Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911.
Brannolte, H. D.; Mangold, H. K.; Stahl, E. *Chem. Phys. Lipids* **1983**, *33*, 297.
Brogle, H. *Chem. Ind.* **1982**, *19*, 385.
Friedrich, J. P.; List, G. R. *J. Agric. Food Chem.* **1982**, *30*, 192.
Hannay, J. B.; Hogarth, J. *Proc. R. Soc. London* **1879**, *29*, 324.
Hubert, P.; Vitzthum, O. G. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 710.
Kaufmann, W.; Biernoth, G.; Frade, E.; Merk, W.; Precht, D.; Timmen, H. *Milchwissenschaft* **1982**, *37*, 92.
Krukonis, V. *JAOCS* **1984**, *61*, 698.
Miki, W.; Toriu, N.; Kondo, Y.; Murakami, M.; Yamaguchi, K.; Konosu, S.; Satake, M.; Fujita, T. *Bull. Jpn. Soc. Fish.* **1983**, *49*, 1417.
Mori, M.; Hikichi, S. *Rep. Cent. Res. Lab. Nippon Suisan Co.* **1976**, *No. 11*, 12.
Needleman, P.; Raz, A.; Mikes, S. M.; Ferrendelli, J. A.; Sprecher, H. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 944.
Paul, P. F.; Wise, W. S. *The Principles of Gas Extraction*; Mills and Boon, Ltd.: London, England, 1971.
Stahl, E.; Schutz, E.; Manglod, H. K. *J. Agric. Food Chem.* **1980**, *28*, 1153.
Wilke, G. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 701.
Yamaguchi, K.; Miki, W.; Toriu, N.; Kondo, Y.; Murakami, M.; Konosu, S.; Satake, M.; Fujita, T. *Bull. Jpn. Soc. Sci. Fish.* **1983**, *49*, 1411.
Zosel, K. Ger. Pat. 2005243, 1974.
Zosel, K. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 701.

Received for review December 9, 1985. Revised manuscript received April 2, 1986. Accepted April 22, 1986.

Isolation of Estrogens in Bovine Plasma and Tissue Extracts Using Alumina and Ion-Exchange Microcolumns

Marjorie B. Medina* and Daniel P. Schwartz

Estrogens (estradiol, estrone) in picogram quantities can be isolated quantitatively from bovine plasma and tissue extracts by a simple procedure. Bovine plasma (0.1–1.0 mL) was extracted with either acetone or ether while tissues (1 g) were extracted with acetone. Extracts were passed through two disposable plastic tubes vertically arranged in tandem. The top column (5-mL pipet tip) contained 1–1.5 g of dry basic alumina and removed interfering substances. The bottom column (transfer pipet) contained 0.3–1.0 g of wet anion-exchange resin in the phosphate form and trapped the estrogens through their phenolic hydroxyl group. The estrogens were then eluted with acetic acid in acetone following a thorough washing of the columns. Recoveries greater than 95% were obtained when extracts of bovine plasma and tissue extracts of liver, kidney, and heart were spiked with either tritiated 17β-estradiol or estrone. This technique offers the advantages of simplicity, rapidity, and accuracy over traditional methods employed routinely in the purification of estrogens.

INTRODUCTION

Partial purification of estrogens extracted from animal tissues and fluids is necessary prior to most methods of quantitation. The methods currently employed in routine analysis of estrogens such as paper chromatography (Shutt, 1969), Sephadex LH-20 (Sjovall and Nystrom, 1968; Murphy, 1970; Mikhail et al., 1970; Murphy and Diez D'Aux, 1975), and Celite column cleanup (Korenman et al., 1969; Abraham et al., 1970) are tedious with reported recoveries of only 65–85%. Aqueous solutions of estrogens had also been purified by ion exchange (Järvenpää et al., 1979) with reported recoveries of 50–90%. Covey and co-workers (1984) also used an anion-exchange resin for purification of diethylstilbestrol and dienestrol.

This paper describes a relatively simple and rapid technique that can quantitatively isolate the estrogens (estradiol, estrone) from acetone extracts of bovine blood plasma and tissues for subsequent chromatographic analysis. This study is a preliminary report on the development of screening methods to detect and measure residues of estrogens in the blood and edible tissues of food-producing animals given growth-promoting hormones such as 17β-estradiol and to ascertain their absence such that safe and wholesome food can be delivered to consumers.

Table I. Column Conditions for Isolation of [^{3}H]Estradiol Added to Bovine Plasma and Tissue Extracts Using the Alumina Ion-Exchange Columns

sample	extr appl to col, mL	basic alumina, g	resin suspensn, mL
A. Bovine Plasma (mL)			
acetone extractn			
0.1	2	1.0 (3)[a]	1.0 (2)[b]
0.5	4	1.0 (3)	2.0 (3)
1.0	8	1.5 (5)	3.0 (4)
1.0	8[c]	1.5 (5)	3.0 (4)
ether extractn			
1.0	4	1.0 (3)	1.0 (2)
B. Bovine Tissues (1 g/8 mL of Acetone)			
liver	2	1.0 (3)	2.0 (3)
muscle	2	1.0 (3)	2.0 (3)
heart	2	1.0 (3)	2.0 (3)
kidney	2	1.0 (3)	2.0 (3)

[a] Total volume 95% acetone (mL) to wash alumina column. [b] Total volume 10% HOAc in acetone (mL) to elute [^{3}H]estradiol. [c] Plasma/acetone mixture added directly to column with glass wool on top of alumina bed.

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118.

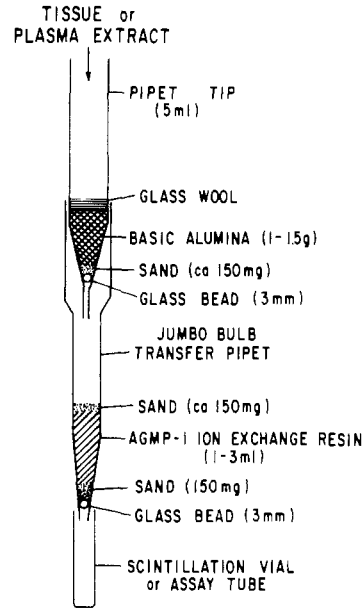

Figure 1. Column setup for purification of estrogens (estradiol and estrone). Glass wool used when acetone and plasma mixture was applied to column without centrifugation.

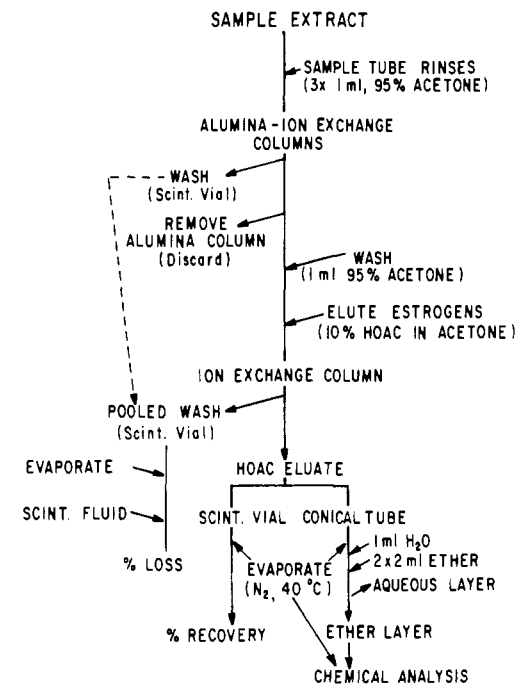

Figure 2. Schematic outline of estradiol and estrone purification using the column setup of Figure 1.

EXPERIMENTAL SECTION

Reagents and Equipment. The following products were used: basic alumina (80–200 mesh, Brockman activity I), polyethylene transfer jumbo-bulb pipets (7.5 mL), sea sand and precision glass beads (3 mm), scintillation vials (7.2 mL), toluene (ACS grade), Fisher Scientific Co., King of Prussia, PA; anion-exchange resin (AG MP-1, 100-200 mesh), Bio-Rad Laboratories, Richmond, CA; acetone (distilled in glass), Burdick and Jackson, Muskegon, MI; polypropylene pipet tips (5 mL), Rainin Instrument Co., Woburn, MA; anhydrous ether, Mallinckrodt, St. Louis, MO; Triton-X 100, Sigma Chemical Co., St. Louis, MO; [2,4,6,7,16,17-^{3}H[N]]-17β-hydroxyestradiol, [2,4,6,7-^{3}H-[N]]estrone and Liquifluor, New England Nuclear, Boston, MA; IKA rotary shaker, Tekmar Co., Cincinnati, OH.

Preparation of Ion-Exchange Column. The rounded top portion of a transfer pipet and 1 cm of the tip were cut, and the pipet was plugged by dropping a glass bead into the tapered end, followed by 150 mg of sea sand. Portions of 1, 2, or 3 mL (Table I) of 30% resin suspension in water, shaken moderately by hand or mechanically with a rotary shaker, were pipetted onto the sand layer. The resin was drained by gravity or vacuum and washed twice with water by filling the pipet each time. Aqueous HOAc (10%, 3 mL) was passed over the bed of resin, followed by rinsing with water until the effluent was neutral. The resin was converted to the phosphate form with saturated aqueous Na_3PO_4 (pH 13.2) that had been purified by recrystallizing 2× from H_2O. A volume of phosphate solution equivalent to 2 volumes of the resin suspension was passed through the column by gravity flow, and the resin was washed with water four times or until the washing was neutral to pH paper or a drop of 10% phenolphthalein solution. Another 150 mg of sand was added on top of the resin.

If more than 10 samples were to be analyzed, conversion of the resin to the phosphate form was carried out batchwise using a Büchner funnel and no. 40 Whatman filter paper allowing the phosphate to drain by gravity and washing until neutral, using a low vacuum. The columns were prepared as outlined above. Prepacked columns by either technique may be stored at 4 °C and used up to 7 days. However, stored columns should be washed twice with distilled water prior to sample application.

Preparation of Alumina Column. A glass bead and ca. 150 mg of sand were used to plug a 5-mL pipet tip. Alumina (1 or 1.5 g) was dispensed from homemade scoops and placed over the sand (Table I). The pipet tip was placed over the transfer pipet containing the phosphate-exchanged resin as is shown in Figure 1.

Extraction and Purification of Estradiol or Estrone in Bovine Blood Plasma. *Ether Extraction.* One milliliter of plasma was spiked with 10 pg of tracer ([^{3}H]estradiol or [^{3}H]estrone) and the resultant solution mixed vigorously in a Vortex mixer with 4 mL of ether. After separation of the layers, the aqueous phase was snap frozen in dry ice/acetone and the ether layer was decanted into a test tube. The extraction was repeated, the ether layers were pooled in each replicate, and then ether was evaporated to dryness under N_2. The residue was dissolved in 4 mL acetone–H_2O (95:5, v/v) and transferred quantitatively to the purification columns (Figure 1), and the estrogens were isolated as outlined in Figure 2. After percolation of all solvent through both beds, the sample test tubes were rinsed with 3 × 1-mL portions of solvent and were then pipetted to the columns while sides of the columns were washed down. The alumina column was removed, and the sides of the resin column were rinsed with 1 mL of solvent. Estrogens were then eluted with 2–4 × 1-mL portions of 10% HOAc in acetone (Table I) and collected in scintillation vials to measure recovery or in conical tubes for chemical analysis. The eluting solvent was evaporated with N_2 at 40 °C. Five milliliters of scintillation fluid (3.78 L of toluene, 160 mL of Liquifluor, 25 g of Triton X-100) was added to the scintillation vial. Distilled water (1 mL) was added to the conical tubes and was further extracted twice with 2 mL of ether. The aqueous phase was snap frozen in dry ice/acetone, and the ether layers were pooled and the ether evaporated off. The dry residues were either measured for recovery or analyzed by TLC or HPLC.

Acetone Extraction. Alternatively, 0.1, 0.5, or 1 mL of plasma, spiked with [^{3}H]estradiol or [^{3}H]estrone was extracted with 2, 4, or 8 mL of solvent, respectively. The extract was either separated by centrifugation at 5000 rpm

(3020g) for 10 min or fractionated by directly applying the entire mixture to the columns without centrifugation. If the latter was used, glass wool was placed on top of the alumina bed to retain the precipitate. In either case, the column purification of plasma estrogens was as described above.

Extraction and Purification of Estrogens (Estradiol, Estrone) in Bovine Tissues. Liver, kidney, heart, or muscle was homogenized in a Waring Blendor for 2 min. Aliquots (1 g) were extracted with 4 mL of acetone and sonicated for 2 min followed by centrifugation at 3020g for 10 min. The supernatant was decanted and the extraction process repeated on the pellet. The supernatants were pooled and the volume adjusted to 8 mL. Aliquots (2.0 mL) were taken and 10 pg of [^{3}H]estradiol or 10 pg of [^{3}H]estrone was added to the tissue extracts and quantitatively transferred to the alumina and ion-exchange columns under conditions described in Table I and purified according to the scheme (Figure 2) used for plasma extraction.

RESULTS AND DISCUSSION

Purification of estrogen (estradiol, estrone) as described utilized basic alumina as a preliminary cleanup column to remove interfering components (e.g., anions stronger than phenols) in blood plasma and tissue extracts while these conditions allowed the estrogens to pass through and subsequently be trapped by the phosphate-exchanged resin contained in the bottom column. The interfering anions could also be trapped on an AG MP-1 resin in the borate form (pH 10.5), which also allowed estradiol and estrone to pass through. Recoveries were near 100%. The borate-exchanged column can be used in tandem or in place of the alumina column for preliminary cleanup of samples. However, the preparation of borate-exchanged resin was more tedious than the use of alumina column, but the nonabsorption of estrogens by the borate-exchanged resin demonstrated the ion-exchange phenomenon between the phosphate-exchanged resin and the estrogens (estradiol, estrone). Estradiol and estrone were retained on AG MP-1 (PO_4^X) resin via their phenolic functional group which has a pK' of 9.89 while phosphoric acid has pK'_3 of 12.67. The phenolic estrogens presumably formed phenoxy conjugates with the cationic functional groups [$-CH_2N(CH_3)_3^+$] and were subsequently eluted with acetic acid and acetone wherein they were regenerated to free phenolic compounds.

Table I summarizes the conditions that gave maximum recovery of estradiol added to plasma and acetone extracts of bovine tissue. Maximum recovery was obtained by varying the amount of resin and alumina to suit the nature and size of samples applied to the purification columns. Larger volume of plasma (e.g., 1 mL) extracted with acetone required 3 mL of the resin suspension and 1.5 g of alumina compared to ether extraction of 1 mL of plasma that required 1 mL of resin suspension and 1 g of alumina for obtaining essentially the same recovery.

Although both ether and acetone extraction of spiked plasma yielded near-quantitative recovery of tritiated estradiol or estrone, use of acetone was preferred due to its greater stability and safety in handling. The use of acetone as extractant also had the advantage of eliminating the need for centifugation of precipitated plasma proteins prior to column purification, thereby reducing analysis time. Acetone extraction of tissue went smoothly, but direct extraction of tissues with ether was not possible. However, one can use ether extraction with an aqueous homogenate of tissues.

Solvent flow rate through the column system was found to influence recovery of estrogens from both plasma and tissue extracts. A flow rate of 20–25 drops/min (ca. 1 mL/min) gave maximum recovery, and flow rates faster or slower than this range resulted in lower yields. Under the conditions specified for packing the columns, the desired flow rate can be obtained.

The stability of the resin in both the chloride and phosphate forms was studied in order to determine whether prepacked columns could be successfully used following storage. Resin in the chloride form stored in distilled water for 40 days at room temperature behaved like freshly prepared resin suspension. Resin in the phosphate form stored for 7 days at 4 °C or frozen for longer periods still gave near-quantitative recoveries. However, frozen resin suspensions can be used only once after thawing. Repeated thawing and freezing significantly ($P < 0.05$) decreased recoveries of the tritiated estrogens. This might be due to fracturing and breakdown of the resin, resulting in decreased ion-exchange capacity.

Experiments on column capacity showed that columns made with 1 g of alumina and 1 mL of 30% aqueous resin suspension can quantitatively trap from 10 pg to at least 10 ng [^{3}H]estradiol dissolved in 95% acetone. However, amounts greater than 1 ng of estradiol applied to the system needed 6 mL of solvent to elute the estrogens from the alumina column. The normal endogenous levels of estrogens in humans and animals are only in picogram amounts per gram of tissue or per milliliter of plasma.

Table II. Recovery of [^{3}H]Estradiol Added to Bovine Plasma and Tissue Extracts When Purified over Alumina Ion-Exchange Columns Using Conditions Described in Table I

samples[a]	no. of replicates	mean % rec	std dev
A. Bovine Plasma (mL)			
acetone extractn			
0.1	3	98.0	0
0.5	6	96.0	0.89
1.0	4	95.8	1.50
1.0[b]	6	95.7	0.82
ether extractn			
1.0	4	96.0	1.73
B. Bovine Tissues (1 g/8 mL of Acetone)			
liver	18	98.9	1.45
muscle	4	96.2	0.50
heart	5	95.4	0.89
kidney	5	96.0	0

[a] Single batch of plasma, muscle, heart, and kidney were used in this study; liver samples were obtained from two animals. [b] Plasma/acetone mixture added directly to column with glass wool on top of alumina bed.

Table III. Studies on Recovery of [^{3}H]Estrone Added to Bovine Plasma and Tissue Extracts When Purified[a] over Alumina Ion-Exchange Columns

sample	extr appl to col, mL	no. of replicates	mean % rec	std dev
A. Bovine Plasma[b] (mL)				
0.1	2	4	95.4	0.78
0.5	4	6	95.3	0.44
1.0[b]	8	4	94.7	0.41
B. Bovine Tissues (1 g/8 mL of Acetone)				
liver	2	6	95.1	1.63
muscle	2	6	95.0	0.76
heart	2	6	94.9	1.16
kidney	2	5	94.6	1.76

[a] Purification conditions used as in Figure 1 and Table I. [b] Plasma/acetone mixture added directly to column modified with glass wool.

Tables II and III show the quantitative recovery of [^{3}H]estradiol and [^{3}H]estrone added to bovine plasma and tissue extracts under the column conditions in Table I and the purification scheme in Figure 2. Statistical analysis demonstrated excellent reproducibility (<3% coefficient of variation) and high accuracy (>95% mean recoveries of [^{3}H]estrogens added to plasma and tissue extracts). Water and ether extraction of the HOAc column effluents did not significantly ($P < 0.05$) change the mean recoveries.

To determine the efficiency of the purification columns, the acetone extracts of the tissues were spiked with [^{3}H]estradiol and yielded near-quantitative (95%) recoveries. However, when tissues were spiked with [^{3}H]-estradiol prior to extraction, recoveries ranged only from 65 to 75% after exhaustive extraction (i.e., sonication and centrifugation three times). The loss of 25–35% is apparently due to irreversible binding of the added estrogen with tissue components. This phenomenon was also reported by Metzler (1981) and Gridley et al. (1983). Therefore, adding this 25–35% loss to actual values measured can overestimate results. The amount of estrogen resulting from this extraction technique can only be interpreted as extractable estrogens, and results in suspect samples should be compared to estrogen levels in tissues of untreated animals.

The use of this cleanup procedure has shown that there is no background interference with 17β-estradiol, DES, or zeranol when these anabolic estrogens were fortified in avian muscle tissue extracts and analyzed by normal-phase HPLC equipped with a UV detector (Medina and Sherman, 1986). A multiresidue TLC screening analysis of anabolic estrogens fortified in avian muscle tissue extracts and purified by this cleanup technique also showed no background interference band with estradiol, DES, or zeranol (Medina and Schwartz, 1986). The procedures for HPLC or TLC analysis of anabolic drugs were also developed in our laboratory and are described in detail in these references.

This cleanup technique offers a rapid extraction and isolation procedure allowing the preparation of at least 10 columns in less than 2 h and sample purification in less than 1 h. The materials used are inexpensive (25¢/column), and the simplicity, speed, and accuracy of this technique make it suitable for routine chemical analysis.

ACKNOWLEDGMENT

We extend our gratitude to James T. Sherman for his technical assistance.

Registry No. Estradiol, 50-28-2; estrone, 53-16-7.

LITERATURE CITED

Abraham, G. E.; Tulchinsky, D.; Korenman, S. G. *Biochem. Med.* **1979**, *3*, 65–368.
Covey, T.; Henion, J.; Maylin, G. Abstracts, Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy, **1984**; p 932.
Gridley, J. C., Allen, E. H., Shimoda, W. *J. Agric. Food Chem.* **1983**, *31*, 292–296.
Järvenpää, P.; Fotsis, T.; Adlercreutz, H. *J. Steroid Biochem.* **1979**, *11*, 1583–1588.
Korenman, S. G.; Perin, L. E.; McCallum, T. P. *J. Clin. Endocrinol. Metab.* **1969**, *29*, 879–883.
Medina, M. B.; Doerr, R. E.; Sherman, J. T.; Fiddler, W. Abstracts, Institute of Food Technologists 45th Annual Meeting, Atlanta, GA, 1985; p 153.
Medina, M. B.; Schwartz, D. P., submitted for publication in *Food Addit. Contam.*, **1986**.
Medina, M. B.; Sherman, J. T. *Food Addit. Contam.* **1986**, in press.
Metzler, M. *CRC Crit. Rev. Biochem.* **1981**, *10*, 171–212.
Mikhail, G.; Wu, C. H.; Ferin, M.; Van de Wiele, R. L. *Steroids* **1970**, *15*, 333–352.
Murphy, B.E.P. *Acta Endocrinol. (Copenhagen), Suppl.* **1970**, *147*, 37–60.
Murphy, B.E.P.; Diez D'Aux, R. C. *J. Steroid Biochem.* **1975**, *6*, 233–237.
Shutt, D. A. *Steroids* **1969**, *13*, 69–82.
Sjovall, J.; Nystrom, E. *Adv. Chromatogr.* **1968**, 119–170.

Received for review May 28, 1985. Revised manuscript received October 18, 1985. Accepted June 9, 1986. Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Monitoring Organic Acids and Carbohydrates in Cotton Leaves by High-Performance Liquid Chromatography

Judy D. Timpa* and John J. Burke

Extraction methodology, sampling procedures, and separations by high-performance liquid chromatography (HPLC) have been developed for analyzing organic acids and carbohydrates found in cotton leaf tissue. The major components sucrose, glucose, malic, and citric acids have been quantitated with calculations based upon fresh weight, dry weight, and leaf surface area. Automated HPLC analyses allow efficient screening and monitoring of large numbers of plant samples for the compounds of interest. Profiles by HPLC of various cottons are being used to characterize responses of plants to stress environments such as drought.

INTRODUCTION

Our research objective is to determine qualitative and quantitative alterations in chemical compositions of crop plants induced by stress environments (such as drought) that correlate with continuance of agronomic growth under stress conditions. Organic acids and carbohydrates have been reported to provide adaptive behavior in response to water-deficit conditions. Among the many acids found in plants, certain ones, namely malic, citric, and oxalic acids, are frequently found in large amounts in mature tissues in plants (Beevers et al., 1966). In cotton leaves, citric and malic acids comprise 5–10% of dry weight (Ergle and Eaton, 1949) and have been reported to be the only two

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70179 (J.D.T.), and Plant Stress and Water Conservation Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Texas Tech University, Lubbock, Texas 79409 (J.J.B.).