μg testosterone. While not as sensitive as GLC or scintillation procedures, the use of TLC and densitometry was shown to be feasible with testosterone. A similar approach to testosterone measurement has been reported by some Russian workers[96,97]. Using muscle tissue, testosterone was extracted with chloroform, partitioned between 70% methanol and hexane, and then into ethyl acetate. The residue was chromatographed on either silica gel thin layers, eluting with cyclohexane–ethanol (1:1), or on an alumina column, eluting with small amounts of ethanol in benzene. Detection was effected on the TLC plate either by reflectance or by extraction and absorbance at 240 nm or by fluorescence at 605 nm after treatment with strong acid (Ittrich[86] and Brown[90]). More recently, testosterone has been determined in human breast tissue by high-resolution MS[92]. In this case, the macerated sample was extracted into acetone, partitioned and defatted between aqueous methanol and petroleum ether, and the TMS derivative was formed. After resolution of the latter by GLC on a 2 m × 2 mm I.D. column of 3% OV-225 and 1% OV-210 at 255°, the eluent was fed into a mass spectrometer where single-ion monitoring of the peak at *m/e* 360.248 was used. Although no correction for recovery was made, the detection limit was about 5 ng per gram of tissue, while cleanup was relatively limited.

Hence a number of methods have been developed for testosterone which could be readily applied to food per se at low levels.

4. PROGESTOGENS

There are several steroidal progestogens which are used in food production, as shown in Fig. 7. In the main, these are employed to control the estrus cycle of female animals either to synchronize dates of conception or to effect greater weight gain. Measurement of the total progestogen content can be effected either by the mouse method of Hooker and Forbes[98] or the Clauberg rabbit test[2]. In the former technique, a suitable meat extract was injected into the uterus of ovariectomized mice and the change of the endometrium noted and, in the latter, injection was made subcutaneously in the rabbit and the change of endometrium noted. Both detect small amounts (~1 ppb) and are specific for total progestogens.

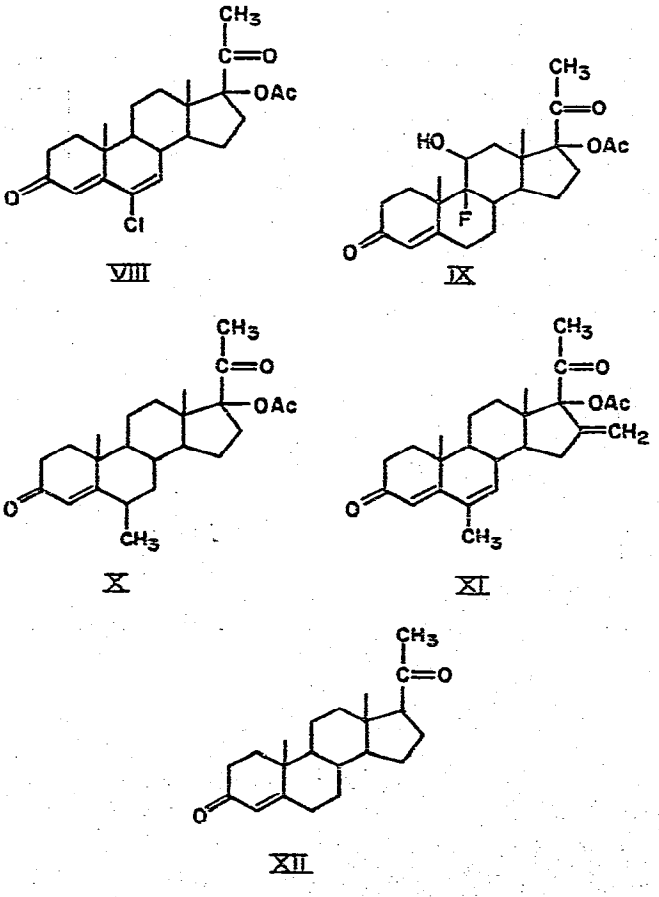


Fig. 7. Structural diagrams of progestogens. VIII = Chlormadinone acetate; IX = fluorogestone acetate; X = medroxyprogesterone acetate; XI = melengestrol acetate; XII = progesterone.

A. Chlormadinone acetate

The steroidal hormone chlormadinone acetate (CMA) has been used in the feed of cattle for the purpose of synchronization of estrus. It is the only progestogen in food animals which contains a chlorine atom. For feeds[99], extraction was carried out in a Soxhlet for 8 h with methylene chloride, after which the concentrated extract was adsorbed on an alumina column and eluted with 2% methanol in methylene chloride. The evaporated extract was then injected on a 30 cm × 4 mm I.D. GLC column of 1.5% XE-60 at 220° and the eluent detected with ECD, taking advantage of the affinity properties of the 3,6-dienone and the chlorine atom. The column was short probably due to the temperature limit of the tritium foil detector. Recoveries in feed samples were high, with a limit of measurement of at least 10 ppm.

For tissue samples, the Eli Lily workers[100] used the same procedure for detection but different reagents for extraction and purification. Lean beef tissues were extracted with methanol and the drug residues partitioned into carbon tetrachloride while, for fat samples, hexane and acetonitrile were used. Muscle, liver and fat were purified by a silica gel column, washing with chloroform–dichloromethane (1:1), and eluting the hormone with chloroform. Kidney samples were purified using an alumina column instead of a silica one but with the same eluants. Recovery of fat samples at the level of 50 ppb was 70–80%, with a limit of detection of 20 ppb. Since the use of CMA is limited, it is not expected that further work will be done on this compound in foods, although a sensitive technique[101] in plasma has appeared recently.

B. Fluorogestone acetate

The progestogen steroid fluorogestone acetate is the only one used in food animals to contain fluorine. It is used basically for estrous control in sheep and goats. A radiolabelling study[102] in ewes showed that milk was not an important route for excretion nor were levels high in tissues. However, no methods have been forthcoming for fluorogestone acetate in these foods.

C. Medroxyprogesterone acetate

A second product found effective in synchronizing the estrous of cattle by oral application is medroxyprogesterone acetate (MPA). A method for this steroid in feeds has been developed by Kramer *et al.*[103]. The sample was extracted for 20 h in a Soxhlet with chloroform, purified by adsorption on a Nuchar carbon column eluting with chloroform, and the dry residue was partitioned between hexane and 70% methanol–water. At this point, the latter solution could be purified further on a silica gel thin layer by developing with ethyl acetate–cyclohexane (3:2), then eluting from the plate with ethanol and detection by the absorbance at 242 nm; or alternatively, injected onto a 61-cm GLC column of 1% OV-1 at 225° with FID. Both procedures gave similar results and 25 ppm of MPA was detectable.

In tissues, the only method available for MPA is one for high levels developed by the Upjohn Co.[104] in an exaggerated studies method. Tissues were treated with ethanol to remove the drug while, for milk, Soxhlet extraction with methanol was used. The residue was taken up in hexane and chromatographed on an acid alumina column

eluting with hexane–chloroform (1:1) followed by partitioning between hexane and 70% methanol–water. The aliquot was purified further by paper chromatography using ethylene glycol–methylcyclohexane and the steroid removed from the paper zones with methanol and the UV absorbance measured at 242 nm. Detection limit of the method was 1 ppm, but this is not adequate for present needs. Adaption of a recently published method[105] for MPA in plasma to tissues involving the GLC detection of the HFB derivative should lead to a lower measurement level for detecting this compound in food.

D. Melengestrol acetate

The last of the four synthetic progestogens to be considered, melengestrol acetate (MGA), enjoys a wide use in the production of feedlot heifers for meat. Inclusion of MGA in the feed at 0.25–0.5 mg/animal/day results in estrous control with the attendant effects of increased weight gain and shorter time to market. Almost all procedures for measurement of MGA take advantage of its high ECD properties due to the 3,6-dienone steroidal system, which necessitates a high thermally stable detector.

In animal feeds, Ogilvie *et al.*[106] have published a short report on MGA. The same was extracted with 3% methanol–chloroform, cleaned up with Nuchar, and partitioned between hexane and 70% methanol–water, and methylene chloride followed by alumina thin-layer and column chromatography, eluting with chloroform–hexane in both cases. Injection of the extract on a 3% QF-1 GLC column with a FID allowed measurement of MGA to be effected at 220°. Recoveries of over 80% were reported for values as low as 0.28 ppm. In a more detailed report, Davis *et al.*[107] extracted the feed with hexane using the same cleanup procedures as above except for the addition of a silica column [elution with ethyl acetate–cyclohexane (3:2)] for dirty samples or very low levels. Detection and measurement of MGA were on a short (61 cm × 3 mm I.D.) 1% OV-17 column at 225° with a ^{63}Ni detector. Recoveries of over 85% were given for values down to 0.05 ppm.

In the case of animal tissues, there have been a number of reports for the determination of MGA and these have been modified and changed over the years. The first of these, by Krzeminski *et al.*[108], was a five-step procedure beginning with extraction of lean tissue with ethanol and partition into chloroform and, for fat tissue, hexane and 90% methanol. Purification was effected by a silica column with ethyl acetate elution, partitioning between 70% methanol and hexane, and chromatography on alumina thin layers with benzene–chloroform–ethyl acetate (10:1:1). The MGA was eluted from the TLC plate and determined colorimetrically at 415 nm by the addition of a suitable reagent. The detection limit of the method was 25 ppb and could also measure progesterone in meat. In a modification of the above[109], GLC with a 3% QF-1 column at 225° and FID was employed instead of the spectrophotometric measurement. In further experiments, to shorten this long procedure, Krzeminski and Cox[110] extracted lean tissue with acetonitrile and fat with hexane followed by partitioning with acetonitrile. The residue was separated on an alumina column with hexane–chloroform (1:1) and measured by GLC with ECD on a 61 cm × 3 mm 1% OV-17 column[1]. Recoveries of MGA were almost quantitative at 25 ppb. Simpson *et al.*[111] modified this procedure by substituting a Florisil column for the alumina and

by using a mixed OV-1/QF-1 column for the GLC but these changes could not be validated at the detection value quoted at 5 ppb.

More recently, the Upjohn group reported[112] further modification of their method using a Florisil column and eluting with hexane–acetone (8:2). For liver tissue samples, additional cleanup was provided by partitioning between hexane and 70% methanol after the Florisil column. This method was studied collaboratively[112] and was considered satisfactory for analyzing samples containing 10 and 20 ppb levels of both endogenous or added steroid.

At the same time, Ryan and Dupont[113] reported their work for screening and confirmation for MGA residues down to 2 ppb in beef tissues. To screen for the hormone, extraction procedures were taken from the Upjohn group[110] but cleanup was by a multistep solvent partitioning consisting of hexane, 70% methanol–water–chloroform. Measurement was carried out by GLC elution on 1% OV-17 at 250° with ECD. By careful attention to solvent purity, the partitioning steps, and the use of hexane as a selective solvent to precipitate out impurities before GLC, detection of as little as 2 ppb was feasible. To confirm and quantitate the drug residue, the sample was further cleaned up on a Florisil column eluting with hexane–acetone (1:1). The MGA was then hydrolysed to its 17-alcohol, melengestrol, with ethanolic potassium hydroxide, which was then measured by GLC on a 1% OV-210 column at 235°. GLC tracings from an extract of beef fat from heifers fed MGA are shown in Fig. 8. The improved GLC pattern after Florisil cleanup and the peak for melengestrol, more poorly resolved than MGA, are both evident. The procedure was used to measure MGA from heifers where levels of 5–10 ppb were found in fat, 1–5 ppb in liver, and lesser amounts in muscle and kidney.

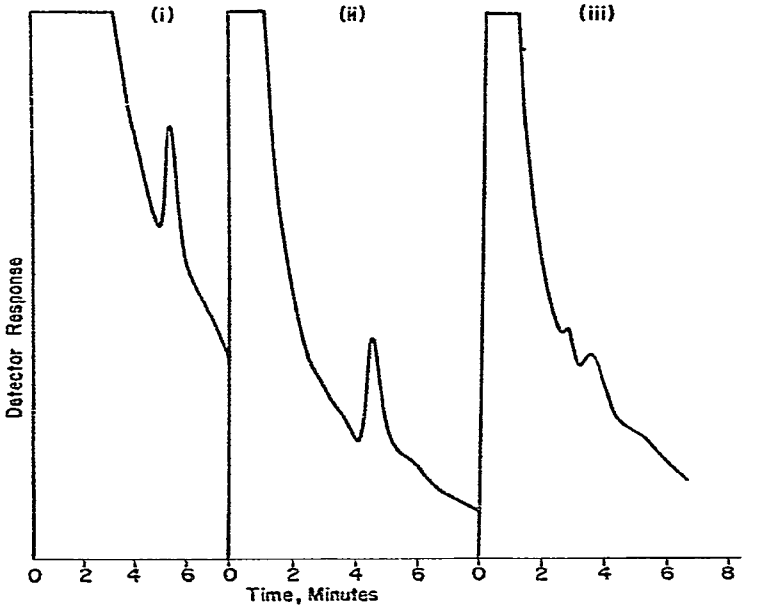


Fig. 8. Gas–liquid chromatograms of an extract of heifer beef fat containing 4 ppb MGA. (i) After extraction and partitioning; (ii) after (i) and Florisil; (iii) after (ii), hydrolysis and Florisil. (Reproduced by permission of the American Chemical Society.)

TABLE 7

GAS–LIQUID CHROMATOGRAPHIC PARAMETERS OF PROGESTOGENS

Compound	*Packing*	*Dimensions, length (cm) × internal diameter (mm)*	*Carrier gas*	*Flow-rate (ml/min)*	*Detection*	*Column retention*		*Reference*
						Temperature (°C)	*Time (min)*	
CMA	1.5% XE-60	30 × 4	nitrogen	170	ECD	220	3	99
			argon–methane (9:1)	190		220	5	100
CMA	3.8% W-98	30 × 3	argon–methane (9:1)	250	ECD	215	3.6	101
	3.0% SE-30 and 1.5% Carbowax M	30 × 3				230	3.6	101
MPA	1% OV-1	61 × 6	helium	120	FID	225	2.5	103
MPA	10% OV-17	61 × 3	argon–methane (9:1)	60	ECD	250	9.6	105
HFB	1% OV-17	61 × 3	—	—	FID	240	1.6	105
MGA	3% QF-1	61 × 3	—	—	FID	220	10–15	106, 109
	1% OV-17	61 × 3	nitrogen	50	ECD	225	4	107
	1% OV-17	61 × 3	argon–methane (19:1)	60	ECD	245	5.5	110
	OV-1; QF-1	—	—	—	ECD	—	—	111
	1% OV-210	91 × 4	argon–methane (9:1)	60	ECD	235	5	113

All of these procedures have resulted in an evolution of methodology for MGA which allows it to be reliably measured in food tissues. A combination of extraction with acetonitrile and/or hexane, liquid partition between hexane–70% methanol and water–chloroform, Florisil chromatography and GLC-ECD on OV-17 or OV-210 suffices to reliably identify this compound.

The GLC properties of the semisynthetic progestogens liable to appear in foods are summarized in Table 7.

E. Progesterone

Again, as with testosterone and estradiol, there is a wealth of chromatography published on progesterone. And, again, little of it is directly applicable to the measurement of progesterone in food samples. However, reviews can be found for GLC analysis of progesterone by Wotiz and Chattoraj[5], for analysis in urine and blood by Petrak[4], and general reviews by Zander[114] and Miyake[115]. A common technique for detecting progesterone is to convert it to the 20β-hydroxy compound enzymatically, form the chloroacetate derivative of the latter, and elute and measure on GLC-ECD. Nevertheless, several experiments have been reported on progesterone in tissue and milk.

Heap[116] treated tissues and plasma with alkali followed by diethyl ether extraction and partition between 70% methanol–water and petroleum ether. The extract was purified by descending paper chromatography with 90% methanol–petroleum ether and the eluted material converted to the 20β-hydroxy steroid, which was purified further by paper chromatography as above. Measurement was effected by treatment with sulfuric acid in ethanol and estimation of the fluorescence produced at about 525 nm. The procedure was specific and recovery losses were estimated through the use of [^{14}C]progesterone.

A double isotope assay for progesterone in blood and muscle tissues has been described by Wiest[117] and was a classic example of the use of the double isotope technique in steroid hormone analysis. [4-^{14}C]Progesterone was added to the tissue extract, after which the extraction, partition, chromatography, and reduction by enzymes were the same as above[116]. The residue was then acetylated with [^{3}H]acetic anhydride and the acetates purified by silica gel paper chromatography, eluting with ethyl acetate–toluene (1:9). The methyl oxime derivative was formed and, after a final purification on paper, the eluted zone was counted for both ^{14}C and tritium. The detection level (~5 ppb) was low and specificity excellent due to the multistep procedures but this long and rather expensive method was not suitable for routine use. A somewhat shorter procedure has been related by Watson *et al.*[118]. In a study of progesterone in biological samples, reproductive tissues were extracted with acetone–ethanol, partitioned between 70% methanol and hexane, and separated by TLC on silica gel with methanol–methylene chloride (2:98). Impurities from the eluted extract were removed by acetylation, and the system further chromatographed in a second solvent system of cyclohexane–ethyl acetate (1:1). The dinitrophenylhydrazone (DNPH) of progesterone was formed and this separated a third time by TLC on silica gel in chloroform–cyclohexane (3:1). Measurement was accomplished by the absorbance of the eluted DNPH at 380 nm. Recoveries of radioactive progesterone were over 60%, detection in the order of 5 ppb, and the design of the assay was such that many sam-

ples could be applied at the same time, although the entire method was not short.

A technique for progesterone in food tissues using absorbance measurement has been described by Krzeminski *et al.*[108] incidental to their assay on melengestrol acetate in food (q.v.). A GLC procedure for high levels of progesterone in reproductive tissues has also been reported[119].

In the case of milk, there have been several reports dealing with progesterone. Since milk is a less complex sample than tissue, the cleanup procedures are less involved and shorter. Thompson *et al.*[120] studied the extraction of radiolabelled progesterone in milk by the addition of ammonium hydroxide and ethanol, and partitioning into petroleum ether–diethyl ether. Further partitioning was performed with 70% methanol and ethylene dichloride. TLC on silica gel in two dimensions in the solvent systems hexane–ethyl acetate (5:2) and dichloromethane–ether (5:2) was used to purify the progesterone; the zone was then scraped off and eluted. Recoveries of added progesterone, either ^{14}C- or tritium-labelled, were over 60% but, unfortunately, this procedure was not combined with a measurement step to determine endogenous progesterone in milk.

Darling *et al.*[121] described their procedure for identifying progesterone in milk samples. Non-saponifiable lipids and steroids were extracted with diethyl ether, oxidized with CrO_3, and the ketonic fraction separated on silica thin layers eluted with chloroform. After elution of the zones, the extracts were separated on a QF-1 GLC column. After collection, the progesterone fraction was re-chromatographed on 1.5% Dexsil 300 at 240° and detected by its mass spectrum using total ion current. Values of 5–15 ng/ml were confirmed in pregnant cow's milk with this highly specific technique. A second TLC procedure[88] in milk for steroids including progesterone involved extraction with chloroform, TLC on silica gel eluting with ethyl acetate–hexane–ethanol (9:9:2), and detection of spots with 254 nm UV. The procedure was rapid but did not detect small amounts (<1 ppm).

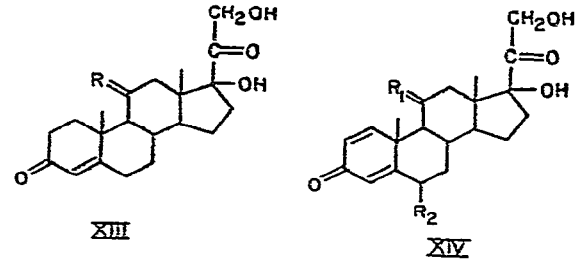
From the above discussion, it appears that the individual steps are available to extract, purify and detect progesterone in food samples at low levels. What is lacking is the combination of these procedures into a rugged method which is short enough to be used on a routine basis.

5. CORTICOSTEROIDS

The fourth class of steroidal hormones which can appear in food above normal levels due to agricultural uses are the corticosteroids or glucosteroids. They are shown structurally in Fig. 9. As they are employed, in combination with other drugs, for the treatment of bovine mastitis and ketosis, their residues are possible mainly in milk and, to a lesser extent, in other tissues. Chemical and chromatographic assays for these steroids in biological samples have been reviewed by Peron[122] and biological assays for corticoid activity have been discussed by Dorfman[2]. However, the lack of specific methods for corticosteroids in food samples is highest compared with any other class of steroids considered so far.

A. Cortisone and cortisol (hydrocortisone)

A number of procedures have been reported for cortisone and cortisol (hydrocortisone), two naturally occurring compounds in milk. Mooney and Pasarela[123] ex-



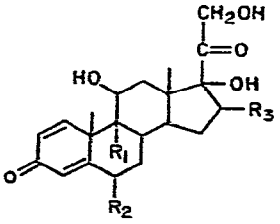


Fig. 9. Structural diagrams of corticosteroids. (XIII) R = O, cortisone; R = OH + H, cortisol. (XIV) R_1 =O, R_2 = H, prednisone; R_1 = OH + H, R_2 = H, prednisolone; R_1 = OH + H, R_2 = CH_3, methylprednisolone. (XV) R_1 = F, R_2 = H, R_3 = β-CH_3, betamethasone; R_1 = F, R_2 = H, R_3 = α-CH_3, dexamethasone; R_1 = R_2 = F, R_3 = CH_3, flumethasone; R_1 = F, R_2 = H, R_3 = OH, triamcinolone; R_1 = F, R_2 = H, R_3 = H, fluoroprednisolone.

tracted free cortisol and its 21-acetate from milk with methylene chloride–hexane (4:1) to minimize emulsion formation, and partitioned the residue between hexane and water and then into methylene chloride. The evaporated extract was then spotted and eluted (7% methanol in methylene chloride) on silica gel thin layers and the scraped zones were dissolved in ethanol. The cortisol was then derivatized with phenylhydrazine in sulfuric acid by heating for 1 h at 55° and the absorbance read at 370, 410, and 450 nm. Recoveries of cortisol acetate were over 80% and those of free cortisol over 75%, and the detection limits, using a blank, were 10 ppb. The possibility of screening for other steroids at the same time was discussed.

In a later publication, Trucksess *et al.*[124] reported their determination of cortisol in milk. The sample was homogenized with chloroform and the concentrated extract transferred to a Florisil column, eluted with methanol–chloroform (1:3), and the eluant washed with sodium hydroxide. The derivatization and measurement were similar as in the above method[123], using the absorbance reading at 410 nm. The recoveries and detection limits were also similar to those obtained by the above procedure but the overall procedure was shorter.

A third procedure[88] has been reported which detected diverse steroids including cortisone and cortisol in milk, and involved extraction with chloroform, TLC on silica gel and detection at 254 nm. While rapid, its limit of measurement (1 ppm) was much higher than that of the two procedures previously discussed.

As far as tissue is concerned, there is little if any literature available on these

two compounds in food. Jansen *et al.*[125] analysed for cortisol in biopsy tissue using a fluorescent measurement, but the technique was not directly applicable to food and, due to lack of chromatographic steps, was non-specific.

B. Prednisone, prednisolone, and methylprednisolone

The synthetic steroids prednisone, prednisolone and methylprednisolone are derived from cortisone and cortisol by dehydrogenation and invariably show a higher potency. A variety of methods exist for their measurement in ointments and pharmaceuticals. A method for the estimation of some synthetic glucocorticoid steroids including prednisolone in rat muscle was reported by Simpson[126]. Macerated tissues containing added water were extracted twice with ethyl acetate and then washed with aqueous acid and base to remove impurities. The organic extract was then partitioned between hexane and 70% aqueous methanol and the residue was silylated with a mixture of N,O-bis(trimethylsilyl) acetamide, trimethylsilylimidazole, and trimethylchlorosilane at room temperature overnight to give a single substance in all cases. The derivatization of all the oxygen functions with the silylation mixture obviated the protection of the C-3 and C-20 ketones and was verified by MS. The mixture was then chromatographed by GLC with a high-temperature ECD on a 1% OV-17 275-cm column at 260°. Recoveries of added prednisolone were from 60–80%, with a detection limit of 20 ppb, although this level was not low enough to measure physiological levels of prednisolone.

One of the first examples of the use of HPLC for the analysis of steroids in food was reported by the Upjohn workers[127]. Methylprednisolone in milk samples was analysed by blending–extracting three times with ethyl acetate, partitioning the evaporated residue between hexane and acetonitrile, then between hexane and water saturated with sodium sulfate, and finally into methylene chloride. The residue from the latter was taken up in methanol and eluted after HPLC on a 1 m × 2.1 mm I.D. DuPont hydrocarbon polymer stationary phase with water–methanol (3:1) at a flow-rate of 0.5 ml/min. Detection was by a precision photometer with 0.005 absorbance units full scale deflection at 254 nm. Recoveries of over 90% for methylprednisolone were reported in the range of 5–50 ppb, with the former value being the detection limit of the method. The procedure was then succesfully used to study the milk of cows receiving the drug.

There have been no procedures developed to date for prednisone in any food tissues.

C. Fluorinated corticoids

Betamethasone, dexamethasone, flumethasone, fluoroprednisolone, and triamcinolone

The fluorinated steroids betamethasone, dexamethasone, flumethasone, fluoroprednisolone, and triamcinolone have been developed from the prednisolones for their greater activity when compared to the mother compounds. The above-named compounds are often used as their acetates or, in the case of triamcinolone, as the acetonide. Only two specific tests have so far appeared for this class in food tissue.

TABLE 8

GAS–LIQUID CHROMATOGRAPHIC RETENTION TIMES OF CORTICOID TMS ETHERS
On a 275-cm 1% OV-17 GLC column at 260°; after Simpson[126].

Steroid	*Retention time (min)*
Cortisol	16.7
Corticosterone	19.5
Prednisolone	17.9
Betamethasone	20.8
Triamcinolone	23.6
Triamcinolone acetonide	31.5

The first of these has been described in the previous section on the work of Simpson[126]. He extracted experimental animal tissues with ethyl acetate, and, after partitioning steps, silylated the mixture to give a single derivative which was separated on GLC and detected by ECD. This method was successfully applied to the fluoroprednisolones, triamcinolone and its acetonide and to betamethasone, amongst several corticoids. Detection limits for both were about 10 ppb in muscle tissue. Application of this relatively simple technique to dosed experimental animals showed no detectable levels after 24 h. The retention times of the several corticoids as their TMS derivatives on a 1% OV-17 275-cm column at 260° taken from Simpson[126] are summarized in Table 8.

A comprehensive study for the analysis of fluoroprednisolone, both the acetate and the alcohol, in several bovine tissues has been reported by Krzeminski *et al.*[128]. Milk samples were extracted with ethyl acetate, fat samples with methylene chloride, and tissue samples with acetone. The evaporated residue was partitioned between acetonitrile and hexane, then between hexane and water, and finally into methylene chloride. The residue from the latter solvent, containing the fluoroprednisolone, either the acetate or the alcohol, was transferred with chloroform to a 4% water-deactivated Florisil column, and the acetate eluted with methanol–chloroform (5:70) followed by the elution of the alcohol in the same solvents (2:8) containing a trace of base. For the acetate, the first fraction from the Florisil column was injected on a 1 m × 2.1 mm hydrocarbon polymer HPLC column, eluted with methanol–water (3:7), and detected at 254 nm using the 0.01 absorbance units full scale deflection range. For the alcohol, the second fraction from the Florisil column was injected on a 31-cm GLC column of 1% OV-17 at 250° and detected by ECD. A study of the method showed high recoveries (greater 90%) for fortified tissues even at the detection limit of 5 ppb and conversion of the acetate to the alcohol took place on storage of the tissues. Residue data showed levels only in liver, kidney, and muscle up to six days and none in milk. The methodology was specific and precise but was somewhat long due to the many evaporative steps.

6. PROSTAGLANDINS

While not strictly true hormones, the prostaglandins (PG) (illustrated in Fig. 10) have a strong and varied physiological effect which often overlaps those of the sex hormones and corticoids. Their isolation and characterization has taken place only in the last 10–15 years. As a result, their use in the production of food has been

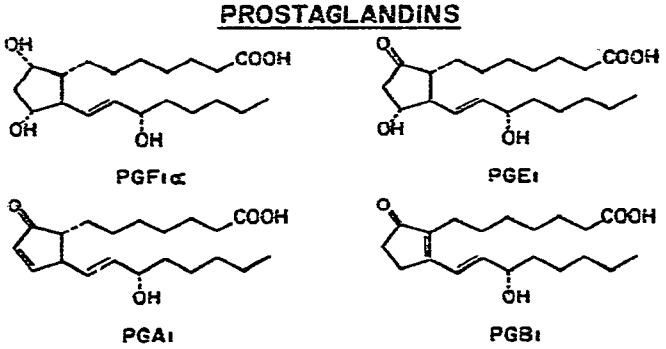


Fig. 10. Structural diagrams of some prostaglandins.

mainly on an experimental basis, but it is to be expected that these compounds will soon be tools of animal production and, hence, liable to occur in foods. To date, the analysis for prostaglandins is mainly limited to isolation techniques and measurement in biological materials in experimental animals and these have been reviewed[129].

Jouvenaz *et al.*[130] reported a sensitive GLC method for the analysis of the PGE class of prostaglandins taking advantage of their facile conversion to PGBs and the high electron capturing properties of the latter. Acidified tissues were extracted with ethanol–diethyl ether (1:1) and the evaporated residue was separated on a silica gel H column by elution with chloroform–methanol (2:1). After conversion to PGB in 0.5 *M* KOH, the prostaglandins were methyl esterified and separated on silica gel thin layers eluting with diethyl ether–petroleum ether (9:1). The hormones in the eluted zones, detected by 254 nm UV, were silylated and analyzed on a 305-cm × 2-mm GLC column of 3% QF-1 at 220° with an ECD. The detection limit of this discrete technique was about 10 ppb. The method was used to analyse the prostaglandin content from experimental animals.

A primising procedure for resolution and measurement of this class is HPLC. An example of the technique was reported by Dunham and Anders[131], who analysed a variety of prostaglandins in small amounts of rat tissue by extraction of phosphate- and EDTA-buffered samples with chloroform–ethanol (1:1). Depending on the levels, the lipid extract was either further purified by solvent partition or injected directly into a 115-cm × 1.8-mm-I.D. Sil-X HPLC column and eluted with chloroform–ethyl acetate (85:15) containing a trace of formic acid. Detection was performed by monitoring the base-treated effluent at 280 nm in a flow-through cell. Of the several types of prostaglandins, PGE and PGF were resolved from PGA and PGB, but the latter two were not separated under these conditions. Injections of from 5–200 ng of PGB_2 gave a linear response although detection levels were not given. A similar procedure was used by Tan and Privett[132] to analyse high levels of PGE in rat glands but no HPLC was necessary. It is to be expected that more chromatographic methods for the separation and measurement of low levels of prostaglandins in food will be forthcoming.

7. POLYPEPTIDES

A few polypeptides are used in animal production for various needs. Among

these are the polypeptides oxytoxin, for mastitis and milk let-down use, corticotropin, for ketosis, and the glycoproteins, gonodotropin and luteinizing hormone. A few procedures are available for these compounds in tissue using competitive binding and bioassay, but none have been published using chromatographic means.

8. ANTITHYROID HORMONES

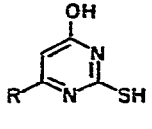
The use of thyroid hormone-inhibiting substances permits a considerable increase in the weight gain of domestic animals namely through augmented water intake. Since this practice is not desirable both from a fraud and health standpoint, some methods for analysis of these so-called antihormones have been published. Amongst the substances of importance are the thiouracils thioimidazole and thioxazolidones and their derivatives (Fig. 11).

An early report by Kreula and Kiesvaara[133] detected the antithyroid 5-vinyl-2-thioxazolidone in milk. Whole milk was extracted with ethyl acetate, the antithyroids partitioned into ammonia–water, and back into the organic solvent. The residue was then spotted on paper by paper chromatography and developed in two dimensions, first with 0.5% acetic acid and then with methanol–diethyl ether. The thioxazolidone in the eluted zone was then measured spectrophotometrically at 240 nm. Recoveries were high, with a measurement limit of 0.1 ppm.

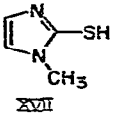
Bruggemann[134] separated the thiouracils from feed by extraction with hot ethanol and spotting and eluting the concentrated extract on paper chromatography with *n*-butanol saturated with 0.2 *N* aqueous ammonia. The spots were visualized by reacting with dichloroquinimide. The R_F values were 0.02, 0.41, and 0.26 for thiouracil, methylthiouracil, and propylthiouracil, respectively.

Van Waes[135] analyzed for methylthiouracil in meat by extraction with methanol and column chromatography on alumina with chloroform–methanol (1:1) as the eluant. Determination was carried out by the color formation at 435 nm with 2,6-dichloroquinonimide. Recoveries of added thiouracil at 0.1 ppm were high if carried out without delay.

As interfering substances were a problem in the above method, Gissil and Schaal[136] proposed a similar procedure for extraction of tissue glands but, for purifica-



XVI



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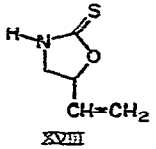

XVIII

Fig. 11. Structural diagrams of some antithyroids. (XVI) Thiouracils: R = H, methyl, *n*-propyl or phenyl. (XVII) methylmercaptoimidazole; (XVIII) vinyl thioxazolidone.

TABLE 9

$R_F \times 100$ VALUES OF ANTITHYROIDS ON SILICA GEL THIN LAYERS IN UNLINED TANKS

Solvents: I = chloroform–ethanol (95:5); II = chloroform–propionic acid (95:5).

Compound	*Solvent*	
	I	*II*
Methylthiouracil	32	48
Propylthiouracil	42	68
Phenylthiouracil	45	76
Mercaptomethylimidazole	67	58
Thiouracil	21	34

tion, used Kieselgel thin layers and elution with trichloroethane–ethanol (8:2). They detected three thiouracils on the TLC plates by UV at 254 nm. The detection limit in tissues was 0.5–1.0 ppm. A more recent comprehensive publication by De Brabander and Verbeke[137] analysed for several antithyroids, including phenyl-, methyl- and propylthiouracil and mercaptomethylimidazole, at the 10 ppb level. Tissue samples were extracted with methanol and the acidified extract defatted with petroleum ether, followed by addition to Dowex 50W-X8 anion-exchange resin. After elution with 5 ml of 75% methanol, the concentrated buffered extract was further defatted with diethyl ether. The sample was derivatized with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD) in acetone at 40°. The derivatives were spotted on a TLC plate of silica and developed in two dimensions, first with chloroform–ethanol (95:5) and then with chloroform–propionic acid (95:5). The results of these separations are shown in Table 9. Estimation was indirect by visualization of the yellow-green fluorescent spots after activation with alkaline cysteine solution. The principle of exchanging the non-fluorescent substance with a more fluorescence-inducing one allowed 50 ng of product to be detected. Recoveries of standards were over 80% at the 10-ppb level but, with 2 g meat samples, recoveries were much lower, even though still measurable.

Wildanger[138] also analysed for the same compounds as above[137] but used HPLC as the determinative technique. Tissues were extracted with methanol–water (1:1) and the evaporated residue taken up in methylene chloride. The extract was then subjected to HPLC on a 30-cm × 3-mm-I.D. column of Kieselgel Merckosorb SI-60, eluted with methylene chloride–ethanol–water (712:18:12), and detection was carried out at 280 nm. This procedure detected levels of 5 ppb of the thiouracils in meat and quantitated at the level of 0.1–0.2 ppm.

9. GENERAL COMMENTS AND FUTURE STUDIES

It is obvious that, for many steroidal hormones, methods are available for their measurement in food samples. It is only in the case of the corticosteroids that few techniques have been developed for their detection and estimation. It is also noteworthy that there are few, if any, general procedures for the simultaneous or multi-detection of hormone residues in food. In this sense, hormone residue analysis is many years in arrears of pesticide residue analysis. In the latter case, multi-residue

and automation procedures have been developed for broad classes of compounds such as the organochlorines and organophosphates. The stage is approaching whereby a single extraction and cleanup will suffice to detect several classes of pesticides. Since these procedures are often automated, the complete scope of analysis should involve minimal effort. As this is not the case in drug residue analysis, this review has considered the individual compounds rather than the separate parts of each method such as extraction, etc. However, it is to be expected that some multi-detection techniques will appear soon in this area. In this regard, both TLC and GLC are useful and, in particular, the newer technique of HPLC should become more evident. There are as yet only a few procedures which use HPLC for separation and, because of its non-destructive and mild nature, it is eminently suited for hormone residue analysis. It has the slight drawback that it lacks sensitive detection systems when compared to GLC or bioassay procedures.

Two possible schemes come to mind for screening of hormone residues. The first of these, involving only chemical and chromatographic techniques, would involve extraction of all hormones with a suitable solvent such as acetone or methanol. The extract could then be separated into estrogens and non-estrogens by extraction with 0.1 *N* aqueous sodium hydroxide. The former could then be purified further by TLC on either silica gel or alumina and detected either by fluorescence or by GLC after derivative formation. The non-estrogens could be further purified by defatting with hexane–70% methanol and treated in a similar manner as the estrogens to detect the androgens, progestogens, and corticoids.

A second possible format for screening for hormones in food would be to apply a sensitive general assay technique such as is done for antibiotic drug residue analysis. In that case, screening for inhibitory substances is carried out with a microorganism after separation using gel electrophoresis. Positive findings are investigated further for quantitation and confirmation using chemical and chromatographic techniques. For hormones, a possible screening would be a suitable general bioassay or competitive binding assay (*e.g.*, radioimmunoassay) followed by the described chemical and chromatographic techniques for confirmation and specificity. Certainly, such an approach could be applied to the estrogen compounds, since the bioassay exists already.

There are a number of other areas also where information is noticeably lacking. Many of the main metabolites of the drugs in question are not known and, if known, the methods usually measureonly the parent compound. This is particularly important whenever the hormone is rapidly metabolized or a metabolite has a high toxicity. There is a need for more research on the effects of temperature as experienced in cooking and pasteurizing, and of prolonged storage on residues of hormones in foods.

The strength of chromatographic assays arises from a combination of both sensitivity and specificity. For the former, the detection limits are in the same range or an order higher than bioassay and competitive binding assays. Their forte over these two bioassays, however, is their high specificity, since the chromatographic steps and confirmatory techniques guarantee a high degree of selectivity for the substance in question. The weakness of some chemical determinations is their length, often involving difficult and intricate manipulations necessitating experienced personnel to ensure high reproducibility.

10. SUMMARY

The analysis of hormone drugs in food samples by chemical and chromatographic techniques has been reviewed. For some of the classes of hormones, adequate methodology exists for their specific and low detection in foods utilizing gas–liquid, thin-layer, and high-pressure liquid chromatography. Many of these procedures, however, are involved and, as yet, no multi-residue technique exists for the analysis of a wide range of hormones in a single assay.

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