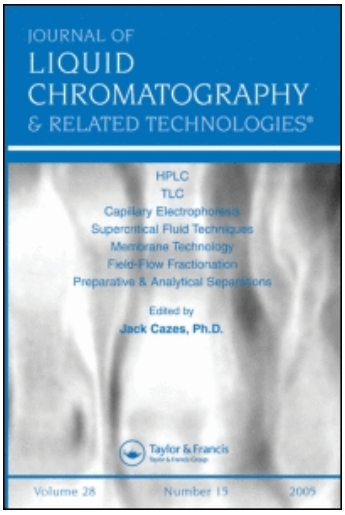

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Application of Supercritical Fluid Extraction and High Performance Liquid Chromatography/Mass Spectrometry for the Determination of Some Anabolic Agents Directly from Bovine Tissue Samples

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APPLICATION OF SUPERCRITICAL FLUID EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY FOR THE DETERMINATION OF SOME ANABOLIC AGENTS DIRECTLY FROM BOVINE TISSUE SAMPLES

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ABSTRACT

The aim of the present work was to evaluate the feasibility of supercritical fluid extraction (SFE) of some growth-promoting anabolic steroids directly from bovine tissue samples with carbon dioxide as a supercritical fluid. The study was divided into two phases. The first phase involved the optimization of SFE parameters versus recovery and repeatability. Results were obtained by high performance liquid chromatography (HPLC) or supercritical fluid chromatography (SFC). The second set of studies consisted of SFE from the real samples monitored by the combination of high performance liquid chromatography

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atmospheric pressure chemical ionization mass spectrometry (HPLC/APCIMS). The limit of detection was about 10 ppb, but 100 ppb is the practical detection limit for SFE-HPLC/MS analysis under the experimental conditions used in this study. The results from this work suggested that SFE may be useful for isolation of target compounds from complex biological samples.

INTRODUCTION

Many samples encountered by the analytical chemist consist of trace components in a complex and interfering matrix. For the identification and quantitation of these components, isolation from the matrix is usually necessary. Sample preparation methods are generally both time and solvent consuming. According to a recent survey, two thirds of the analysis time is devoted to sample preparation and this step accounts for at least one-third of the errors generated during the performance of an analytical method.[1] Extraction with supercritical fluids is one of the most rapidly developing methods for separation substances from various matrices. The term dense gas is also used, but it covers both the liquid and supercritical states of a fluid and the term supercritical is applied only when the critical parameters have been exceeded. Comprehensive reviews on principles, theory, techniques and applications of supercritical fluid extraction (SFE) have been published.[2-6]

Supercritical fluid extraction is a relatively new analytical isolation technique which offers advantages of low temperature processing, recovery of a solvent-free extract, rapid extraction resulting from high mass transfer due to higher diffusivity and lower viscosity in comparison to organic solvents.[7] The selectivity and solvent power depend on the density, which can be varied during the extraction procedure by controlling pressure and temperature. By changing the extraction conditions class-selective extractions and fractionation of the extract can be achieved. By far the most widely used extraction fluid has been supercritical carbon dioxide (CO_2)[2]. Its preferential use is due to the fact that it is an chemically inert, inexpensive, non-toxic and non-flammable extraction gas with mild critical parameters (31.1°C critical temperature and 7.38 MPa critical pressure).[8] This enables the application of low extraction temperatures thereby reducing the risk of analyte degradation during the extraction. Due to the gaseous nature of CO_2 at low pressure SFE can be combined with chromatographic techniques reviewed e.g., by Chester et al.,[6] Hawthorne et

al.,[9] Jinno and Saito.[10] By adding a modifier (*e.g.*, MeOH, H_2O) it is possible to adjust the solvating power of CO_2. Many other supercritical fluids have been also used *e.g.*, N_2O, SF_6, CH_3OH, H_2O and $CHClF_2$.[11-13]

Supercritical fluid extraction has been used for industrial-scale separations and the isolation of a variety of compounds.[2,4] On the analytical scale SFE has also attracted considerable attention and a variety of techniques have been used for on-line analyses combined with other chromatographic methods.[6] An increasing number of quantitative applications of SFE especially for the extraction of environmental pollutants has been reported in recent years.[14-22] SFE is utilized also in the field of food science to isolate not only natural food components such as fat,[23] cholesterol[24] and volatiles[25-26] but also natural but not desirable components like mycotoxins[27] and organic contaminants such as pesticides and PAHs.[28-32]

Only limited studies have been published on the use of supercritical fluids for the extraction of polar drug residues in the bovine tissues. Ramsey *et al.*[33] have used the combination of SFE/SFC/MS/MS for the determination of residues of a small group of veterinary drugs in freeze-dried pig's kidney. The results showed the detection of 1 mg/kg of spiked samples for the four drugs diethylstilbestrol, dienestrol, hexestrol and trimethoprim.

The combination of SFE-HPLC has been used for the determination of polar drugs, steroidal compounds and sulfonamides from animal food, blood plasma and chicken tissue.[34-37] Also, solubility's of some steroidal compounds have been conducted by SFE-HPLC.[38] The concentration of drugs investigated in most previous studies were, however, so high that true residue analysis is out of question.

The aim of the present study was to develop a unified multiresidue extraction procedure for the isolation of estrogenic and anabolic agents from bovine tissues using supercritical fluid extraction techniques. The goal of this work was also to evaluate the feasibility of extracting these growth-promoting target compounds at low parts per billion level directly from bovine tissue samples with carbon dioxide as a supercritical fluid. The method is compared with a traditional three-phase liquid-liquid extraction procedure used previously for related studies. The analytes were monitored by HPLC and identified by UV detection and mass spectrometry.

MATERIALS AND METHODS

Chemicals

All solvents, buffers and common reagents were purchased from Fisher Scientific (Rochester, NY, USA) unless otherwise noted. A standard stock solution of the seven drugs (500 μg/ mL dichlromethane) was prepared from pure standards purchased from Sigma Chemical Co (St. Louis, MO, USA). The drugs were dexamethasone (DEX), diethylstilbestrol (DES), medroxy progesterone (MDP), melengestrol acetate (MGA), trenbolone (TBOH), triamcinolone acetate (TACA) and zeranol (ZER). Figure 1 shows structures of these seven growth promoting drugs investigated in this study.

Tissue samples

About 200 g of beef steak, purchased from a local supplier, was homogenized to a smooth paste in a Waring blender (2 min/full speed). The paste was spread onto Petri trays and freeze-dried using a VirTis Unitrap II (VirTis, Gardiner, NY, USA). The lyophilized tissue sample was then ground using a mortar & pestle. Portions of the powdered muscle or liver (1 g) were mixed with methanol (10 mL), spiked with the appropriate quantity of each anabolic compound by using the standard stock solution, allowed to equilibrate for one hour whilst being sonicated and then evaporated to dryness under a stream of dry nitrogen. All samples were thoroughly mixed prior to being loaded into the SFE extraction cell.

Three-phase liquid-liquid extraction

The liquid-liquid extraction was carried out according to the procedure reported by Hsu et *al.*[39] and Covey et al.[40] Muscle or liver tissue were homogenized in the same way as described above. Five-gram aliquots of bovine liver or muscle homogenate were then vortex-mixed with 10 mL of 0.04 M NaOAc. The pH of the solution was brought to 4.2 - 4.7 with glacial acetic acid. The enzymatic hydrolysis was initiated by adding 100 μL of β-glucuronidase (Sigma Chemical Co., St. Louis, MO, USA). After 8 hour of enzymatic hydrolysis at 37°C, 20 mL of acetonitrile was added followed by mixing on a vortex mixer for 30 seconds. The homogenate was then centrifuged (5000 rpm for 20 min) and the supernatant (30 mL) was then

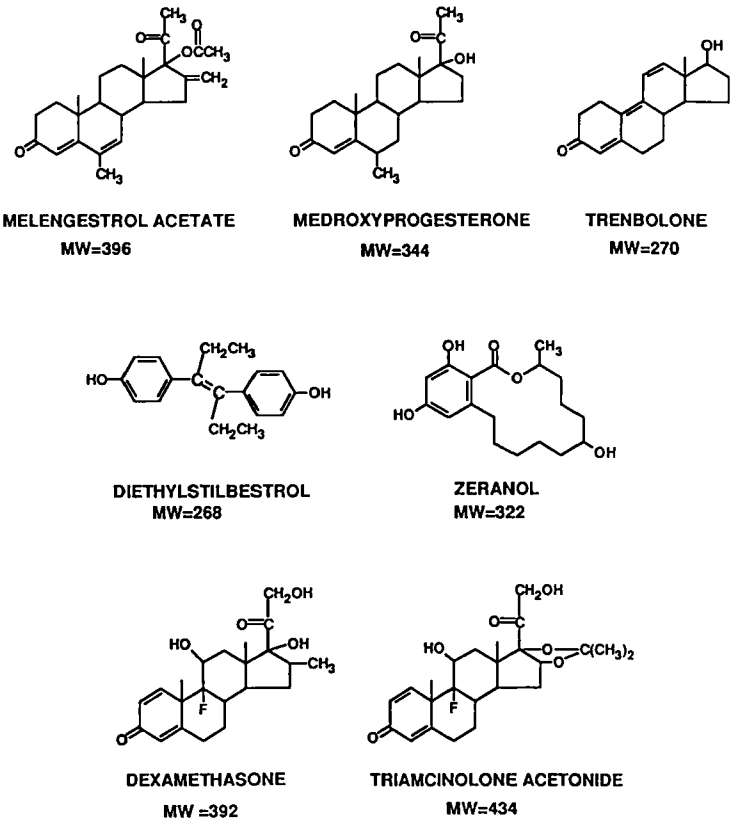

Figure 1. Structures of growth promoting drugs investigated in this study.

transferred to a clean test tube. Hexane (8 mL) and dichloromethane (2 mL) were added and mixed by rotation for 3 minutes. Samples were then centrifuged (2000 rpm for 2 min). A three-phase liquid system was obtained. The middle layer (15 mL in acetonitrile) was transferred to a 20 mL scintillation vial and evaporated to dryness under nitrogen. The liver and muscle extracts were each dissolved in 1 mL of dichloromethane prior to analysis.

Supercritical fluid extraction (SFE)

A Lee Scientific Series 600 micro-scale Extraction System (Lee Scientific, Inc., Salt Lake City, UT, USA) was used for sample preparation while the analytes were analyzed by high performance liquid chromatography (HPLC) combined with UV-detection or mass-spectrometry (MS).

Samples were injected (standards in the solvent) or weighed (spiked tissue samples) into a 2 mL extraction cell (Valco guard column, Valco Instruments Co. Inc. TX, USA) located external to the instrument. A restrictor was directed through the oven into the collection system external to the capillary SFC instrument (Lee Scientific, Inc., Salt Lake City, UT, USA). During the SFE procedure the temperature of the oven was kept constant at 60°C. Restrictors used in this study were cut to the desired length from fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA). The Guthrie-type restrictor was homemade. A schematic of the supercritical fluid extraction system is shown in the owner's manual of the instrument. SFC-grade CO_2 in an aluminum cylinder with helium head pressure from Scott Specialty Gases (Plumbsteadville, PA, USA) was used for all SFE experiments.

Analytes were collected by placing the restrictor into a 2 mL auto sampler vial containing 1 mL of precooled (+ 5°C) methanol (MeOH). The cooling effect of expanding CO_2 was sufficient to keep the vial cool to prevent the loss of analytes. After the extraction samples were evaporated to dryness under nitrogen and prior to HPLC analysis they were dissolved in 100 μL of eluent containing 30% of methanol/acetonitrile (50/50) and 70% mM ammonium formate.

High performance liquid chromatography (HPLC)

Dissolved tissue extract residue as well as standard mixtures were injected into a Waters HPLC system, Model # 510 (Waters, Inc. Milford, MA, USA) using a Rheodyne injector, Model # 7125 (Rheodyne Inc., Cotati, CA USA) with a 20 mL sample loop. A fixed-wavelength (245 nm) UV-detector, Model #440 (Waters, Inc. Milford, MA, USA) was used for monitoring the seven drugs. The data were recorded on a Hewlett-Packard integrator, Model # 3390A (Hewlett-Packard, Palo Alto, CA, USA).

The HPLC flow rate used was 1.0 mL/min and the column utilized in this

system was 5 cm x 4.6 mm ID, 5 μm, Supelcosil (Supelco, Inc. Bellefonte, PA, USA). The mobile phase was composed of methanol, acetonitrile and 20 mM ammonium formate controlled by a Waters automated gradient controller, Model # 680 (Waters, Inc. Milford, MA, USA). The linear gradient program of 19 minutes was initiated with the low organic solvent composition (5%) while the final mobile phase composition was mainly the mixture of methanol and acetonitrile (95%).

Quantitation of the seven drugs recoveries by SFE from tissue samples was accomplished by comparison of HPLC peak areas between the analytical standards of the drugs and fortified tissue samples (100 ppb of the each drug). Triplicate samples were used for recovery and repeatability studies.

Supercritical Fluid Extraction-Supercritical Fluid Chromatography (SFE/SFC)

The micro-scale Extraction System Series 600 based on cryogenic solute focusing combined to capillary Supercritical Fluid Chromatography Series 600 (Lee Scientific, Inc., Salt Lake City, UT, USA) was used to determine the optimum extraction time. Samples were placed into the 2 mL extraction cell (Valco Instruments Co. Inc., TX, USA). During the SFE procedure the temperature of the SFC oven was maintained at 60°C, and the cryofocusing T-piece located inside the SFC oven was cooled by the external CO_2 tank. The temperature of extraction cell was 60°C. Three successive 20 minute extractions were accomplished from the same sample at 400 atm (1 atm = 0.10132 MPa). After the extraction, the 10-port valve was switched to the column position and the normal SFC run was commenced. A SB-cyanopropyl-50, 50 mm x 10 m fused-silica capillary column coated with a 25 μm film thickness (Lee Scientific, Inc., Salt Lake City, UT, USA), was used for the separation. SFC-grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, USA) was used as the extraction fluid as the mobile phase. The oven temperature was kept constant at 120°C. The frit restrictor was used for the mobile phase restriction providing an average linear velocity of 1.2 cm/sec (at 100 atm). The temperature of the flame ionization detector (FID) was 375°C. The separation was accomplished by applying a pressure program as follows: 100 atm initial pressure held constant for 10 min, then ramped to 415 atm at a constant ramp rate of 10 atm/min.

Mass Spectrometry (MS)

A Sciex TAGA 6000E tandem triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with a standard atmospheric pressure ionization (API) source was used in these experiments. The heated pneumatic nebulizer interface was used for combined high performance liquid chromatography/mass spectrometry (LC/MS) experiments. To produce atmospheric pressure chemical ionization (APCI) mass spectra the mass ranged scanned was from m/z 250-450 while the $[M+H]^+$ ion was monitored for each individual solute in the selected ion monitoring (SIM) mode.

RESULTS AND DISCUSSION

The goal of this work was to investigate the use of supercritical fluid extraction (CO_2) for the isolation of growth promoting anabolic steroids directly from fortified bovine tissue samples and to determine them by high performance liquid chromatography coupled with UV or mass spectrometry detection.

The present study was divided into two phases. The first phase of investigation involved studies to optimize the extraction parameters (temperature, pressure, time, restrictor) versus recovery and repeatability. The matrix was an inert filter paper or fortified freeze-dried bovine muscle tissue sample. Results were obtained by HPLC-UV or SFC-FID. The second set of studies consisted of SFE from three-phase liquid-liquid liver extracts and from freeze-dried muscle tissue sample monitored by HPLC/MS. In order to get satisfactory recovery and repeatability results, a good reference material was required. Spiking directly onto the sample can cause too high recoveries.[35] Therefore, spiked samples were prepared according to Ramsey et al.[33] The drugs were first diluted in dichloromethane and the solution was then thoroughly mixed with freeze-dried matrix

SFE conditions were optimized for the seven drugs by segnentially varying the following parameters. Temperature: 50, 60, 80 and 100°C; extraction pressure: 250, 300, 350 and 400 atm (1 atm = 0.10132 MPa); extraction time: 10, 20, 30, 40, 50, 60 and 120 min; restrictor: Guthrie-type (homemade), 20 cm long x 15 or 21 mm ID linear. The optimization was done by keeping three of the variables constant while varying the other. The collected analytes were

then measured by HPLC with UV or MS detection. SFC-FID was also used to determine the optimum extraction time.

Table 1

Effect of Temperature and Restrictor on the Recovery of the Drugs Investigated

Compound	60°C 21 mm a) %	80°C 21 mm a) %	80°C 15 mm a) %	100°C 15 mm b) %	100°C 21 mm b) %
DEX	59	93	98	37	65
TBOH	69	109	102	110	68
TACA	70	80	68	58	69
ZER	77	85	80	69	72
DES	68	84	100	67	64
MDP	74	76	68	55	55
MGA	71	77	80	63	61

a) extraction time: 60 min; pressure: 400 atm.
b) extraction time: 30 min; pressure: 400 atm.

In the optimization studies the stock solution (500 ng of each compound) was deposited onto a piece of filter paper located in the extraction vessel. Table 1 and Figure 2 show the results from optimizing SFE conditions for recovery of the seven drugs. SFE/SFC-FID data shown in Figure 2 show that the 20 min extraction is not sufficient to extract all the analytes and at least 40 min is required. A 60 min extraction produced about 80% recoveries. The three extracts were carried out successively from the same sample. Table 1 shows that, by increasing the extraction temperature to 80°C, the recoveries (e.g. DEX) were also increased compared to the results obtained at 60°C. At the same time, however, the fat contained in the samples caused difficulties with UV-detection. In this study alumina or other absorbents were not used for retaining fat in the extraction vessel. Because of this it might be that the recoveries at 100°C were even lower than at 80°C even through the extraction time at 100°C was only 30 min. The effect of pressure on the SFE recoveries of seven drugs was also conducted. Although the data are not presented, it was

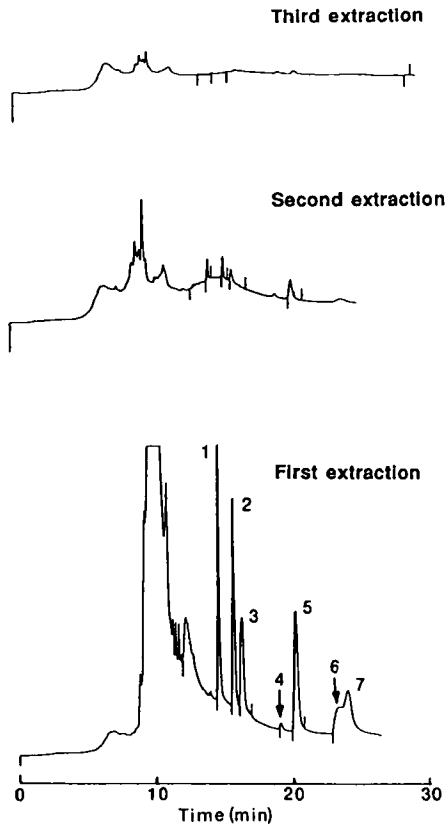

Figure 2. SFE/SFC-FID chromatograms of spiked (500 ng of each compound) three-phase liquid-liquid extract residue of bovine muscle after three successive 20 min extractions at 400 atm and 60°C.
1 = melengestrol acetate, 2 = medroxyprogesterone, 3 = trenbolone, 4 = diethylstilbestrol, 5 = dexamethasone, 6 = zeranol, 7 = triamcinolone acetonide.

found out that 400 atm gave the best recoveries for all the drugs. The pressure limit of the system was 415 atm.

To maintain the pressure in a supercritical fluid extraction system, restrictors have to be used at the outlet. This restrictor is often a piece of fused-

silica capillary tubing of 5-25 μm i.d.. These might, however, cause plugging when the sample matrices contain high concentrations of water or fat. To avoid this problem heated variable restrictors have been designed which "sense" the plugging before it happens.[41] Despite our use of linear restrictors in this work plugging rarely occurred. This may be due to our use of freeze-dried samples prior to SFE. The restrictor type also has an effect on recoveries. The linear restrictor, 20 cm length x 21 μm i.d. produced the best recoveries ranging from 57 % (DEX) to 112 % (TBOH).

Improved results were obtained if the restrictor was prerinsed with a small amount of methanol (100 mL). This was especially true with TACA, ZER, DES and MDP, where recoveries improved approximately 10% by prerinsing the restrictor. In practice this procedure, however, became impractical and was discontinued. The Guthrie-type restrictor plugged easily, especially when tissue samples were used even though it gave quite good results with pure standards.

To determine the optimum conditions for isolating all seven compounds, a compromise must be met. The optimized conditions were as follows: extraction time 60 min, extraction temperature 60°C, extraction pressure 400 atm with a linear restrictor 20 cm x 21 μm i.d. Using these optimum conditions, the recoveries and repeatabilites shown in Table 2 were obtained. SFE was done from spiked (100 ppb of each drug) freeze-dried bovine muscle tissue sample. It is shown that repeatability and recovery were poor with polar drugs such as dexamethasone (DEX), triamcinolone acetonide (TACA) and zeranol (ZER) with recovery varying from 44% to 58%.

The more non polar drugs including melengestrol acetate (MGA), medroxyprogesterone (MDP) and diethylstilbestrol (DES) recoveries were quite good varying from 83 to 91%. The overall recovery was 69% with an R.S.D. of 19.7%. Trenbolene (TBOH) could not be measured in these experiments, due to interference from coeluting compounds. One reason for the variable recoveries may be due to the position of the extraction cell. In this work it was positioned horizontally rather than the preferred vertical position to achieve better recoveries and especially more repeatable results. At the 100 ppb concentration level repeatability was not acceptable, but at 1 ppm concentration level the R.S.D. was 5%. Recovery of DES was only 34.0 % which is quite consistent with the results of Karlsson et al.[36]

Table 2

Recovery and Repeatability of SFE from Freeze-Dried Muscle Tissue Sample. Fortified with 100 ppb of the Drugs.

Compound	Recovery %	R.S.D. %	n
DEX	57	13.6	3
TACA	44	17.7	3
ZER	58	12.1	3
DES	83	34.0	3
MDP	91	11.2	3
MGA	83	29.5	3
$\bar{x}$	$\overline{69}$	$\overline{19.7}$	

$\bar{x}$ = mean of recoveries

Extraction of pure steroids from the filter paper using 5% of MeOH as a modifier gave a recovery of *ca.* 50% for DEX. It should be noted that individual sample matrices affect the extraction parameters.[38] In this work, the matrix was freeze-dried bovine muscle. The matrix effect is clearly shown in Figure 3, where A) shows the HPLC-UV chromatogram after SFE of pure drugs from filter paper, B) shows the same trace after SFE of spiked freeze-dried muscle tissue sample and C) shows the HPLC-UV chromatogram after SFE of spiked three-phase liver extract. Even if MeOH is not used as a modifier fat and fat-related compounds coelute and interfere with the interpretation of the HPLC-UV chromatogram especially in the case of the liver sample.

Quite often analytes are collected directly into organic solvent,[42] but they can be trapped first on sorbents and after that diluted with a small amount of an organic solvent.[43] Analytes could be collected also directly into a SPE mini-column for further sample preparation.[44] In this study the compounds were collected directly into MeOH to simplify the collection system. No loss of the target compounds appeared to occur using this collection procedure.

Figure 4 A), B) and C) show the HPLC/API/MS analysis of the SFE extract from freeze-dried muscle tissue sample and three-phase liquid-liquid extract

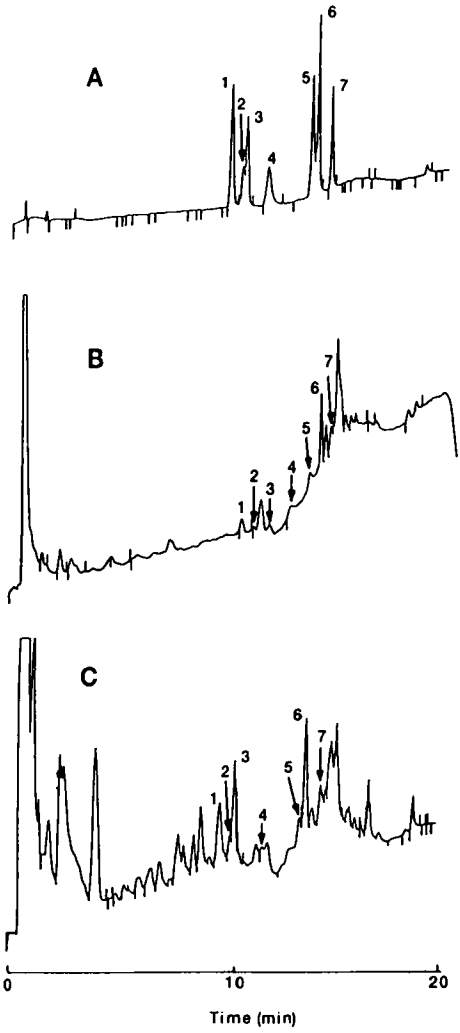

Figure 3. HPLC-UV chromatograms of A) SFE of the stock solution (20 ng of each compound) in filter paper, B) SFE from spiked (500 ppb) freeze-dried muscle tissue sample, C) SFE from three-phase liquid-liquid extract from liver residue spiked to 100 ppb. SFE conditions: 60 min/ 60°C/400 atm.
1 = dexamethasone, 2 = trenbolone, 3 = triamcinolone acetonide, 4 = zeranol, 5 = diethylstilbestrol, 6 = medroxyprogesterone, 7 = melengestrol acetate.

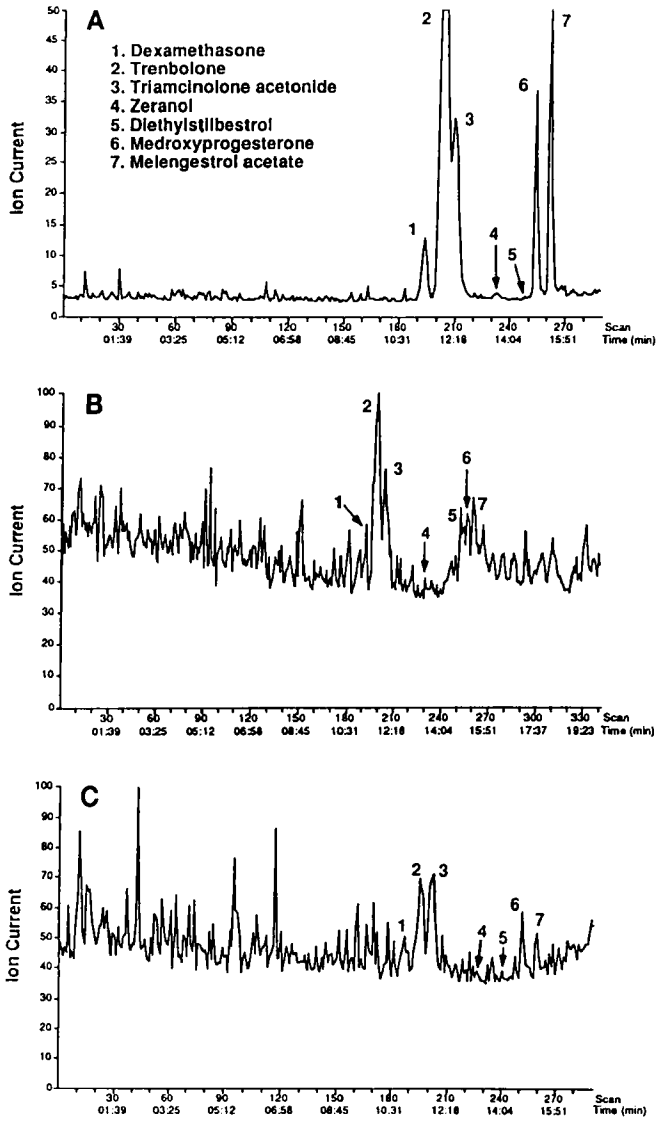

Figure 4. (SIM)HPLC-APIMS analysis of A) freeze-dried muscle tissue sample spiked to 100 ppb, B) three-phase liquid-liquid liver extract spiked to 100 ppb, and C) three-phase liquid-liquid liver extract spiked to 10 pbb of the seven drugs.

under selected ion monitoring (SIM) conditions. In Figure 4A) the muscle sample was spiked with the seven drugs at the level of 100 ppb and in Figure 4B) the three-phase liver extract residue was spiked at the same level. The liver sample (three-phase extract) in the study of Figure 4C) was fortified with 10 ppb of the seven drugs. As expected the liver sample was more complex than the muscle sample. It appears that the 100 ppb level is the practical detection limit for HPLC/MS analysis under these experimental conditions for both samples. The 10 ppb level (Figure 4C) was reached, but the S/N ratio was unsatisfactory. The identification of zeranol and diethylstilbestrol was less certain, but for trenbolene, triamcinolone acetate, medroxy progesterone and melengestrol acetate the 10 ppb level could be detected. In previous literature reports the detection limit has been reported only to 1 ppm for diethylstilbestrol by SFE/SFC/MS/MS with no data from recovery and repeatability.[33]

CONCLUSIONS

The goal of this work was to study the feasibility of supercritical fluid extraction using CO_2 as an extraction fluid to isolate some growth promoting compounds directly from bovine tissue sample and to analyze them by (SIM)HPLC/API/MS. Conventional methods for the determination of these analytes exist, but they are time-consuming with excessive use of organic solvents and low recoveries.

The results of this work show that (SIM)HPLC/MS provides an alternative method for monitoring selected anabolic compounds isolated from tissue samples by SFE. The 100 ppb level was the practical detection limit for all the drugs investigated while the 10 ppb level could be reached for some. The disadvantage of the single quadrupole mass spectrometer operation is that there is little structural information available. However, for target analyses single quadrupole instruments operated under selected ion monitoring (SIM) conditions can offer a fast and reliable method. For quantitative identification tandem mass spectrometry offers additional analytical capabilities.

Supercritical fluid extraction has some desirable analytical advantages so that this approach still deserves more investigation. One should not expect SFE to replace all other forms of sample preparation steps. Some sample cleanup may still have to be done after or before supercritical fluid extraction. It seems, however, that modern SFE instrumentation with multiple extraction cells, higher pressure limits and modifier addition systems will provide more

versatility for the isolation of target compounds from complex biological samples.

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