Feasibility of On-Line Supercritical Fluid Extraction of Steroids from Aqueous-Based Matrices with Analysis via Gas Chromatography–Mass Spectrometry

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Abstract

The solubility of testosterone, boldenone, androstenone, etiocholanolone, and epitestosterone are measured in pure supercritical CO_2. Testosterone exhibited the highest solubility in supercritical CO_2. The solubility of all steroids except epitestosterone increased by one order of magnitude with increasing pressure from 100 to 400 atm. Epitestosterone had the lowest solubility in supercritical CO_2 and its solubility was not affected by pressure. The extraction efficiency of steroids from an aqueous saline environment exceeded 95%. Because of the partial solubility of water in supercritical CO_2, the addition of a moisture trap after the aqueous vessel is necessary to prevent the plugging and deterioration of the gas chromatographic (GC) column. It is demonstrated that on-line supercritical fluid extraction–GC–mass spectrometry is feasible for the quantitative extraction and analysis of steroids from both saline and urine solutions. However, it is determined that the adsorbent vessel filled with Hydromatrix is not sufficient to trap all the moisture, and after 3 to 4 extractions, the GC column efficiency lowered.

Introduction

Anabolic steroids are abused especially by adolescents and competitive-sport athletes as physical performance-enhancing agents. Considerable health risks in humans ranging from cancer to coronary disease has been attributed to the abuse of these agents. The illegal use of anabolic steroids is not limited to humans. For example, they are widely used as growth promoters in meat-producing animals (1).

The lack of simple, rapid, and sensitive techniques for the extraction, separation, and detection of anabolic steroids in the body has been of interest to government and health laboratories. The extraction, recovery, and analysis of these steroids require extreme care and long hours and generates large amounts of hydrocarbon and chlorinated waste solvents. Screening for the presence of anabolic steroids in the body is commonly conducted by urinalysis (2,3). General analytical techniques involve the treatment of urine with β-glucuronidase in order to cleave the conjugates of acids of carbohydrates, initial clean-up via reversed-phase solid-phase extraction (SPE), and further extraction into an organic solvent such as methylene chloride. The extracts are then derivatized with a suitable reagent that enables the application of gas chromatographic (GC)–mass spectrometric (MS) methods. These methods usually require extensive sample preparation and use of organic solvents. Replacing the current methods with a simple and less tedious and costly procedure would be a significant advantage for government and private laboratories.

On-line supercritical-fluid extraction (SFE)–GC is a potential solution to the problem of the sample preparation, sample handling, and analysis of drugs in different matrices. The on-line coupling of extraction and separation techniques can be highly beneficial. The resulting process is far less labor intensive than off-line analysis because it is not necessary to collect, concentrate, and inject the extract. The opportunity for the sample to become contaminated or for analytes to volatilize or degrade is also minimized.

Burford et al. (4) have noted that extractions with supercritical fluids are ideal in many ways for on-line coupling. Because these fluids can have liquid-like solvent strengths, they extract nonvolatile compounds particularly well from a wide variety of matrices. Under ambient conditions, these fluids become gaseous and hence are easy to eliminate from the on-line analysis system. In addition, SFEs tend to be more selective than extractions with conventional solvents.

The status of SFE–GC has been thoroughly reviewed in a recent report (4). Table I summarizes the on-line (internal trap) SFE–GC studies that were found by our research group in a recent search of chemical abstracts. This is believed to represent the majority of

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studies that have been published on the subject. In some individual cases, extraction parameters were not provided. Researchers using internal trapping have generally used one of two approaches. The first is to depressurize the effluent in a conventional injection port with the split valve open (referred to in Table I as the internal/split approach) (5–8). The alternative to decompression in the injection port is to place the restrictor directly into the analytical column (referred to as the internal/on-column approach) (9–16). There have been three studies that have used this approach and reported quantitative transfer of the analytes (14–16).

Table I. Survey of On-Line SFE–GC Studies

	Vessel volume (mL)	Extraction flow rate (mL/min)	Dynamic extraction time (min)	Trapping temperature (°C)	Reference
Internal/	0.41	n/a	7	30	3
split	2.5	0.6	10	–50	4
	n/a	1	10	–50 to –25	5
	2	0.16	n/a	–30	6
Internal/	~0.5*	0.13*	10	0	7
on-column	0.1	0.2	10	–30	8
	0.2–0.3	n/a	time	0	9
	0.17*, 0.75*	≤ 0.4	time	–30	split: 3 s 10 split: 10 s
	0.015	0.01*	1	30	11
	0.3	n/a	10–15	–30 to –5	12
	0.1	n/a	10	5	13
	0.3	0.4	10	–30 to –5	14

* Value was calculated.

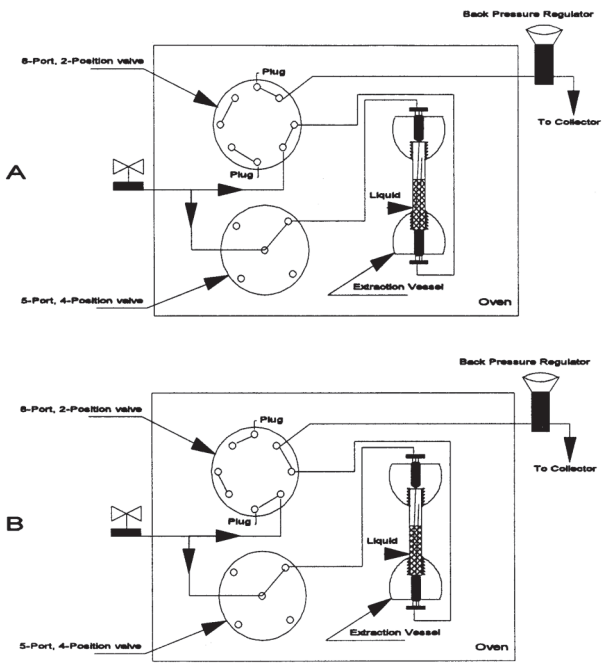


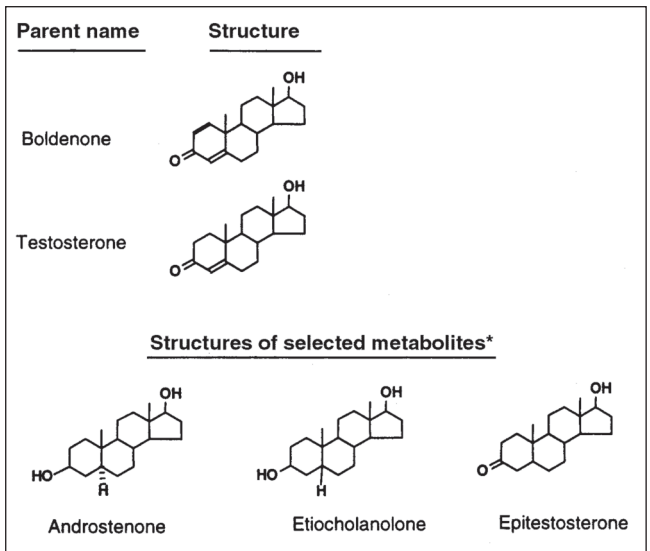
Figure 1. SFE of liquid in static mode (A) and SFE of liquid in dynamic mode (B).

Although the technique of off-line SFE has been demonstrated as capable of meeting these ideal conditions for the analytical extraction of solid and liquid matrices, an on-line coupled SFC–GC system that takes full advantage of these conditions for liquid matrices has lagged in development. It can be argued here that an SFE system coupled directly with GC may be a logical candidate to serve as a hyphenated system for the direct extraction and analysis of anabolic steroids in aqueous matrices. The following issues relevant to this hypothesis are addressed in this report, namely: (*a*) to determine the solubility of selected anabolic steroids in supercritical CO_2 and (*b*) to demonstrate that pure supercritical CO_2 can quantitatively extract drugs and their metabolites from spiked saline and urine solutions.

Experimental

An Isco-Suprex (Lincoln, NE) Prepmaster equipped with an Accutrap and variable flow restrictor was used for all extractions. A schematic of the off-line SFE system modified for the extraction of analytes from a liquid matrix in both the static and dynamic mode is shown in Figure 1. Liquid–fluid extractions were accomplished using a 10-mL extraction vessel that was modified in a similar fashion as reported in previous studies (17–19). The stainless steel frits (which were part of the fitting seals) were removed so that 1/16-inch stainless steel tubing (0.01-inch i.d.) could pass through either end of the vessel. The stainless steel tube extended within 1 cm of the top and bottom of the vessel. A standard 1/16-inch fitting and ferrule (Valco, Houston, TX) were used to obtain a reliable seal.

In the static mode, supercritical CO_2 entered the extraction vessel from both the inlet and outlet of the extraction vessel (Figure 1A). This design prevented the entrance of water into the outlet tube during vessel pressurization, which could have caused restrictor plugging. The system was designed so that after equilibrium (i.e., static extraction), the 8-port/2-position valve could



* R. Messe et al. *J. Chromatog. (Biomed. Applic.)* **489:** 23–50 (1989).

Figure 2. Structure of studied steroids.

Table II. Solubility of Different Steroids in Helium-Saturated Supercritical CO_2*

Pressure (atm)	Etiocholanolone	Boldenone	Epitestosterone	Androstenone	Testosterone
100	7.27E – 05	3.44E – 05	4.06E – 05	7.86E – 05	8.76E – 05
200	9.00E – 05	5.47E – 05	3.77E – 05	7.19E – 05	1.28E – 04
300	1.25E – 04	7.46E – 05	4.19E – 05	1.09E – 04	1.50E – 04
400	1.63E – 04	9.19E – 05	4.53E – 05	1.51E – 04	2.02E – 04

*Temperature, 40°C; units for solubility, µg/µL.

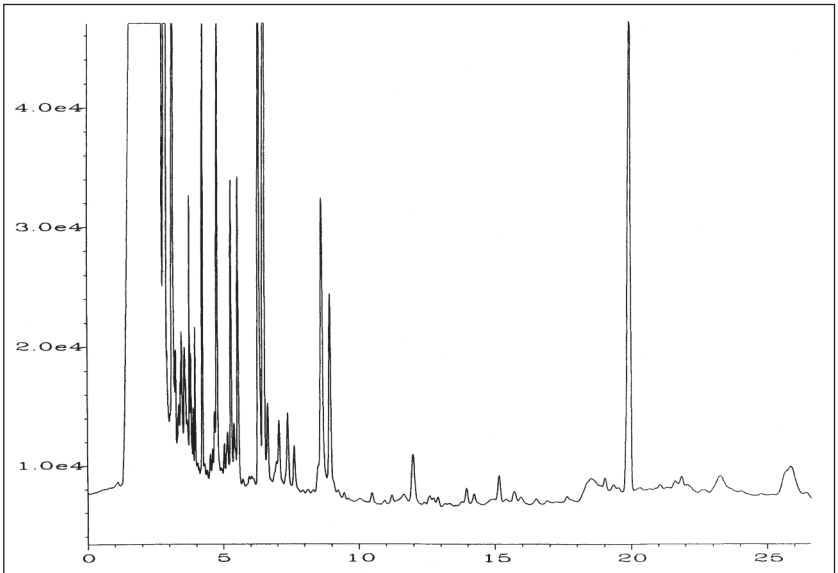


Figure 3. SFE–GC–FID trace of the blank saline solution. Extraction conditions: 400 atm, 40°C, 2-min static, 20-min dynamic at flow rate of 0.2 mL/min. GC conditions: oven temperature during extraction, 50°C; GC injection port temperature, 280°C; GC temperature programming after extraction, 50 to 200°C at rate of 50°C/min, then from 200°C to 270°C at rate of 4°C/min, and then held for 5 min at 270°C. Column: RTx–5 (30 m × 0.5 mm, 3.0-µm df)

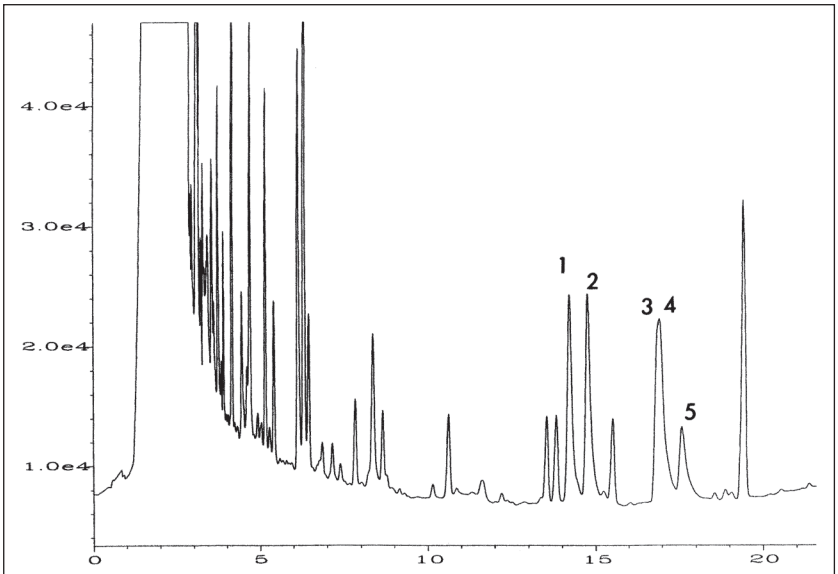


Figure 4. SFE–GC–FID chromatogram of extracted steroids spiked into the saline solution. Etiocholanolone, 1; boldenone, 2; epitestosterone, 3; androstenone, 4; and testosterone, 5. SFE and GC conditions are the same as Figure 3.

be switched to the dynamic mode (Figure 1B). In this mode, supercritical CO_2 passed only through the 5-port/4-position valve into the vessel. The extracted analytes were collected in a 10-mL flask partially filled with methanol. The final volume of the trapped extract was adjusted to 10 mL with additional methanol at the end of the extraction.

A similar system was used for the on-line SFE–GC experiments. Previous studies have shown that water has a solubility of approximately 0.3% (w/w) in supercritical CO_2 (20). Therefore, in order to prevent the transfer of CO_2 extractable moisture into the GC column, a 5-mL vessel filled with an adsorbent (Hydromatrix) was placed directly after the extraction vessel without any modification.

A Hewlett Packard (Little Falls, DE) model 5890 series II GC equipped with a model 5971A mass-selective detector or a flame ionization detector (FID) or both was used. A DB-5 (J&W Scientific) capillary column (30 m × 0.25 mm, 0.25-µm df) and a Rtx-5 (Restek) capillary column (30 m × 0.53 mm, 3.0-µm df) were used for analytical separations. Samples from off-line extractions were injected onto the GC column using a Hewlett Packard model 7673 autosampler. The GC oven temperature program was set to start at 200°C for 1 min then increase at 4°C/min to 270°C and hold for 5 min. For all on-line SFE–GC experiments, the oven temperature was set to 50°C during the analyte collection. After collection, the oven temperature was ramped to 200°C at 50°C/min, followed by an increase at 4°C/min to 270°C and then held for 5 min. The injector temperature was set to 280°C and the MS interface temperature was 270°C. Masses were scanned from 33 to 500 amu. For the analysis of all off-line extracts, 1 µL was injected (splitless) onto the GC column.

A Suprex model 200A (Lincoln, NE) supercritical fluid chromatographic (SFC) pump, a Waters (Milford, MA) model 6000 high-performance liquid chromatographic (HPLC) pump, a Micropump (Vancouver, WA) model 1805R-346 recirculating pump, a 6-port/2-position valve, a 4-port/2-position valve, and a Kratos Spectroflow model 757 UV-absorbance detector and evaporating light-scattering detector (ELSD) (Alltech Associate, Deerfield, IL) were used for on-line solubility measurements. A 0.5-mL stainless steel extraction vessel (Isco-Suprex) was used to contain the analyte whose solubility was to be determined. The apparatus and positions of the valves at each step for measuring the solubility of the analytes under supercritical conditions have been previously described (21).

Testosterone, boldenone, androstenone, etiocholanolone, and epitestosterone (Figure 2) were purchased from Sigma Chemical (St. Louis, MO).

A stock solution of each steroid at 1 µg/µL was prepared in methanol. HPLC-grade solvents were purchased from EM Science (Gibbstown, NJ). The saline solution was obtained from a local pharmacy. The Hydromatrix drying agent was obtained from Varian (Harbor City, CA). SFE/SFC-grade CO_2 pressurized with 2000 psi helium (Air Products and Chemicals, Allentown, PA) was used for all SFE and solubility studies.

For the off-line extractions of steroids from a liquid matrix, 5 mL of saline solution was added directly to the modified extraction vessel that was discussed previously. Then, 100 µL of the previously prepared steroid mixture (1 µg/µL) was spiked directly into the saline solution. The system was operated at either 200 or 400 atm and 40°C for a 10-min static and 30- or 45-min dynamic period using pure CO_2 at a liquid flow rate of 2 mL/min.

For all on-line SFE–GC experiments, 5 mL of saline solution was added directly to the modified extraction vessel. Then, 3 µL of the steroid mixture was spiked directly into the saline solution. The small trapping capacity of the on-line trap necessitated a much smaller mass spike. The system was operated at 400 atm and 40°C for 2 min at static conditions and then for a 20-min dynamic period using pure CO_2 at a liquid flow rate of 0.2 mL/min. For all on-line SFE–GC experiments, the restrictor was placed directly into the GC injection port. Preliminary studies showed that 50°C was sufficient to focus the analytes on the tip of the capillary column.

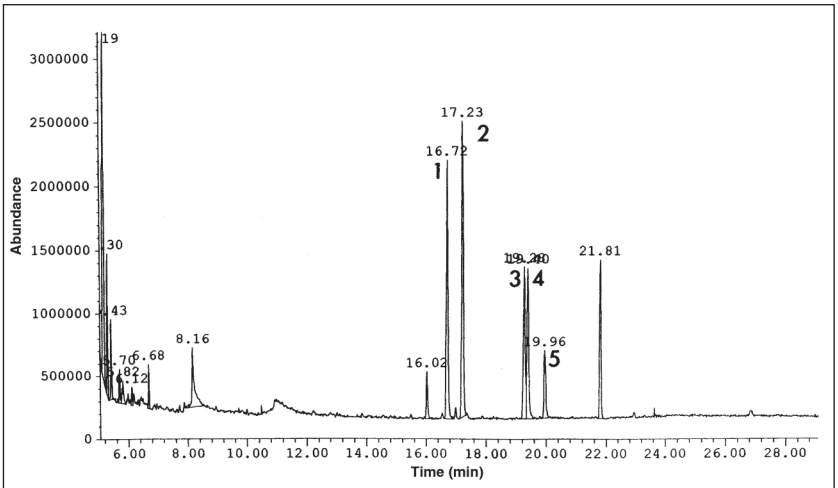


Figure 5. SPE–GC–MS of extracted steroids from saline solution. SFE conditions same as Figure 3. GC conditions: column DB-5 (30 m × 0.25 mm, 0.25-µm df), remaining conditions the same as Figure 3.

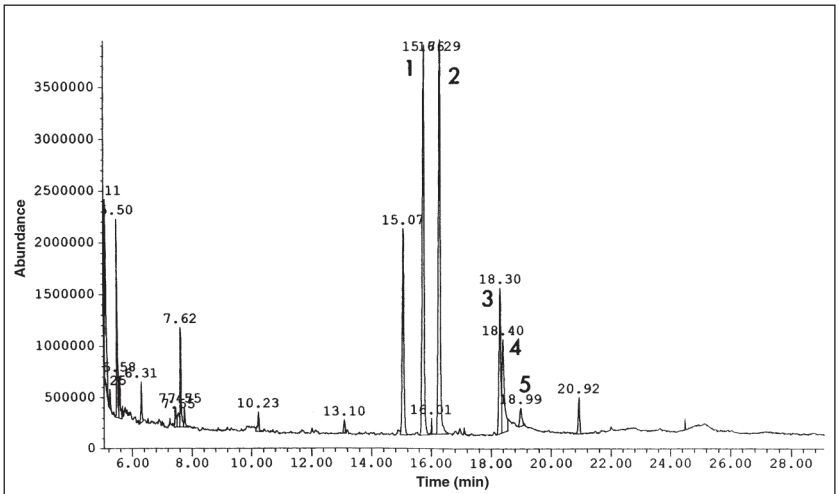


Figure 6. SFE–GC–MS of extracted steroids from urine–saline solution (50:50). Extraction and GC conditions are the same as Figure 5.

Results and Discussion

Solubility measurements

Our solubility measurement system was first validated by measuring the solubility of anthracene in supercritical CO_2 with a 2000-psi helium head pressure at 40°C in the pressure range of 100 to 340 atm. Our results (21) compared favorably with previously reported data in the literature (22). Then, the solubility of boldenone, testosterone, androstenone, etiocholanolone, and epitestosterone in supercritical CO_2 at different pressures (100–400 atm) and a constant temperature (40°C) was measured. Because of the lack of a UV chromophore for boldenone, androstenone, etiocholanolone, and epitestosterone, ELSD was used as the detection method for the solubility measurements. Table II shows the measured solubility of each steroid versus CO_2 pressure. Testosterone had the greatest solubility in supercritical CO_2 regardless of pressure. The solubility of epitestosterone had the least solubility and did not change significantly with increasing CO_2 pressure. Our results showed that testosterone, boldenone, andro-stenone, and etiocholanolone solubility doubled by increasing the pressure from 100 to 400 atm at 40°C. Another research group has reported similar results for the solubility of testosterone and cholesterol (23).

Sufficient solubility of each steroid was demonstrated so that a failure to extract from liquid would not be because of a lack of solubility in supercritical CO_2. Furthermore, the extraction efficiency of all steroids spiked on solid Hydromatrix at only 200 atm was found to be approximately 100% using pure CO_2.

Off-line SFE recovery of steroids from saline solution

Initially, the extraction of steroids spiked into saline solution using pure CO_2 at 200 and 400 atm and 40°C was attempted. In this part of the study, no H_2O adsorbent trap was placed in-line after the extraction vessel. Increasing the pressure from 200 to 400 atm caused extraction efficiencies of analytes to increase by more than 10%. Extraction efficiencies greater than 80% were obtained for all analytes regardless of the pressure. At a higher pressure (400 atm), the relative standard deviation (RSD) for the recovery of analytes varied from 1 to 3%.

In comparison, extraction efficiencies of the same steroids that were spiked into the saline

solution were obtained with a second vessel (5 mL) filled with an adsorbent (Hydromatrix) placed after the liquid vessel. Because it is known that water can remove and damage the stationary phase in a GC column, this experiment was necessary for future work regarding the use of the on-line SFE–GC. Extraction efficiencies of steroids at 400 atm, 40°C, and a 45-min dynamic period with an adsorbent trap in-line were near 100% for all steroids. The RSD for recovery of these steroids varied from 2 to 5%.

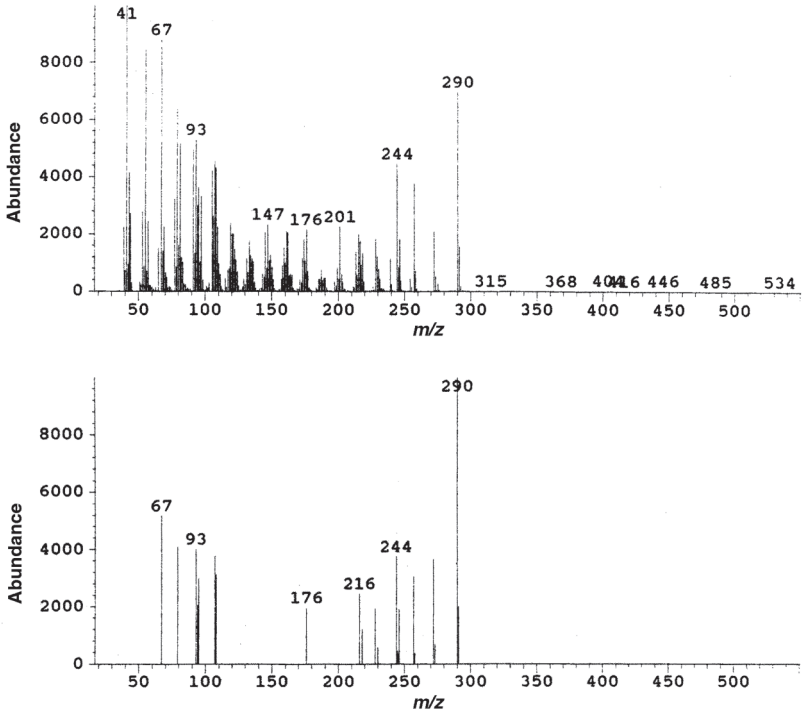


Figure 7. Mass spectrum of component eluting as peak 1 in Figure 6.

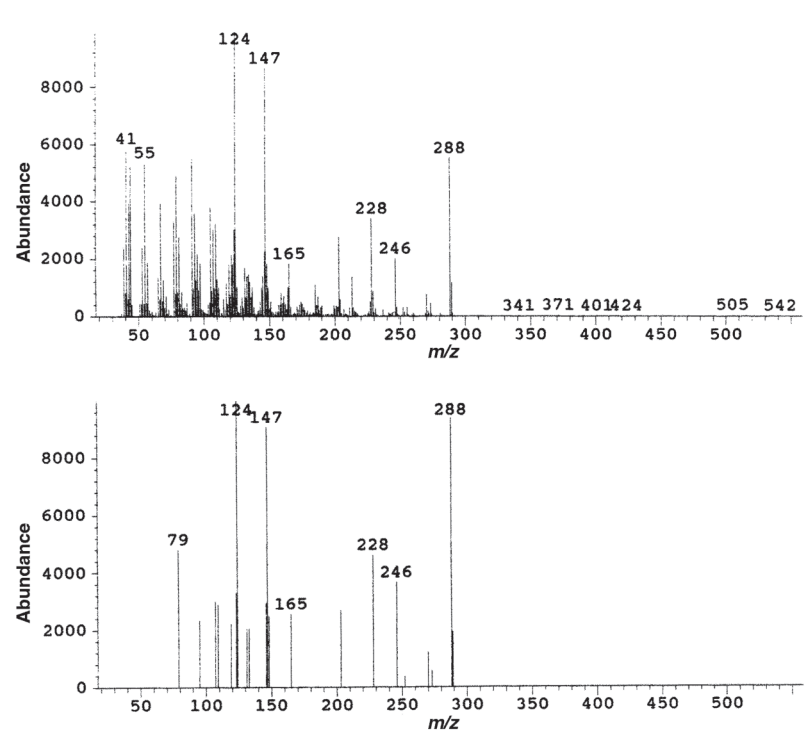


Figure 8. Mass spectrum of component eluting as peak 3 in Figure 6.

On-line SFE–GC of steroids from saline solution

SFE–GC–FID extraction of an empty vessel using a 30-m × 0.530-mm capillary column was initially performed. Results showed the system to be free of any impurities besides those that are normally found in liquid CO_2. Then, the on-line SFE–GC–FID of a saline solution spiked with steroids was performed after the SFE–GC of a blank saline solution was initially ascertained. For this purpose, 5 mL of the saline solution was transferred into the aqueous vessel, and another 5-mL vessel filled with Hydromatrix was placed after the aqueous vessel. Figure 3 shows the SFE–GC–FID trace of the blank saline solution. It is believed that the observed GC peaks were because of the extractable analytes that were added to the saline solution as stabilizers (e.g., ascorbic acid).

The on-line SFE–GC–FID of a saline solution spiked with steroids was then obtained under the exact extraction and analysis conditions. Figure 4 shows the on-line SFE–GC–FID chromatogram of extracted steroids spiked into the saline solution. As can be observed, epitestosterone and androstenone (peaks 3 and 4) were eluted together under these conditions, and the remaining analytes were separated from each other with some peak tailing (which may be a result of low column efficiency). It is believed that the adsorbent trap filled with Hydromatrix was not totally sufficient for trapping all the extracted moisture and the water that was mechanically inadvertently transferred, thus causing deactivation of the column and lower column efficiency. A second extraction of the same aqueous sample without recharging the extraction or trapping vessel showed that less than 10% of the analytes remained in the vessel after the initial extraction.

In order to confirm the peak assignments, MS detection was put in place of FID. To begin with, the on-line SFE–GC–MS of a blank saline solution was obtained. No major peaks were observed after 6 min, and those that eluted before 6 min did not interfere with our analysis. Then, the on-line SFE–GC–MS of a steroid spiked saline solution was performed. Again, 3 µL of the steroid standard was spiked into 5 mL of the saline solution that was added to the liquid extraction vessel. After pressurization, analytes were extracted and collected directly at the head of the GC column. Following the 20-min extraction and collection procedure, the GC was temperature-programmed as previously stated.

Figure 5 shows the on-line SFE–GC–MS chromatogram of steroids from saline solution. A second extraction of the same sample without

recharging the vessel showed that more than 30% of the analytes remained in the vessel. Also, the loss of column efficiency was believed to be because of moisture present in the CO_2, which causes column deactivation and removal of the stationary phase. This problem was usually observed after 3 or 4 extractions. The effect and its impact could be eliminated by cutting away the first meter of the column. However, after another 3 to 4 extractions, the same problem recurred.

On-line SFE–GC–MS of steroids from urine sample

Finally, the on-line extraction and analysis of steroids from a urine sample using SFE–GC–MS was obtained. For this experiment, a mixture of urine–saline solution (50:50) was prepared. The urine sample was obtained from a steroid-free source. The extraction and analysis of the unspiked urine sample confirmed the absence of any steroid. Then, a fresh sample of urine–saline solution was spiked with 3 µL of the steroid mixture. The sample was extracted for a 2-min static and 20-min dynamic period at 400 atm and 40°C and a flow rate of 0.2 mL/min of liquid CO_2. The extracted analytes were trapped on the head of a GC column at near-ambient temperature (50°C). After extraction, the GC oven was heated using a similar program that was previously discussed.

Figure 6 shows the on-line SFE–GC–MS chromatogram of steroids extracted from urine–saline solution. All steroids were extracted and detected. Figures 7 and 9 show the mass spectra of components that eluted as peaks 1 and 3 in Figure 6, thus confirming the presence of etiocholanolone and epitestosterone. Again, loss of column efficiency was observed after 3 to 4 extractions of the urine samples because of the moisture present. If moisture could be efficiently retained via an in-line trap, then the extraction of steroids from aqueous-based matrices would appear to be an analytical method that needs more serious consideration.

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References

1. A. Paris, L. Dolo, L. Debrauwer, D. Rao, and M. Tergui. Analysis of [H-3] estradiol-17 beta metabolites in calf perirenal fat. *Analyst* **119:** 2623–26 (1994).
2. S. Calvarese, P. Rubini, G. Urbani, N. Ferri, V. Ramazza, and M. Zucchi. Experimental administration of 19-nortestosterone and dexamethasone in cattle—elimination of the two drugs in different biological matrices. *Analyst* **119**: 2611–15 (1994).
3. D. Hooijerink, R. Schilt, E. van Bennekom, and B. Brouwer. Determination of anabolic esters in oily formulations and plasma in husbandry using high performance liquid chromatography and gas chromatography–mass selection detection. *Analyst* **119:** 2617–22 (1994).
4. M.D. Burford, K.D. Bartle, and S.B. Hawthorne. Directly coupled (online) SFE–GC: instrumentation and applications. *Adv. Chromatogr.* **37:** 163–204 (1997).
5. J.H. Raymer and G.R. Velez. Development of a flexible, on-line supercritical fluid extraction–gas chromatographic (SFE–GC) system. *J. Chromatogr. Sci.* **29:** 467–75 (1991).
6. M.D. Burford, S.B. Hawthorne, and D.J. Miller. Analysis of volatile organics by supercritical fluid extraction coupled to gas chromatography, I. Optimization of chromatographic parameters. *J. Chromatogr. A* **685:** 79–94 (1994).
7. S.B. Hawthorne, D.J. Miller, and J.J. Langenfeld. Quantitative analysis using directly coupled supercritical fluid extraction–capillary gas chromatography (SFE–GC) with conventional split/splitless injection port. *J. Chromatogr. Sci.* **28**: 2–8 (1990).
8. J.M. Levy and A.C. Rosselli. Quantitative supercritical fluid extraction coupled to capillary gas chromatography. *J. Chromatogr. Sci.* **28**: 613–16 (1990).
9. S.B. Hawthorne and D.J. Miller. Extraction and recovery of organic pollutants from environmental solids and Tenax-GC using supercritical CO_2. *J. Chromatogr. Sci.* **24:** 258–64 (1986).
10. S.B. Hawthorne, M.S. Kreiger, and D.J. Miller. Analysis of flavor and fragrance compounds using supercritical fluid extraction coupled with gas chromatography. *Anal. Chem.* **60**: 472–77 (1988).
11. M. Lohleit and K. Bächmann. Integrated analysis of solid samples by online supercritical fluid extraction gas chromatography. *J. Chromatogr.* **505**: 227–35 (1990).
12. M. Lohleit, R. Hillmann, and K. Bächmann. The use of supercritical-fluid extraction in environmental analysis. *Fresenius J. Anal. Chem.* **339**: 470–474 (1991).
13. B.W. Wright, S.R. Frye, D.G. McMinn, and R.D. Smith. On-line supercritical fluid extraction-capillary gas chromatography. *Anal. Chem.* **59**: 640–644 (1987).
14. S.B. Hawthorne, D.J. Miller, and M.S. Krieger. Coupled SFE–GC: a rapid and simple technique for extracting, identifying, and quantitating organic analytes from solids and sorbent resins. *J. Chromatogr. Sci.* **27:** 347–54 (1989).
15. S.B. Hawthorne and D.J. Miller. Directly coupled supercritical fluid extraction–gas chromatographic analysis of polycyclic aromatic hydrocarbons and polychlorinated biphenyls from environmental solids. *J. Chromatogr.* **403**: 63–76 (1987).
16. S.B. Hawthorne, D.J. Miller, and M.S. Krieger. Rapid and quantitative extraction and analysis of trace organics using directly coupled SFE–GC. *J. High Resolut. Chromatogr.* **12**: 714–720 (1989).
17. J.L. Hedrick and L.T. Taylor. Quantitative supercritical fluid extraction/supercritical fluid chromatography of a phosphonate from aqueous media. *Anal. Chem.* **61**: 1986–88 (1989).
18. A. Daneshfar, M. Barzega, and M. Ashraf-Khorassani. Supercritical fluid extraction of phenoxy acids from water. *J. High Resolut. Chromatogr.* **18**: 446–49 (1995).
19. M.T. Combs, M. Ashraf-Khorassani, and L.T. Taylor. pH effects on the direct supercritical fluid extraction of phenols from aqueous matrixes. *J. Supercritical Fluids* **9:** 122–27 (1996).
20. W. Francis. Ternary system of liquid CO_2. *J. Phys. Chem.* **58**: 1099–1106 (1954).
21. M. Ashraf-Khorassani, M.T. Combs, L.T. Taylor, F.K. Schweighardt, and P.S. Mathias. Solubility study of sulfamethazine and sulfadimethoxine in supercritical carbon dioxide, fluoroform, and subcritical freon 134A. *J. Chem. Eng. Data* **42**: 636–640 (1997).
22. J.W. Hampson. A recirculating equilibrium procedure for determining organic compounds solubility in supercritical fluid anthracene in CO_2. *J. Chem. Eng. Data* **41:** 97–100 (1996).
23. E. Kosal, C.H. Lee, and G.D. Holde. Solubility of progesterone, testosterone, and cholesterol in supercritical fluids. *J. Supercritical Fluids* **5**: 169–79 (1992).

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