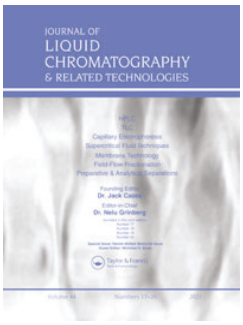

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Streamlined stationary phase selection facilitated by a "sample-plug retention test" in supercritical fluid extraction-supercritical fluid chromatography-mass spectroscopy (SFE-SFC-MS) method development for on-line extraction of anabolic agents

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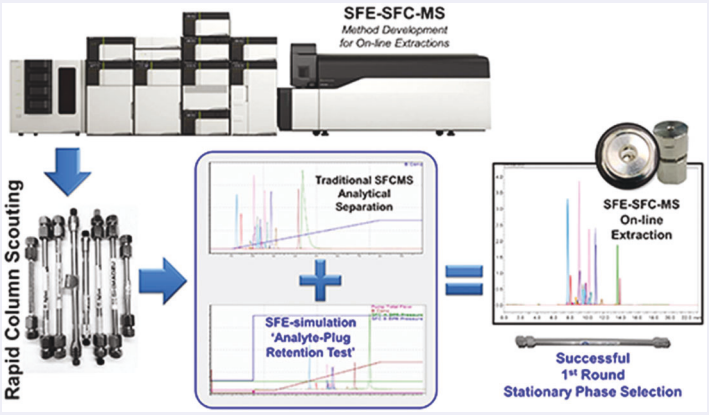
ABSTRACT

A novel method development (MD) approach was established to aid column selection for online supercritical fluid extraction-supercritical fluid chromatography-mass spectrometry (SFE-SFC-MS). An adapted stationary phase (SP) selection procedure was used to expand traditional SFC-MS screening by inclusion of an "Analyte-Plug Retention Test." This test enabled an early evaluation of "trapping" capability for a plug of targeted analytes on the SP. Using an SFE-simulation method, the on-line trapping of an SFE-based extract-plug was mimicked by trapping an SFC-based injection-plug under instrument conditions similar to an actual on-line extraction. Thirteen SPs were screened using the adapted MD procedure as part of the SFC-separation optimization in the development of an on-line SFE-SFC-MS extraction method for anabolic agents (AAS) in anti-doping testing. A cyano SP was chosen for further development, based on chromatographic separation potential and high plug-retention potential for targeted analytes. The chosen column was used in "proof-of-concept" on-line extractions using a generic SFE-SFC-MS method. Successful extract-plug trapping was demonstrated. Retention times and peak areas (4–20%RSD) were reproducible for targeted AAS. Successful first round SFE-SFC-MS SP selection was demonstrated to circumvent tedious re-optimization in future development stages and streamlining of the hyphenated SFE-SFC-MS MD process.

KEYWORDS

Anabolic androgenic steroids; extraction simulation; hyphenated method development; on-line extraction; SFC column scouting

GRAPHICAL ABSTRACT



Introduction

Supercritical fluid extraction (SFE) has become a popular sample preparation technique for biological and environmental samples, by providing a green alternative compared to traditional liquid-based extractions. Toxic organic solvents are replaced with compressed carbon dioxide (CO_2) as the extraction solvent.[1,2] Off-line SFE has been dominantly adopted in large-scale industrial applications. On-line extractions offer many advantages, such as limited sample

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handling, direct analysis, and shorter preparation time.[3,4] Therefore, on-line SFE is particularly suited for labile sensitive samples and/or situations where small sampling sizes are the main focus. Supercritical fluid extraction-supercritical fluid chromatography (SFE-SFC) is especially attractive for on-line coupling, since both techniques utilize dense CO_2 as the main solvent component (i.e., as the mobile phase or extraction solvent). Thus SFE is more feasible for on-line coupling than any other sample-prep technique.[5–7] On-line supercritical fluid extraction coupled with supercritical fluid chromatography-mass spectrometry (SFE-SFC-MS) combines extraction, separation, and detection in a single rapid analysis, while eliminating extensive sample preparation.[8,9] Exhaustive comparisons of on-line versus off-line SFE, as well as reviews of the benefits of coupled SFE systems, to off-line sample preparation techniques (such as liquid-liquid extraction, solid-phase extraction, filtration and protein precipitation, etc.) are provided by the literature.

Historically on-line or in-line SFE-SFC instrumentation have been made up of separate SFE and SFC instruments, which were coupled by an interface or some variety of trap column.[10–14] The design of the coupling between the techniques is a crucial aspect of developing a hyphenated method.[6,15] Recent rejuvenation in the implementation of technical equipment associated with on-line SFE-SFC-MS, using dual back pressure regulators (BPRs), provides an on-line system flow splitter. This eliminates the need for an additional interface between the SFE and SFC and instead utilizes the analytical column to trap a plug of analytes for on-line analysis.[16–18] As a result the past few years have seen resurgence in the use of on-line SFE-SFC-MS with applications in biological,[9] food,[7] and soil samples.[5,8] Nevertheless, difficulties stem from limited knowledge of how the multitude of extraction parameters affect the on-line analysis and detection, and lack of understanding of the variable interactions, continues to limit the routine application of on-line SFE-SFC-MS systems.[19]

Hyphenated SFE-SFC-MS instrumentation

The Shimadzu Nexera UC on-line SFE-SFC-MS instrument (Figure 1) consists of an SFE-extractor, an SFC-chromatograph, and an MS-detector. The SFE and SFC share a solvent delivery system (Figure 1; *Solvent supply and pumping system*), with two pumps. One preconditions and pumps CO_2 as a dense liquid. The second pump delivers degassed organic solvent to increase the polarity of the CO_2. The two liquids are mixed prior to sample introduction. SFC-specific modules (Figure 1; *SFC*) also must include a back pressure regulator [BPR_A], which controls the system outlet pressure. A stirred air oven controls the temperature of the column. Detection is performed using a mass spectrometer (Figure 1; *MS*), which is often facilitated by an additional solvent pump for makeup flow.

The Nexera SFE-SFC-MS system can be operated in either SFC-only mode or alternatively, in on-line extraction-mode. The main difference lies in the source of sample introduction. In SFC-only mode, sample is introduced by a liquid injector (via an injection-valve with external injection-loop [Figure 1; *Injector*]). For on-line extraction mode, sample is introduced by diverting the mobile phase through an extraction chamber (containing a solid sample) using an SFE-extractor unit (via the extraction-valves and extraction-loop [Figure 1; *SFE*]). An overview of the general extraction process is provided in section S_1.1. Supplemental Discussion: General On-line Extraction Process and S_Figure 1.[20] The resulting "extract-plug" is swept by the mobile phase onto the column during one of the extraction steps. This "plug" of extracted analytes must be "trapped"/retained in a narrow band at the head of the column (previous to the start of the analysis).[17,18] Such "plug-retention" becomes a crucial factor in the development of hyphenated methods.

The on-line loading of the extract-plug simplifies the physical coupling of the extraction to the separation technique. Yet, complexities in the hyphenated method

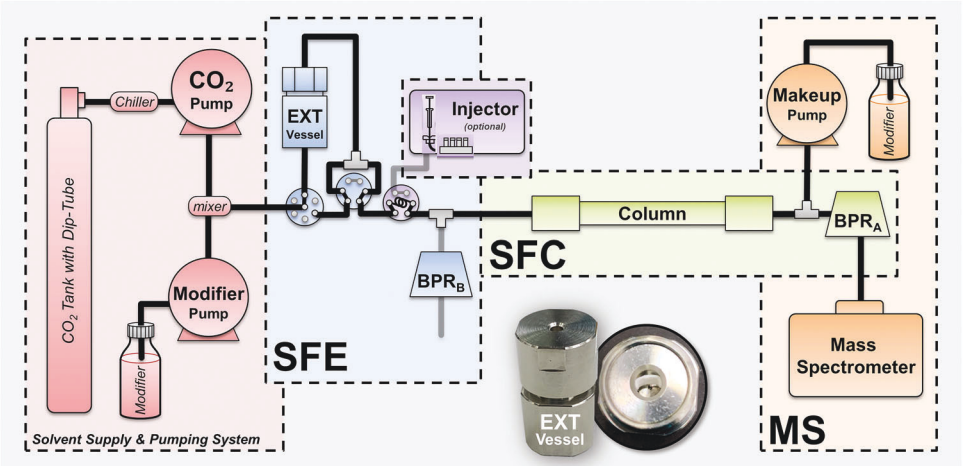

Figure 1. Nexera UC™ SFE-SFC-MS instrument schematic, showing typical system tubing connections between modules, and highlighting components of the hyphenated instrument specific to [solvent supply, *red*] the shared solvent supply and pumping system; [SFE, *blue*] the SFE-extractor unit (with extraction-loop, extraction-valves, and extraction chamber [EXT-vessel]), and optional system flow splitter (*pre-column*, back-pressure regulator [BPR_B]); [Injector, *purple*] the optional automated liquid injector for SFC-only operation (with injection-valve, and injection-loop); [SFC, *green*] the SFC chromatograph (with column oven and outlet pressure regulator (*post-column*, [BPR_A]); and [MS, *orange*] the detector (triple quadrupole mass spectrometer and solvent pump for makeup flow). Bottom picture showing 0.2-mL extraction vessel [left] and internal view [right] with a 6-mm paper sample core placed inside.

development process, slow the techniques penetration into many arenas in which it is ideally suited to provide solutions for difficult sample considerations.

Hyphenated SFE-SFC-MS method development

Method development (MD) for SFE-SFC-MS involves the systematic optimization and hyphenation of the individual detection, separation, and extraction techniques. Therefore, hyphenated SFE-SFC-MS MD is a multistep process, involving stepwise optimizations: first for MS-based detection optimizations; followed by SFC-based separation optimizations; then SFE-based extraction optimizations; and finally sample matrix-specific optimizations. These steps are intrinsically gapped by intermediate method-hyphenation steps, systematically adding the next technique, and therefore building the on-line hyphenated method. These hyphenation steps often times require re-optimization and this process can become quite involved, where once one set of parameters are optimized, complications found during hyphenation with the next technique requires the user to go back and re-optimize. This circle of re-optimizations can be time consuming, and interactions between instrument variables can make determining the source of an undesirable effect difficult.

The availability of systematic examples of observed effects would provide a multitude of information to help guide development of novel SFE-SFC-MS methods. For example, a recent study using multivariate optimization provides a better understanding of the synergistic relations between the hyphenated extraction and separation processes.[19] These types of reports continue to be limited and little information is available to new users to help guide the development of hyphenated methods. Therefore, complications due to synergistic effects continue to fuel the circle of re-optimization that plague the hyphenated MD process.

One of the most significant re-optimization routes is based on poor column selection choices, due to an uninformed decision process. As in any separation method, column selection must occur early in the MD process.[21] Ordinarily, the SFE-SFC-MS column scouting process has usually been the same as in SFCMS-only MD, with focus solely on the "best" separation of the targeted analytes. However, this approach oversimplifies the hyphenated method. As a result, the transfer of an established SFC-separation method directly to an on-line extraction method has proven difficult.[22–24]

Ultimately, choosing the correct analytical column means the difference between the success or failure of a hyphenated method. The column is the bridge between the hyphenation of the SFE-extraction and the SFCMS-analysis and therefore is crucial not only to the analytical separation of targeted analytes. The ability of the stationary phase to effectively act simultaneously (in parallel) as the trap column during the extraction, in the end determines the success of the on-line process. However, the demands of the SFC-separation may differ greatly than those required by the SFE-extraction.[19] These two demands can have very opposing requirements, which are not often compatible to provide the conditions required for the effective on-line trapping of a sample-plug and subsequent separation.

Conventional column selection for on-line SFE-SFC-MS MD

SFC is a normal phase technique, where the mobile phase composition is programmed from low to high polarity. Generally, more polar solutes will require more polar mobile phases (MP) and stationary phases (SP) (S_Figure 2).[25,26] Stationary phases traditionally implemented in SFC range from non-polar phases (such as C18) to much more polar phases (such as amino and ethylpyridine). SFC MD often starts by simply using a silica column and methanol as a modifier to obtain an initial evaluation of the separation of targeted analytes. This is a reasonable starting point. If the analytes are not retained, or alternatively, if they are excessively retained, the user has options to move to higher or lower polarity phases to compensate. In standard SFCMS MD, changing the column is not normally a first tool used to try to improve a separation.[21,25–27]

In on-line SFE-SFC-MS MD, more emphasis is often placed on column selection. Extracts often contain a more complex range of solute types consisting of both the solutes of interest and matrix components that must be separated. More importantly, the phase chosen must also be able to trap and retain a "sample-plug" during the on-line extraction process.[28] Therefore, you will often see a screening of a wide range of columns in on-line MD.[29]

SFE-SFC-MS MD flow charts are shown in Figure 2. The steps most relevant to column selection are highlighted. Panel-A depicts the traditional approach to developing hyphenated methods, which normally involved an approach similar to the SFC-only column screening (focused solely on the separation of targeted analytes). This approach does not take into consideration the trapping of the analyte-plug, and in the event that the phase chosen is not capable of retaining the "extraction-plug," it is not uncommon, to need to re-start the MD process (Figure 2A; *Thick red dashed lines*).[19]

Proposed column selection approach for on-line SFE-SFC-MS MD

The aim of the current work is to provide an alternative approach to column screening, specifically tailored to aid in on-line SFE-SFC-MS column selection. The proposed, alternative MD flow (Figure 2; panel-B) involves using a "plug-retention test" in addition to traditional SFC-only column scouting. This plug-retention test would use an SFE-simulation method in order to provide an early evaluation of each phases "plug-retention" capabilities. Allowing for uni-directional flow of the on-line MD process, would circumvent the need to re-start MD at later stages and should save large amounts of time normally spent on re-optimizations (the sample plug-retention test is described in detail in the experimental Section "Plug-retention test purpose").

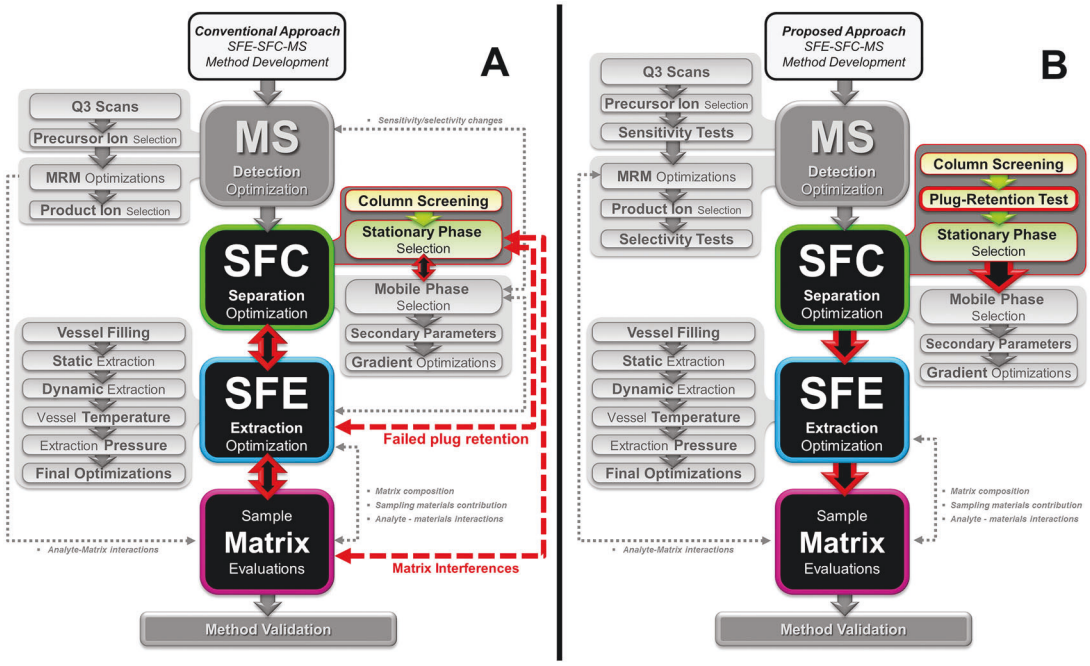

Figure 2. SFE-SFC-MS method development flowcharts, highlighting the steps most relevant to column selection: (A) conventional method development flow for coupling SFC methods to on-line SFE showing common re-optimization paths [*dashed lines*] requiring re-starting SFC column screening in the event the phase chosen is not capable of sufficiently retaining the "extraction-plug" (figure adapted from Wicker et al.[19]); (B) proposed novel method development flow adding a "sample-plug retention test" during SFC column screening to evaluate plug retentivity at an early method development step.

AAS as a model target analyte group

Ideally, on-line extraction evaluations are accomplished using a model sampling technique that could be applicable to a wide range of biological samples. Furthermore, the target group of analytes needs to be relevant to a field where it may provide solutions for difficult sample considerations. Drug doping remains a prevalent and potent form of drug cheating among elite athletes.[30] At the core of anti-doping policies and regulations is the ever evolving need for rapid, accurate, and sensitive techniques to detect abuse of established and newly regulated substances in a time-sensitive and reliable manner. Such an environment requires rapid, large scale, high-throughput analytical methods, compatible with biological samples collected in the field, such as saliva, urine, and blood,[31–33] with preferably minimal sample preparation. With such, hyphenated techniques such as SFE-SFC-MS should be ideal.

The world anti-doping association (WADA) publishes a list of prohibited substances.[34] Anabolic agents (category S1) continue to be the most frequently abused substances according to the "adverse analytical findings" (AAFs) listed by WADA annual anti-doping testing figures.[35,36] This category provides a special challenge to controllers, as they include endogenously produced hormones (androgenic anabolic steroids [AAS]), such as testosterone, as well as synthetic analogs specifically designed to avoid detection.[37,38] The AAS, largely composed of highly similar compounds, are a good target group for SFC, which is excellent at separating similarly shaped compounds, including isomers.[26] Although most separation methods are based on either liquid chromatography (LC) or gas chromatography (GC), SFC has been shown to be a highly sensitive and selective tool for the analysis of AAS.[35,39–46]

AAS analysis by SFE-SFC-MS was chosen as a model system for choosing the chromatographic SP. The current work centers on the SFC-based separation optimization (mainly step 2 in the hyphenated MD process) as part of the development of an on-line SFE-SFC-MS method for the extraction of anabolic agents in anti-doping testing. Optimization focused on using an adapted approach to streamline SFE-SFC-MS column selection. The proposed approach involved a rapid SFC-only screening (using a generic screening gradient), plus a "Sample-Plug Retention Test" (using an SFE-simulation method). The best performing phase was then used in a "proof-of-concept" on-line extraction.

Materials and methods

Materials

Solvents, reagents and gases

All solvents used for instrument mobile phases, spiking solutions, and injection solutions were of LCMS-grade. Methanol [MeOH] and ammonium formate were purchased from VWR International (Radnor, PA, USA). Analytical-grade carbon dioxide gas ([CO_2]; 60 lbs tanks with siphon) and argon gas ([Ar]; 60 lbs tanks) were obtained from Airgas Corporation (Grand Prairie, TX, USA). ACS-grade MeOH was utilized exclusively for cleaning extraction vessels.

Analytical standards

Twenty-three anabolic androgenic steroids [AAS] were chosen (Figure 3) from the 2021 WADA prohibited substances list: category S1, anabolic agents.[34] All analytical standards (S_Table 1) were purchased from Sigma Aldrich (St.

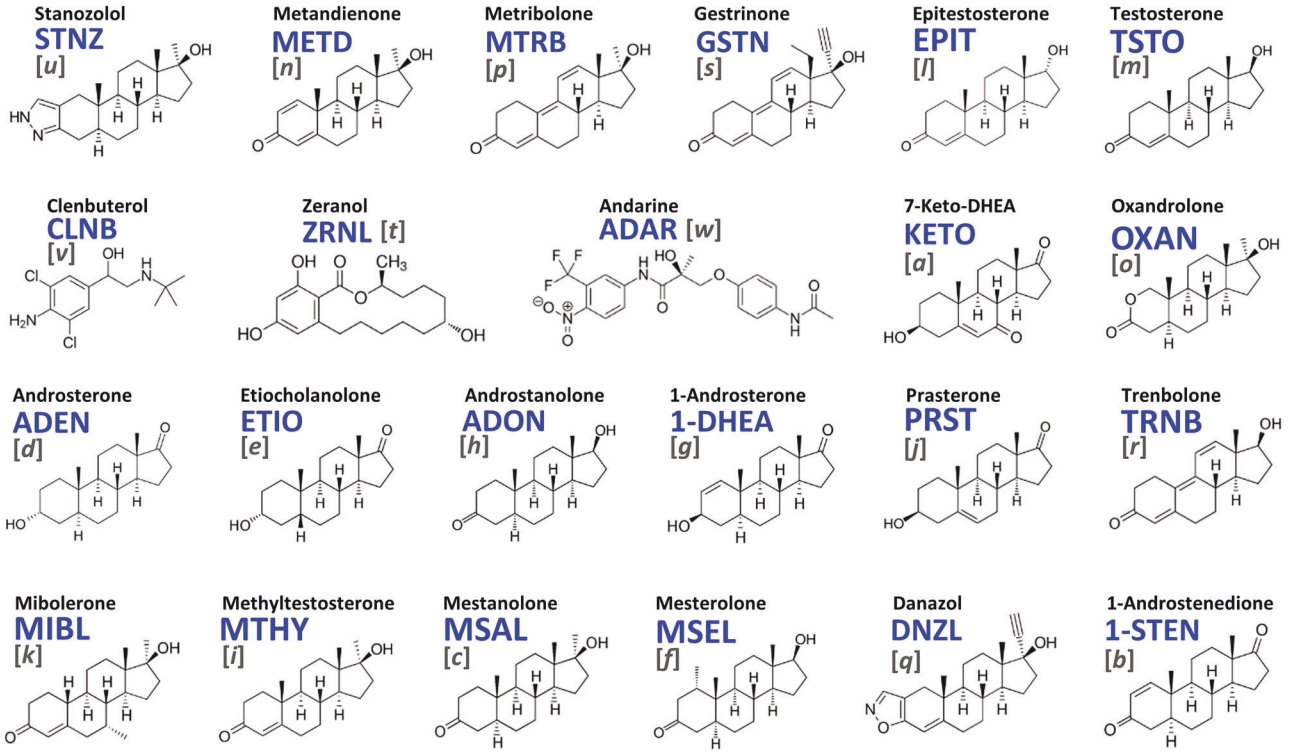

Figure 3. Structures of targeted anabolic agents.

Louis, MO, USA), and included: 1-androsterone [1DHEA]; andarine [ADAR]; androstenedione [1STEN]; androsterone [ADEN]; androstanolone [ADON]; clenbuterol [CLNB]; danazol [DNZL]; epitestosterone [EPIT]; etiocholanolone [ETIO]; gestrinone [GSTN]; 7-keto-DHEA [KETO]; mibolerone [MIBL]; metandienone [METD]; mestanolone [MSAL]; mesterolone [MSEL]; methyltestosterone [MTHY]; metribolone [MTRB]; oxandrolone [OXAN]; prasterone [PRST]; stanozolol [STNZ]; trenbolone [TRNB]; testosterone [TSTO]; and zeranol [ZRNL]. Deuterium labeled testosterone-d_3 [T-d_3] was also obtained for use as an internal standard.

Collection/preservation cards

Whatman® FTA®, classic, cellulose-based, sample collection/preservation cards (P#:WHAWB120205) were obtained from Sigma Aldrich (St. Louis, MO, USA). Standard, single-hole punch devices (Pen + Gear brand) were obtained from local sources (Walmart, Arlington, TX, USA).

Instrumentation

Nexera UC™ SFE-SFC-MS

A Nexera UC on-line supercritical fluid extraction/supercritical fluid chromatograph was used and directly coupled to an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Scientific Instruments, Kyoto, Japan) operated with an electrospray ionization source. The system was equipped with a liquid carbon dioxide delivery unit (Nexera UC [LC-30AD_{SF}] CO_2 pump) and a quaternary modifier infusion pump (Nexera X2 [LC-30AD] Liquid chromatograph) with degasser (DGU-20A_{3R}); a binary modifier pump (Nexera XR [LC-20AD_{XR}] liquid chromatograph) with degasser (DGU-20A_{5R}) for make-up flow; dual backpressure control units (Nexera UC [SFC-30A] backpressure regulators), one post-column [BPR_A] and the other pre-column [BPR_B]; a thermostatted column oven (Prominence [CTO-20AC]); an automated liquid injector (Nexera X2 [SIL-30AC] autosampler); a supercritical fluid extraction unit (Nexera UC [SFE-30A] auto extractor) with 48-vessel automated rack changer II; and communications bus (CBM-20A). The system was accessorized with 0.2-mL extraction vessels and racks. Instrument control and data acquisition were achieved using RealtimeAnalysis, and data post-processing using DataAnalysis (LabSolutions® chromatography workstation software, v.5.98, Shimadzu Corporation, Japan). Further data evaluation was accomplished using Excel (Office 2010, v.14.0.7268.5000; Microsoft Corp., Redmond, WA, USA).

Extraction vessels and accessories

Vessel racks [P/N: 228-58648-41], a torque wrench closing tool (1.5 N-m), and screen-type vessel filters [P/N: 228-59264-84] for 0.2-mL extraction vessels [P/N: 228-58079-84] were used and obtained from Shimadzu Corp. (Tokyo, Japan).

Other equipment

A Genius 1051 PSA nitrogen gas generator provided a continuous flow (up to 25 L/min) of dry air and high purity nitrogen gas (≥97%) for instrument operation (Peak Scientific, Billerica, MA. USA). A Sartorius CP64 analytical

Table 1. Targeted anabolic agents (AAS): analyte and peak IDs, MS-critical groups and solution concentrations.

			MS-critical		Stock solutions		Injection solution	Extraction cores
Anabolic agent (AAS)	Analyte ID	Peak ID	Group	Pair	HC-stock (μg/mL)	LC-stock (μg/mL)	AAS-mix (ng/μL)	AAS-core (ng/spot)
1-Androsterone	1DHEA	[*g*]	1	–	500	50	20	20
1-Androstenedione	1STEN	[*b*]	2	–	100	50	2	2
Andarine	ADAR	[*w*]	–	–	500	50	20	20
Androsterone	ADEN	[*d*]	1	–	500	50	20	20
Androstanolone	ADON	[*h*]	1	–	100	50	2	2
Clenbuterol	CLNB	[*v*]	–	–	500	50	20	20
Danazol	DNZL	[*q*]	2	–	500	50	20	20
Epitestosterone	EPIT	[*l*]	3	D	100	50	2	2
Etiocholanolone	ETIO	[*e*]	1	–	500	50	20	20
Gestrinone	GSTN	[*s*]	–	–	500	50	20	20
7-Keto-DHEA	KETO	[*a*]	–	–	500	50	20	20
Methandienone	METD	[*n*]	–	–	100	50	2	2
Mibolerone	MIBL	[*k*]	2	B	500	50	20	20
Mestanolone	MSAL	[*c*]	2	C	500	50	20	20
Mesterolone	MSEL	[*f*]	2	C	500	50	20	20
Metribolone	MTRB	[*p*]	–	–	500	50	20	20
Oxandrolone	OXAN	[*o*]	3	–	500	50	20	20
Prasterone (DHEA)	PRST	[*j*]	3	E	500	50	20	20
Stanozolol	STNZ	[*u*]	–	–	100	50	2	2
Trenbolone	TRNB	[*r*]	3	E	100	50	2	2
Testosterone	TSTO	[*m*]	3	D	100	50	2	2
Zeranol	ZRNL	[*t*]	–	–	1	0.1	0.04	0.04
Testosterone-d_3	T-d_3	[-]	–	–	10	1	–	–

balance (60 g × 0.1 mg; ± 0.1 mg) precision scale was obtained from DWS (Wood Dale, IL, USA).

Solutions and sample preparation

Stock solutions

Stock solutions (Table 1; *stock solutions*) were prepared for each analyte at two concentrations with MeOH, using 10-mL (class A) volumetric flasks. After being brought to volume, solutions were sonicated for 20 min before being transferred to glass scintillation vials for storage at −18 °C until the time of use.

Standards obtained as powders. A high concentration (500 μg/mL) stock [p-HCstock] was prepared by weighing 5.0 mg of powder, which was transferred to a 10-mL flask and dissolved with MeOH. A low concentration (50 μg/mL) stock [p-LCstock] was prepared by transferring 1.0 mL of the p-HCstock in a second 10-mL flask and diluting with MeOH.

Standards obtained in ampules (1.0 mg/mL). A high concentration (100 μg/mL) stock [a-HCstock] was made by quantitatively transferring the ampule contents to a 10-mL flask and diluting with MeOH. A low concentration (50 μg/mL) stock [a-LCstock] was prepared by transferring 5.0 mL of the a-HCstock in a second 10-mL flask and diluting with MeOH.

Zeranol obtained as a (10 μg/mL) solution. A high concentration (1.0 μg/mL) stock [Z-HCstock] was made by quantitatively transferring the ampule contents to a 10-mL flask and diluting with MeOH. A low concentration (0.1 μg/mL) stock [Z-LCstock] was prepared by transferring 1.0 mL of the Z-HCstock in a second 10-mL flask and diluting with MeOH.

Testosterone-d_3 internal standard obtained as a (100 μg/mL) solution. A high concentration (10 μg/mL) stock [Td_3-HCstock] was made by quantitatively transferring the ampule contents to a 10-mL flask and diluting with MeOH. A low concentration (1.0 μg/mL) stock [Td_3-LCstock] was prepared by transferring 1.0 mL of the Td_3-HCstock to a second 10-mL flask, where it was diluted with MeOH.

Injection and spiking solution [AAS-mix]: AAS test mixture

A test mixture containing all 23 analytes [AAS-mix] was created by combining 0.100 mL of each of the stock solutions as follows: using the high concentration stock for each standard that originated as a powder (*p-HCstock*), the low concentration stock for each ampule (*a-LCstock*) and the high concentration stock for ZRNL (*Z-HCstock*). The resulting mixture was diluted with an additional 0.300 mL of MeOH to a final volume of 2.6 mL, for a final concentration of 20 μg/mL for each standard originating from a powder and 2.0 μg/mL for each standard originating from an ampule; with the exception of ZRNL, where the concentration was 0.04 μg/mL (final concentrations are listed per analyte in Table 1; *AAS-mix*).

Extraction cores [AAS-core]: spiked (cellulose-based) paper cores

For on-line extractions, cellulose-based collection cards were used to make quality control cores (with spots of spiked standards). One microliter aliquots of the *AAS-mix* were spotted (quantitatively applied [1.0 μL/spot]) onto Whatman collection cards and allowed to dry for more than 3 h. The entire spot was then excised (cored) using a standard single

hole (6 mm) punch [AAS-core] (final spiked concentrations are listed per analyte in Table 1; *AAS-core*).

System configurations and sample introduction

To expedite the MD process, three different instrument configurations were used to isolate only the required modules for each analysis-type (S_Figure 3). Regardless of the configuration used, the hyphenated instrument utilized the shared solvent delivery system (*Solvent supply*), and carbon dioxide (CO_2) was utilized as the main component of the MP, modified with a polar organic solvent (*MOD*). Additionally, no matter the configuration used, the oven was utilized to control the temperature of the column (*SP*), the post-column pressure regulator (BPR_A) functioned as the system outlet pressure, and the triple quadrupole mass spectrometer (*MS*) was used for detection.

SFC-separation optimization configuration

The SFC-only configuration (SFC-MS Mode) was used for column scouting (S_Figure 3; A). Sample introduction was via the liquid injector (*INJ*) via external loop injections. The SFE-extractor unit (extraction-loop [*EXT*]) and the system split [BPR_B, *pre-column*] were bypassed.

SFE-simulation configuration

The SFE-simulation configuration (SFE-sim mode) was used for the sample-plug retention tests (S_Figure 3; B). Sample introduction was via the liquid injector (*INJ*). The SFE-extractor (and extraction-loop [*EXT*]) was bypassed. Therefore, the system setup was nearly identical to that used during SFC-only operation, except the second back pressure regulator ([BPR_B] *pre-column*) could be optionally used for split-mode simulations.

SFE-SFC-MS on-line extraction configuration

The on-line extraction configuration (SFE-SFC-MS mode) was utilized for proof-of-concept on-line extractions (S_Figure 3; C). The system setup was nearly identical to that used in SFE-sim mode, except importantly the liquid injector (*INJ*) was bypassed, and instead sample introduction was via the SFE-extractor unit and extraction-loop (*EXT*). Samples were placed inside 0.2-mL vessels and extracted on-line. Two switching valves facilitated the introduction of the resulting "extract-plug" to the system flow path. The "extract-plug" was carried along with the MP to be delivered to and trapped at the head of the analytical column for subsequent on-line analysis and detection.

Instrument methods

Pre-optimized methods for MS-detection

Detection was accomplished via ESI-MS analysis in MRM-mode using the triple-quadrupole mass spectrometer. The method used in the current work for detection was developed in previous works.[47]

MS parameters. An electrospray ionization (ESI−) source was used in positive (+) and negative (−) ionization mode under the following conditions: Interface voltages were set to 4.0 kV (for positive mode) and −3.0 kV (for negative mode) and temperature set to 300 °C. Nitrogen gas was used for both drying and nebulizing gas; with flow rate of 2.0 L/min for nebulizing gas and 10.0 L/min for drying gas. Desolvation and DL temperatures were 526 °C and 250 °C, respectively. Heat Block temperature was set to 400 °C, and heating gas used was dry air. Gas used for collision induced dissociation (CID) was argon at 270 kPa.

Multiple reaction monitoring (MRM) method. In previous works, MRM-optimizations were performed using the LabSolutions "optimize MRM event from product ion search" function, to determine optimal product ions, voltages, and collision energies for the target analytes. MRM-transitions, voltages, and collision energies are given in S_Table 2. Groups of analytes flagged in previous work as requiring chromatographic separation are indicated Table 1; MS-critical groups and pairs and (similar) MRM transitions in S_Figure 4.[47]

Mobile phases (MP) and stationary phases (SP)

Mobile phases. All work utilized SFC/CO_2-based MPs, where up to 40% modifier was mixed with carbon dioxide via the instrument solvent delivery pumps using isocratic or gradient elution. Modifier was either methanol with no additive [MeOH] or methanol + 5 mM ammonium formate [AmFo].

Columns. Thirteen chromatographic columns, of various phases and dimensions, were screened and are listed in Table 2. The Shim-pack UC-Cyano column (5.0 µm, 4.6 × 150 mm) from Shimadzu Corp. (Tokyo, Japan) was utilized for the proof-of-concept on-line extractions.

Parameters held constant

The following settings were held constant as default parameters unless otherwise specified. A methanolic mixture of 23 anabolic agents [*AAS-mix*] was injected onto a 5-µL external loop, using partial-loop (1.0 µL) injections with 0.10 µL air gaps. The injector needle was washed with 500 µL of MeOH, before and after, each injection. Mobile phase [A] was CO_2 and mobile phase [B] was MeOH. A column temperature of 50 °C was used and the system outlet pressure ([BPR_A] *post-column*) was set to 150 bar. Blank runs were performed between each injection to ensure system equilibration. Retention times and elution order were recorded for each analyte and resolution were calculated. Chromatographic resolution was compared between columns according to the following criteria: (A) Overall separation (i.e., resolution between all 22 targeted analytes); (B) MS-critical group separation (e.g., resolution between all five analytes in critical group 3); and (C) MS-critical pair separation (e.g., resolution between EPIT and TSTO [critical pair-3D]).

Table 2. Screened stationary phases: vendor information and column dimensions.

	ID	Phase	Vendor (location)	Brand	Phase trade-name	Particle size (μm)	Dimensions (mm)
A	[2EP]	2-Ethylpyridine	Princton Chrom. (Cranbury, NJ. USA)	PrincetonSFC	2-Ethylpyridine	3.0	3.0 × 100
	[NH2]	Amino	Agilent Technologies (Santa Clara, CA, USA)	Zorbax®	NH_2	5.0	4.6 × 150
	[Diol]	Diol	Daiso Fine Chem USA (Torrance, CA, USA)	DAISO	SP-60-5-Diol-P	5.0	4.6 × 150
	[HIL-Sil]	Silica (HILIC)	Restek Corp. (Belefonte, PA. USA)	Raptor	HILIC-Si	2.7	4.6 × 150
	[RX-Sil]	Silica	Agilent Technologies (Santa Clara, CA, USA)	Zorbax®	RX-SIL	1.8	3.0 × 100
	[CN]	Cyano	Agilent Technologies (Santa Clara, CA, USA)	Zorbax®	Cyano	3.5	4.6 × 150
	[UC-CN]	Cyano	Shimadzu Corp. (Tokyo, Japan)	Shim-pack	UC-Cyano	5.0	4.6 × 150
	[C18]	C18	Phenomenex, Inc. (Torrance, CA, USA)	Luna®	C18(2)	5.0	4.6 × 100
B	[UC-Choles]	Cholesteryl	Shimadzu Corp. (Tokyo, Japan)	Shim-pack	UC-Choles	5.0	4.6 × 250
	[UC-HyP]	Hydroxyphenyl	Shimadzu Corp. (Tokyo, Japan)	Shim-pack	UC-HyP	5.0	4.6 × 250
	[UC-Triazole]	Triazolyl	Shimadzu Corp. (Tokyo, Japan)	Shim-pack	UC-Triazole	5.0	4.6 × 250
	[UC-PBr]	Pentabromobenzyl	Shimadzu Corp. (Tokyo, Japan)	Shim-pack	UC-PBr	5.0	4.6 × 250
	[PolarX]	*np*[a]	Restek Corp. (Belefonte, PA, USA)	Raptor	PolarX	2.7	4.6 × 100

Panel-A = Phases traditionally implemented in SFC method development; Panel-B = Nontraditional phases less commonly implemented in conventional SFC method development. [a]*np* = not provided (proprietary).

Methods for SFC-MS column scouting

For column scouting, the instrument was operated in SFC-MS mode using the "SFC-separation optimization configuration." One microliter of the AAS-mix was injected per run via the liquid autosampler (and external injection-loop).

Instrument method: isocratic runs. On each column screened, isocratic runs were performed using 40%, 20%, and 10% modifier. All other parameters were constant as described in Section "Parameters held constant."

Instrument method: "generic screening gradient". A generic gradient was used for the SFCMS-only column screening and was as follows: initial concentration of 5% modifier was held for 1.0 min (5% [B], 0.00–1.00 min), before ramping from 5% to 40% modifier over 7 min (5–40% [B], 1.01–8.00 min), and finally held at 40% for 2 min (40% [B], 8.01–10.00 min). All other parameters were held constant as described in Section "Parameters held constant."

Plug-retention test (SFE-simulation) methods

For the "plug-retention test," the instrument was operated in SFE-sim mode using the "SFE-simulation configuration." One microliter of the AAS-mix was injected per run via the liquid autosampler (and external injection-loop).

Instrument method: SFE-simulation parameters. Prior to each run, the column was equilibrated using 30% modifier for 2 min (30% [B], pre-run, [−2.00]–0.00 min). The switching of the injector-valve to the "inject" position indicated the start of the run (SFC-inject, 0.00 min). The initial concentration of 30% modifier was then held constant for an additional 0.3 min (30% [B]; 0.00–0.03 min), before being stepped down to 5% modifier which was held for 2 min (5% [B]; 0.04–2.00 min). The system was operated in split-mode, where the split was open for the first 2 min ([BPR_B, *pre-column*], set to 152 bar, 0.00–2.00 min). Finally, the split was then closed ([BPR_B, *pre-column*], set to 400 bar, 2.01 min) and an 8-min analysis gradient was started ("SFE-simulation gradient" [detailed below], 2.01–10.00 min).

SFE-simulation gradient. The gradient program used for the separation of the "trapped injection-plug" was as follows: initial concentration of 5% modifier was held for 1.0 min (5% [B]; 2.01–3.00 min), before ramping from 5% to 40% modifier over 5 min (5–40% [B]; 3.01–8.00 min), and finally held at 40% for 2 min (40% [B]; 8.01–10.00 min). All other parameters were held constant as described in Section "Parameters held constant."

Methods for SFE-SFC-MS 'proof-of-concept' on-line extractions

For on-line extractions, the instrument was operated in SFE-SFC-MS mode using the "SFE-SFC-MS on-line extraction configuration." On-line extractions were performed using 0.2-mL vessels. A single AAS-core (1.0-μL AAS-mix/spot) was extracted and analyzed per run via the on-line extractor unit (and extraction-loop).

Instrument method: generic proof-of-concept on-line extraction. Triplicate AAS-cores were extracted on-line using the "on-line SFE extraction parameters." The resulting extract-plug was trapped and subsequent chromatographic separation achieved on the Shimadzu Corp., Shim-pack, UC-cyano column, using the "SFE-SFC-MS Analysis gradient." Pump [B] delivered AmFo as modifier. The system was operated in splitless-mode ([BPR_B, *pre-column*], set to 400 bar) for the duration of the extraction and analysis.

On-line SFE extraction parameters. The parameters used specific to the extraction portion of the on-line SFE-SFC-MS method included a 1-min vessel filling using 2% modifier at 2.0 mL/min (2% [B], 0.00–1.00 min), followed by a 2-min static extraction using 0% modifier at 3.0 mL/min (0% [B], 1.01–3.00 min). The SFE-valve was then switched to dynamic (SFE-dynamic, 3.00 min), which initiated a 2-min dynamic extraction also using 0% modifier at 3.0 mL/min (0% [B]; 3.01–5.00 min). The extraction step ends at the end of the dynamic extraction (SFE-end; 5.01 min), and a 15-min analysis gradient was started ("SFE-SFC-MS analysis gradient" [detailed below], 5.01–20.00 min). Finally, a 3.0-min wash of the extraction-loop was performed using 100% modifier at 3.0 mL/min (SFE-wash, CO_2 shutoff valve closed, 100% [B], 20.01–23.00 min) and a 1.0-min system re-equilibration using 2% modifier at 3.0 mL/min (SFE-static, CO_2 shutoff valve open, 2% [B], 23.01–24.00 min). The

extraction pressure was held constant ([BPR_A, *post-column*], set to 150 bar]). The rack changer held the temperature of vessels constant at 20 °C until just prior to extraction when each vessel was heated to 35 °C.

SFE-SFC-MS analysis gradient. The gradient program used for the separation of the "trapped extract-plug" was as follows: initial concentration of 2% modifier was ramped to 12.5% over 8 min (2.0–12.5% [B]; 5.02 – 13.00 min), and finally the modifier concentration was stepped to 30% and held constant for 7 min (30% [B]; 13.01–20.00 m). A flow rate of 3.0 mL/min, a column temperature of 60 °C, and a system outlet pressure [BPR_A, *post-column*] of 150 bar were set for the duration of the analysis step.

Methods specific to sample and extraction vessel preparation workflow. On-line extractions were performed using 0.2-mL vessels. Supporting information describing the sample prep approach and workflow for on-line extractions can be found in section S_2.4. Supplemental Discussion: On-line Extraction Sample Prep Workflow and S_Figure 5. In short, for each extraction, a single AAS-core was placed within an (empty, clean, and dry) extraction chamber, with no desiccant added. The vessel was then closed using a 1.5 N-m torque wrench and set to the automated rack changer for the SFE-extractor unit.

Results and discussion

Model sampling technique and target analyte selection

Ideally, method evaluations for hyphenated on-line extractions would be accomplished using a model sampling technique that could be applicable to a wide range of biological samples. This model must also provide blank material in which targeted analytes can be spiked at known concentrations, in order to adequately evaluate developed methods. Furthermore, the target group of analytes needs to be relevant to a field where it may provide solutions for difficult sample considerations.

AAS as a model target analyte group

Anabolic agents were chosen as the target "model" group due to their high relevance in the active anti-doping field that is continuously adapting/adopting novel analysis approaches. AAS were chosen due to established familiarity to techniques utilizing CO_2-based MPs (i.e., applicability of SFC has already been widely demonstrated for steroids); as well as for the potential for providing ample opportunity for method transfer between a wide range of biological applications (e.g., between biological samples; such as saliva, urine and blood). Twenty-three anabolic agents were targeted, including endogenous and exogenous AAS (such as 1DHEA, 1STEN, ADEN, ADON, EPIT, ETIO, KETO, MIBL, MSAL, MSEL, MTHY, PRST, TRNB, and TSTO). Other anabolic agents that were included in the study were either synthetic steroids (DNZL, GSTN, METD, MTRB, STNZ, and OXAN) or selective androgen receptor modulators [SARMS] (ADAR, and ZRNL) and CLNB. This group includes 21 compounds that share either molecular weight and/or LogP (S_Table 1; MW and LogP), making their separation a challenge.

Sampling approach

The same steroid mixture (*AAS-mix*) was used in all analyses of the current work. In both the SFC-only screening and SFE-simulation, 1.0-µL liquid injections of the *AAS-mix* were performed. Therefore, to allow for direct comparison in the proof-of-concept on-line extraction, 1.0-µL aliquots of the same steroid mixture were used to create quality control cores (spiked paper cores). Specifically, in the current work, on-line extractions were performed directly from cellulose-based sample collection cards. These cards can be used with urine, blood, plant, and tissue samples and therefore extend the opportunity for rapid method transfer between a wide range of biological applications.

Pre-optimized detection method

Pre-optimized detection method for AAS

MS-based optimizations were performed for the detection of AAS in previous steps of the on-line MD process. Therefore, the method used in the current work for detection was developed in previous works.[47] First, optimal precursor ions were chosen for each of the targeted steroids based on the effect of SFC/CO_2-based MP compositions on characteristic ionization patterns using Q3-scans. In further steps, optimal product ions, voltages, and collision energies were chosen via MRM-optimizations. For one of the target analytes, ADON, it was determined that further MS-optimization was necessary (beyond the scope of the work). Therefore, although the analyte ADON was present in the steroid test mixture (the AAS-mix), due to difficulties in detection (e.g., unreliable MRM-stability across SFC/CO_2-based MP compositions), ADON was not included for the evaluation of chromatographic performance in the current work.

MS-selectivity tests and critical groups

Each analyte was then individually reinjected using the pre-optimized MRM method. Steroids that produced signal for two or more MRMs were investigated further for structural similarities and their Q3 scans compared. This identified three groups of compounds as "MS-critical," where due to fundamental similarities in structure and/or molecular weight, differentiation with MS-alone was impractical/impossible. These were flagged in previous work as MS-critical groups that would require chromatographic separation.[47] Comparison of the resolution between MS-critical analytes were used throughout the current work to evaluate the chromatographic method performance for each stationary phase and as selection criteria in identifying best performing columns.

General approach to the proposed SFE-SFC-MS column scouting

In total thirteen SPs were evaluated using a rapid screening approach. Separations were performed on each column, first using the traditional SFC-only scouting approach, with isocratic runs (at 40, 20, and 10% modifier) and a "generic screening gradient." Next, separations were performed on each column, for a "plug-retention test" using an "SFE-simulation" method.

Although, in standard SFCMS MD, changing the column is not normally the first tool used to try to improve a separation and in consideration that commonly online MD, requires screening of a wide range of columns. Since the proposed alternative MD flow is a novel approach, as support for the demonstration, and for proof-of-concept; a large range of columns (larger than most labs would normally include) were included in the current study. In order to establish the validity of the alternative MD flow, the approach was evaluated using "traditional phases" (representing the historically demonstrated polarity range of phases for SFC), as well as several 'nontraditional' or proprietary phases.

SFCMS-only column scouting approach

Column scouting was performed using the SFC-MS mode configuration (S_Figure 3; A) to provide an optimized flow path for SFC-only operation (i.e., bypasses the SFE-extractor unit and system splitter [BPR_B]). This setup is meant to reduce the time for each run to enable rapid SFC-method screening and optimization. Separations of liquid injections were performed using the "generic screening gradient." This gradient ramped from low (5% B) to high (40% B) modifier over 7 min to enable a rapid evaluation of the potential separation capabilities of each phase for the target analytes. The column temperature and system outlet pressure were held constant between each column screened (50 °C and 150 bar, respectively) in order to minimize the effect on secondary parameters on retention and resolution.

SFC-only scouting results: traditional SFC SPs

Generic screening gradient: traditional SFC phases. Eight SPs, traditionally used in SFC, were screened to evaluate the effect of phase polarity on the separation of the steroid mixture. Traditional SPs (Table 2; A) ranged in polarity from C18 [non-polar] to more polar phases such as amino and ethyl-pyridine. Resulting chromatograms were compared (Figure 4), with close attention given to resolution between MS-critical groups (Table 3). Note that ideally the set of traditional columns would have the same dimension (e.g., length, diameter, and particle size), but due to availability, the set of traditional SFC columns used in this work were not of the same column dimensions. Although the normal comparison between phase polarities was hard to make, some trends could clearly be identified based on differences in polarity.

Traditional phases: overall performance for generic gradient. The full runtime is compared in panel-A of Figure 4. Improved retention was observed for all compounds on the higher polarity phases. The majority of the compounds were virtually unretained on the non-polar C18 phase, with most of the target analytes (especially those having the characteristic steroid backbone) eluting in the first 1.5 min of the run and producing very low resolution between all MS-critical groups. Alternatively, using the more polar cyano columns (CN and UC-CN) with the same generic gradient, the majority of the target analytes were much more retained, spreading the earliest eluters out across the first 5 min of runtime. This resulted in much improved resolution within each critical group; baseline resolution was achieved for many critical compounds, even with the generic gradient. The silica columns (HIL-Sil and RX-Sil) were even more retentive, where the latest eluting compounds (e.g., mainly the non-steroid, androgen mimics, CLNB and ADAR), were retained beyond the runtime of the generic gradient. The silica columns had similar selectivity to the cyano columns, but as the phase polarity increased, a notable gap in elution formed between the first two steroids (earliest eluting, most non-polar analytes) and the bulk of the remaining analytes (mid-eluters). Another interesting note is that the latest eluters (CLNB [*dark green-MRM*] and ADAR [*maroon-MRM*]), became less retained on the most polar columns. This is a result of a peak reversal between CLNB and ADAR. CLNB elutes later on C18, co-elutes on silica, and elutes earlier than ADAR on the most polar phases.

Traditional phases: resolution within MS-critical groups. Detailed discussion of the resolution of critical group compounds can be found in section S_3.1. Supplemental Discussion: *Traditional Phases: Resolution within MS-Critical Groups*. Generally, members of critical group 1 appeared to be the most difficult to separate (Figure 4; panel-B; *left* [*Group-1*]). Only on cyano and diol were three distinct peak apex observed; and therefore, these were considered to be the most promising phases for the potential separation of group 1. For critical group 2 (Figure 4; panel-B; *middle* [*Group-2*]), the RX-Sil column produced the best overall group separation, where all six compounds were nearly baseline resolved ($Rs > 1.3$). The separation of MIBL [*peak-i*] and MTHY [*peak-k*] may be difficult, as these two compounds co-eluted on all phases except for the RX-Sil and amino columns, the later giving the greatest resolution, which was just barely baseline resolved ($Rs_{[i:k]} = 1.5$). Critical group 3 (Figure 4; panel-B; *right* [*Group-3*]) although generally showed increasing group resolution with increased phase polarity and adequate resolution between the pair-3D (PRST [*peak-j*] and TRNB [*peak-r*]) was observed on all stationary phases regardless of polarity. The remaining members of this group appear to be much more difficult to separate. The pair-3E, epimers, TSTO [*peak-l*] and EPIT [*peak-m*], although not resolved on the non-polar C18 column, showed increasing resolution with increasing SP polarity, but the amino phase gave surprisingly low resolution, possibly due to a peak reversal between this pair. The

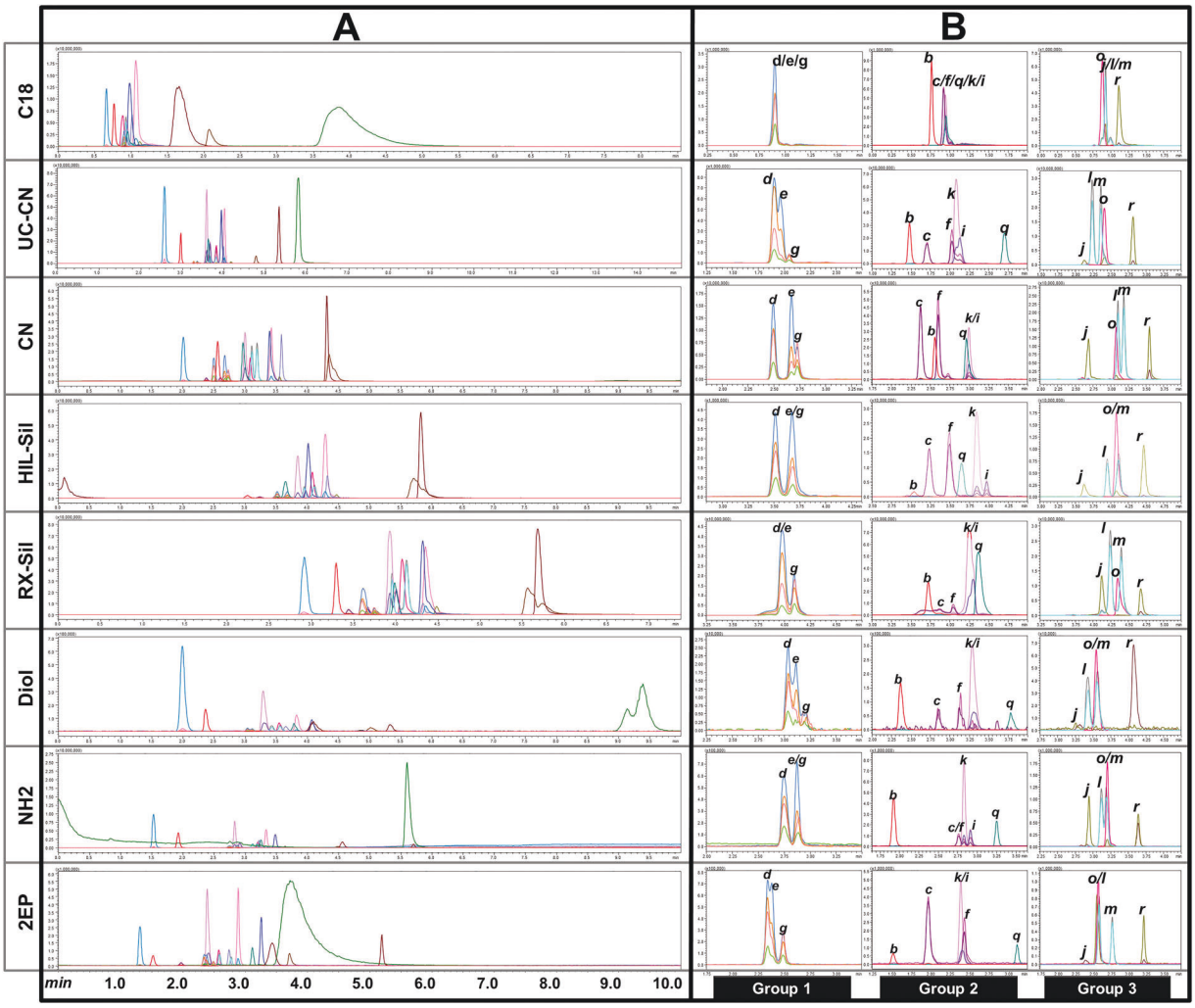

Figure 4. SFC-MS chromatograms for scouting traditional SFC phases using the generic screening gradient, showing: (A) full runtime (0–10 min) *highlighting the overall separation between all 22 targeted anabolic agents*; and (B) zoomed retention time range for the separation of MS-critical group analytes, [*left*] Group-1, [*middle*] Group-2, and [*right*] Group-3. *Conditions*: generic screening gradient (5–40% MeOH over 8 min); with column temperature of 50 °C and outlet pressure of 150 bar (*post-column*, [BPR_A]); using 1.0 µL liquid injections of the steroid mixture [AAS-mix]. Overlaid MRM-TIC chromatograms displayed on the same intensity scale. *Column ID key*: [C18] Luna, C18(2) column; [UC-CN] Shim-pack, UC-Cyano column; [CN] Zorbax, Cyano column; [HIL-Sil] Raptor, HILIC-Silica column; [RX-Sil] Zorbax, RX-Silica column; [Diol] Daiso, SP-60-5-Diol-P column; [NH2] Zorbax, Amino column; and [2EP] PrincetonSFC, 2-Ethylpyridine column. *Peak ID key MS-critical analytes*: Group-1 (ADEN [*peak-d*, *orange-MRM*], ETIO [*peak-e*, *blue-MRM*], and 1DHEA [*peak-g*, *pink-MRM*]); Group-2 (1STEN [*peak-b*, *red*], MSAL [*peak-c*, *magenta*], MSEL [*peak-f*, *rose*], MIBL [*peak-k*, *purple*], MTHY [*peak-l*, *light-pink*], and DNZL [*peak-q*, *turquoise*]); and Group-3 (EPIT [*peak-m*, *cyan*], TSTO [*peak-l*, *gray*], OXAN [*peak-o*, *hot-pink*], PRST [*peak-j*, *gold*], and TRNB [*peak-r*, *brown*]).

most significant difficulties for this group resided in the resolution of OXAN [*peak-o*], which eluted very close to both EPIT and TSTO across all phase polarities. UC-Cyano [UC-CN] was the only phase where OXAN eluted later than TSTO and also gave the best (albeit poor) resolution between the two compounds ($Rs_{[o:m]} = 0.6$) when using the generic screening gradient. Special focus on the separation of these three analytes will be required in further SFC-based separation optimizations in future work.

SFC-only scouting results: nontraditional SFC SPs

Generic screening gradient: nontraditional phases. Five nontraditional SPs (Table 2; B) were screened using the "generic screening gradient." Resulting chromatograms are compared in Figure 5 and resolution between critical groups is given in Table 4.

Nontraditional phases: overall performance for generic gradient. Of the nontraditional phases, the cholesterol column [UC-Choles] gave little overall retention for the majority of the compounds. Alternatively, the pentabromobenzyl column [UC-PBr] was excessively retentive with nearly half the compounds requiring high modifier concentration (>40%) for elution. The PolarX and the triazole [UC-Triazole] columns showed good potential for overall resolution between all 22 anabolic agents. Tailing was observed for many of the later eluting compounds on PolarX and it was less retentive than triazole.

Nontraditional phases: resolution within MS-Critical groups. Detailed discussion of the resolution of critical group compounds can be found in section S_3.2. Supplemental Discussion: *Nontraditional Phases: Resolution within MS-Critical Groups*. Generally, resolution for critical group 1 was much better on the nontraditional phases (Figure 5;

Table 3. Resolution of MS-critical groups during screening of traditional SFC stationary phases.

	MS-critical group resolution (Rs)													
	Group-1		Group-2					Group-3						
Column	Elution order	Overall group Rs	Elution Order		Overall group Rs		Pair-2B Rs	Pair-2C Rs	Elution order	Overall group Rs		Pair-3D Rs	Pair-3E Rs
C18	*d/e/g*	**<0.1**	**<0.1**	*b, c/f/i/l/k/q*	**<5.0**	**<0.1**	**<0.1**	**<0.1**	<0.1	*o, j/l/m, r*	**<3.0**	**<1.0**	**<0.1**	**<10**	<10	<0.1
UC-CN	*d, e, g*	**<3.0**	**<1.0**	*b, c, f, k, i, q*	**<3.0**	**<5.0**	**<1.0**	**<10**	<5.0	*j, l, m, o, r*	**<3.0**	**<3.0**	**<1.0**	**<10**	<10	<3.0
CN	*d, e, g*	**<3.0**	**<1.0**	*b, c, f, q, k/i*	**<3.0**	**<1.0**	**<5.0**	**<1.0**	**<0.1**	<3.0	*j, o, l, m, r*	**<10**	**<1.0**	**<3.0**	**<10**	>10	<3.0
HIL-Sil	*d/e, g*	**<0.1**	**<1.0**	*b, c, f, k/l, q*	**<3.0**	**<3.0**	**<1.0**	**<1.0**	<3.0	<1.0	*j, l, o, m, r*	**<3.0**	**<3.0**	**<1.0**	**<5.0**	<10	<3.0
RX-Sil	*d, e/g*	**<3.0**	**<1.0**	*b, c, f, q, k, i*	**<3.0**	**<3.0**	**<5.0**	**<3.0**	<3.0	<3.0	*j, l, o, m, r*	**<5.0**	**<3.0**	**<1.0**	**<5.0**	<10	<3.0
Diol	*d, e, g*	**<1.0**	**<1.0**	*b, c, f, k/l, q*	**<10**	**<5.0**	**<3.0**	**<1.0**	**<3.0**	<5.0	<1.0	*j, l, o, m, r*	**<3.0**	**<3.0**	**<1.0**	**<5.0**	<10	<3.0
NH2	*d, e/g*	**<3.0**	**<1.0**	*b, c/f, k, l, q*	**<10**	**<0.1**	**<1.0**	**<3.0**	**<5.0**	<0.1	<3.0	*j, l, o, m, r*	**<3.0**	**<1.0**	**<1.0**	**<10**	<10	<1.0
2EP	*d, e, g*	**<1.0**	**<3.0**	*b, c, k/l, f, q*	**<5.0**	**<5.0**	**<1.0**	**<1.0**	**<5.0**	<10	<1.0	*j, o, l, m, r*	**<1.0**	**<3.0**	**<10**	**<10**	<10	<3.0

Peak ID key: Group-1, [*d*] ADEN; [*e*] ETIO; [*g*] 1DHEA; Group-2, [*b*] 1STEN; [*c*] MSAL; [*f*] MSEL; [*i*] MTHY; [*k*] MIBL; [*q*] DNZL. Pair 2-B, MSAL:MSEL [*c:f*]. Pair 2-C, MTHY:MIBL [*i:k*]; Group-3, [*j*] PRST; [*l*] EPIT; [*m*] TSTO; [*o*] OXAN; [*r*] TRNB. Pair-3D, PRST:TRNB [*j:r*]. Pair-3E, EPIT:TSTO [*l:m*].

panel-B; *left* [*Group-1*]). Hydroxyphenyl [UC-HyP] and UC-PBr gave the best overall resolution for group 1, but out of all the screened columns, only UC-PBr gave extended baseline separation ($Rs_{[d:e:g]} > 3$) for all compounds of this group. For critical group 2 compounds, elution order was very different in some of the nontraditional phases (Figure 5; panel-B, *middle* [*Group-2*]). Generally, none of the nontraditional phases out-performed the traditional phases for the resolution of group 2. For critical group 3 (Figure 5; panel-B; *right* [*Group-3*]), out of all the phases screened, the baseline separation (Rs > 1.5) of OXAN (peak-*o*) from TSTO (peak-*m*) and EPIT (peak-*l*) was only accomplished on two phases, the UC-Choles and the UC-PBr columns.

Nontraditional phases: summary. Triazole had the least resolution for the main pairs using the generic screening gradient (all having Rs < 1). Although the best resolution for most of the critical compounds was observed on UC-PBr (Rs > 3), elution for CLNB and ADAR required > 60% modifier concentration, and peak shape of STNZ and DNZL deteriorated at lower % modifier.

"Plug-retention test" purpose

SFE-simulation method approach

During on-line SFE-extractions, solid samples are extracted on-line and the resulting "extract-plug" must be "trapped"/retained at the head of the column during the extraction process. One significant challenge for hyphenated method development is predicting a column's capability to adequately retain this "plug" of extracted analytes. A poorly retained "plug" will result in broad peaks and poor peak symmetry, especially for early eluting compounds. With that in mind, a simulation was performed, using SFC-based liquid injections of the steroid mixture. The "SFE-simulation" was performed on each of the thirteen columns to evaluate the potential plug-retentivity of each phase.

SFE-simulation instrument setup. The extraction simulations utilized the SFE-sim mode configuration (S_Figure 3; B). The biggest difference from SFC-only mode was in the system operation. The simulation method resembled a time program that would be used during on-line extractions, but the liquid injector is the source of sample introduction. Therefore, the simulation time program is instead set up around the injection.

SFC injections were performed while the system was operated in a fashion similar to as if the extraction-loop had been being utilized. This type of operation was set to mimic as many of the conditions as possible of an on-line extraction, including changes in flow rates and MP compositions that would normally occur during an actual on-line extraction process.

Simulated extraction factors

The method used for this simulation was devised to simulate system conditions during on-line extraction. The normal

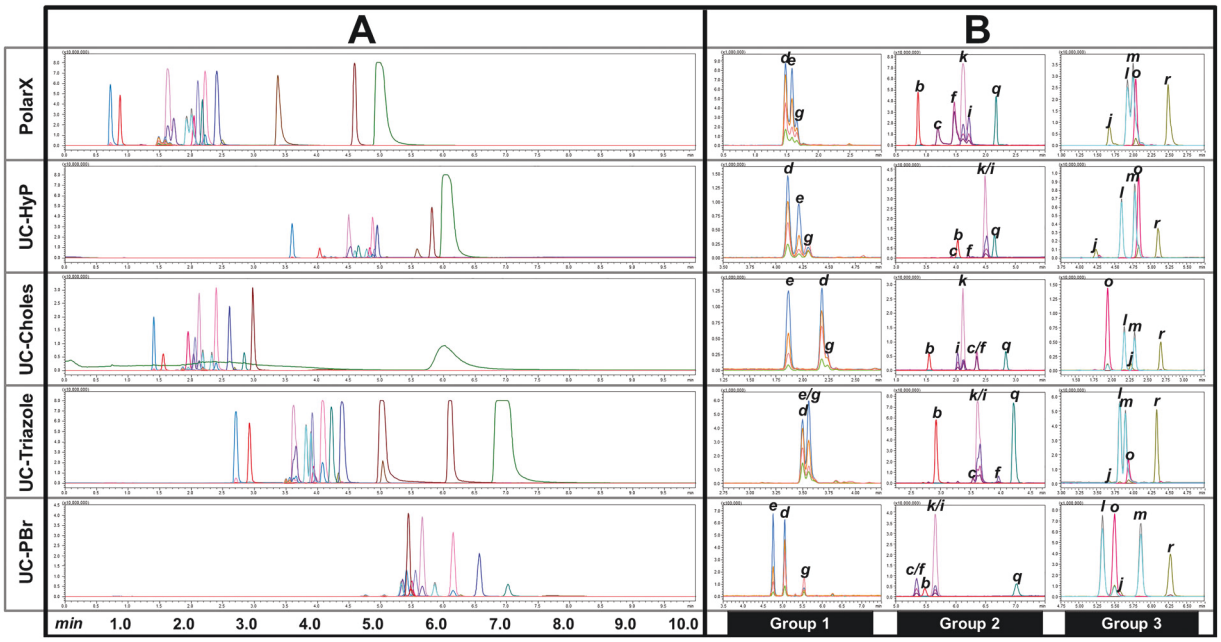

Figure 5. SFC-MS chromatograms for scouting nontraditional phases using the generic screening gradient, showing (A) full runtime (0–10 min) *highlighting the overall separation between all 22 targeted anabolic agents* and [B] zoomed retention time range for the separation of MS-critical group analytes, [*left*] Group-1, [*middle*] Group-2, and [*right*] Group-3. *Conditions*: generic screening gradient (5–40% MeOH over 8 min); with column temperature of 50 °C and outlet pressure of 150 bar (*post-column*, [BPR_A]); using 1.0 µL liquid injections of the steroid mixture [AAS-mix]. Overlaid MRM-TIC chromatograms displayed on the same intensity scale. *Column ID key*: [PolarX] Raptor, PolarX column; [UC-HyP] Shim-pack, UC-Hydroxyphenyl column; [UC-Choles] Shim-pack, UC-cholesteryl column; [UC-Triazole] Shim-pack, UC-triazolyl column; and [UC-PBr] Shim-pack, UC-Pentabromobenzyl column. *Peak ID key for MS-critical analytes*: Group-1 (ADEN [*peak-d, orange-MRM*], ETIO [*peak-e, blue-MRM*], and 1DHEA [*peak-g, pink-MRM*]); Group-2 (1STEN [*peak-b, red*], MSAL [*peak-c, magenta*], MSEL [*peak-f, rose*], MIBL [*peak-k, purple*], MTHY [*peak-l, light-pink*], and DNZL [*peak-q, turquoise*]); and Group-3 (EPIT [*peak-m, cyan*], TSTO [*peak-l, gray*], OXAN [*peak-o, hot-pink*], PRST [*peak-j, gold*], and TRNB [*peak-r, brown*]).

on-line extraction process consists of four main steps: (1) Vessel Filling; (2) Static Extraction; (3) Dynamic Extraction; and (4) Analysis. A detailed description of the extraction process is provided in section S_4.1. Supplemental Discussion: *Detailed On-line Extraction Process*. The conditions for the SFE-simulation method involved multiple steps, each simulating a different "extraction step," before ultimately starting a generic gradient similar to that used in the SFC-only column screening.

Fill-factors simulated. In normal on-line extraction, vessel filling is the first step of the extraction process (S_Figure 6; B and C). System flow is split between the extraction-loop and the on-line SFC system. Mobile phase enters the extraction-loop and fills the vessel containing the sample. The vessel is normally filled at high modifier concentration. Excess flow is diverted over the column stabilizing system wide pressure at BPR_A. In the SFE-simulation (Figure 6; Filling Simulation [*orange*]), the injection-plug (being a 1.0-µL slug of 100% modifier) was used to simulate the high modifier concentration normally used in the "filling step" of on-line extractions.

Static-factors simulated. In on-line extraction, static extraction is the second step of the extraction process (S_Figure 6; D). During static extraction, the instrument flow path is the same as in the vessel filling step. The sample (now exposed to fill solvent) is left for a duration of time (allowing for "passive" extraction). During this "passive" extraction step, the extraction chamber is deadheaded; the vessel contents are mostly isolated from the main flowpath. Static extraction is also normally performed at high modifier concentration and occurs just prior to the transport and loading of the "extract-plug" to the analytical column. Due to the deadheading of the extraction-loop, excess system effluent continues to flow through the column out thru BPR_A. Synonymous to a pre-conditioning step, where the SP is effectively equilibrated at high modifier concentration just prior to plug-loading, static conditions often also affect the integrity of the "trapping and retention" of the "extract-plug," which potentially makes it harder to "trap"/retain analytes at the head of the column (especially less polar compounds). In the SFE-simulation, in order to simulate static conditions (Figure 6; Static Simulation [*red*]), the column was equilibrated at high modifier concentration (30% B) for 2 min before and for a brief time during the injection.

Dynamic-factors simulated. In normal on-line extraction, dynamic extraction is the third step of the extraction process. During dynamic extraction (S_Figure 6; E, F, G, and H), the extraction valve directs the system flow through the extraction vessel and the flow is now deadheaded at the extraction-valve. So just as in static, all system effluent eventually flows through the column to the system outlet thru BPR_A. Since the MP flows through the extraction vessel, the solvent in the vessel that has been exposed to the sample. The "extract-plug" is pushed (carried) along with the system flow and ultimately, is delivered to the analytical column. This plug of analytes must be trapped and retained at the column head. Dynamic extraction is also called "active"

Table 4. Resolution of MS-critical groups during screening of nontraditional SFC stationary phases.

	MS-critical group resolution (Rs)													
	Group-1		Group-2						Group-3					
Column	Elution order	Overall group Rs	Elution order		Overall group Rs			Pair-2B Rs	Pair-2C Rs	Elution order	Overall group Rs		Pair-3D Rs	Pair-3E Rs
PolarX	*d, e, g*	**<3.0**	**<1.0**	*b, c, f, k, i, q*	**<5.0**	**<5.0**	**<3.0**	**<1.0**	**<10**	**<5.0**	**<1.0**	*j, l, m, o, r*	**<3.0**	**<1.0**	**<10**	**<10**	**<1.0**
UC-HyP	*d, e, g*	**<3.0**	**<3.0**	*c, b, f, k/i, q*	**<3.0**	**<3.0**	**<5.0**	**<1.0**	**<3.0**	<5.0	<1.0	*j, l, m, o, r*	**<5.0**	**<3.0**	**<1.0**	**<5.0**	>10	<3.0
UC-Choles	*e, d, g*	**<5.0**	**<1.0**	*b, i, k, c/f, q*	**<10**	**<3.0**	**<5.0**	**<0.1**	**<10**	<0.1	<3.0	*o, l, j, m, r*	**<5.0**	**<3.0**	**<1.0**	**<10**	<10	<3.0
UC-Triazole	*d, e/g*	**<0.1**	**<1.0**	*b, c, k/i, f, q*	**<10**	**<1.0**	**<3.0**	**<5.0**	**>10**	<10	<1.0	*j, l, m, o, r*	**<3.0**	**<1.0**	**<1.0**	**<10**	<10	<1.0
UC-PBr	*e, d, g*	**<5.0**	**<10**	*c/f, b, k/i, q*	**<0.1**	**<0.1**	**<3.0**	**<3.0**	**>10**	<0.1	<5.0	*l, o, j, m, r*	**<3.0**	**<0.1**	**<5.0**	**<5.0**	<10	<10

Peak ID key: Group-1, [*d*] ADEN; [*e*] ETIO; [*g*] 1DHEA; Group-2, [*b*] 1STEN; [*c*] MSAL; [*f*] MSEL; [*i*] MTHY; [*k*] MIBL; [*q*] DNZL. Pair 2-B, MSAL:MSEL [*c:f*]. Pair 2-C, MTHY:MIBL [*i:k*]; Group-3, [*j*] PRST; [*l*] EPIT; [*m*] TSTO; [*o*] OXAN; [*r*] TRNB. Pair-3D, PRST:TRNB [*j:r*]. Pair-3E, EPIT:TSTO [*l:m*].

extraction. As for a duration of time, flow continues through the vessel, allowing fresh MP solvent to "actively" extract from the sample (and therefore building [i.e., adding to] the plug of analytes). The success of an on-line extraction method is highly dependent on the trapping of this "plug," which must be retained at the column head for the entire duration of the dynamic step. In order to effectively trap analytes, the modifier concentration must be low enough and/or the column must be retentive enough to allow retention of all the analytes present. For these reasons, the dynamic extraction has the largest effect on the integrity of the trapping of the "extract-plug."

In the SFE-simulation (Figure 6; Dynamic Simulation [*blue*]), the "injection-loop" replaces the "extraction-loop" and therefore the "injection-plug" replaces the "extract-plug." In the simulation, prior to run start, the automated injector loads a plug of liquid onto the external injection-loop. As in any SFC-only operation, at the run start the injector valve switches, exposing the injection-loop to the system flow path. Since the MP flows thru the injection-loop, the "injection-plug" is pushed (carried) along with the system flow and ultimately is delivered to the analytical column. In the simulation timetable, in order to mimic dynamic extraction conditions, the composition of the MP was changed to a much lower modifier concentration (5% B), immediately after the injection. This lower concentration was held for a duration of 1 min before ultimately starting the gradient for analysis. This 2-min hold simulates the transport and delivery of the "plug" of analytes to the SP and the proceeding active flow of MP over the column (where the "plug" must be retained at the column head for the entire duration of this time) that would normally occur prior to analysis in on-line extractions. This acts as the "plug-retention test."

SFE-simulation analysis. In on-line extraction, analysis is the fourth step of the extraction process and follows the end of the on-line extraction (S_Figure 6; I and J). At extraction end, the extraction-valves switch to bypass the vessel and extraction-loop. This directs all MP flow directly toward the column and the chromatographic method is started for the separation of the trapped "extract-plug." In the SFE-simulation, the chromatographic method is used instead for the separation of the trapped "injection-plug" (Figure 6; Analysis [*green*]). The gradient program used for the simulation closely resembled the "generic screening gradient" used in the SFC-only column screening. This enabled direct comparison for each phase between the results obtained from the SFC-only column screening to those obtained from the "plug-retention test."

"Plug-retention test" results

Interpreting SFE-simulation results

Chromatograms obtained using the plug-retention test are compared to those obtained using the SFC-only screening gradient in Figure 7. The resulting chromatograms are overlaid on the gradient used. An example of a column

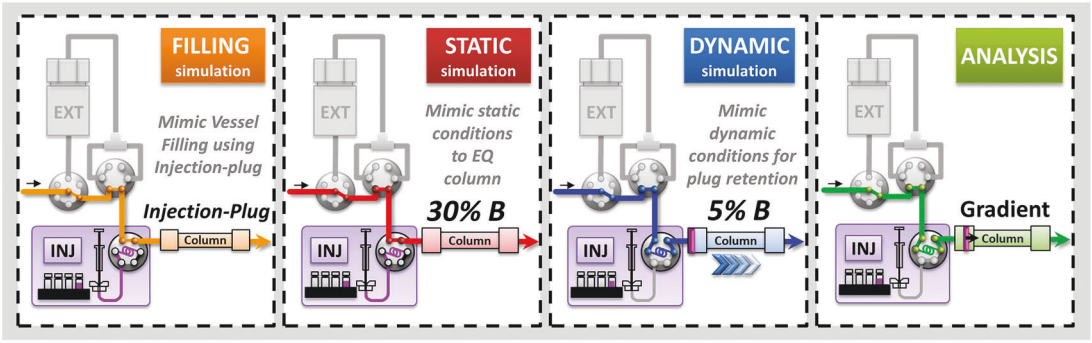

Figure 6. Instrument flow diagrams for the main steps of the SFE-simulation method for the "plug-retention test," showing conditions for sample-plug trapping using SFC-injections.

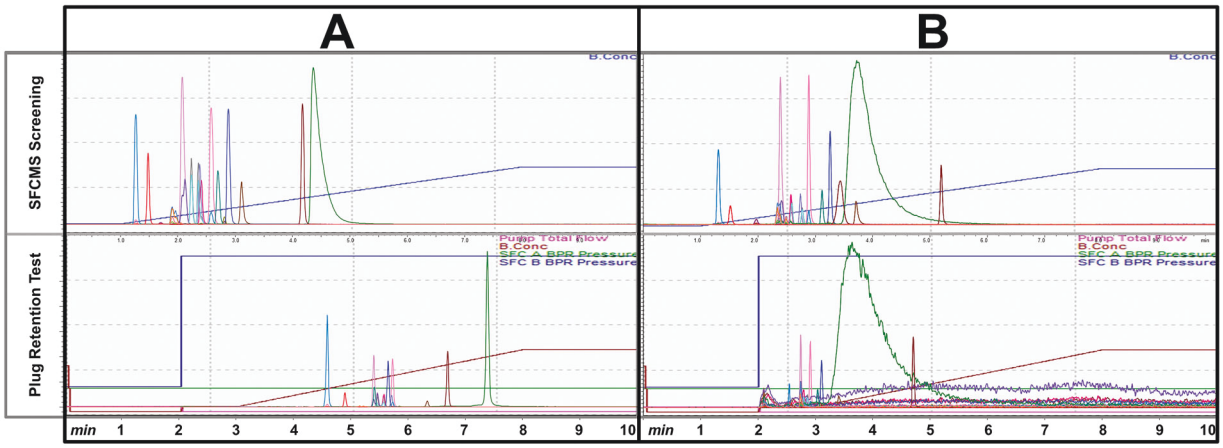

Figure 7. Example chromatograms for the interpretation of the "plug-retention test" results on two different phases: (A) Cyano column (UC-CN) and (B) 2-ethylpyridine column (2EP); comparing separations produced using [Top] the SFC-only "generic screening gradient" to [Bottom] separations produced by the plug-retention test using the "SFE-simulation method." Common conditions: 1.0 µL liquid injections of the steroid mixture [AAS-mix]; with column temperature of 50 °C; and system outlet pressure of 150 bar (*post-column*, [BPR_A]). *Overlaid MRM-TIC chromatograms displayed on the same intensity scale.* Generic screening gradient shown overlaid on top in blue (0–1 m, *isocratic* [5% MeOH]; 1–8 m, *ramp* [5–40% MeOH]) and SFE-simulation method shown overlaid on bottom in red (0–2 m, *extraction-simulation*; 3–8 m, *SFE-simulation Gradient* [5–40% MeOH]).

exhibiting good potential extract-plug retention is shown in panel-A. Alternatively, panel-B shows a column exhibiting poor plug-retention. For both columns, the top chromatogram was produced using the generic screening gradient (showing the ramp in modifier concentration in blue). The bottom chromatogram for each column was produced using the SFE-simulation gradient (modifier ramp shown in red). Note that in the SFE-simulation, the gradient does not start until 2 min into the run. This 2-min period is the duration where the SFE-simulation is performed. Once the simulation is complete, the same generic gradient ramp (as in the top chromatogram) was started. Theoretically, if the SFE-simulation had little/no effect, the only difference between the top and bottom chromatograms should be a delay in elution (of approximately 2 min).

The column showing good plug-retention was the UC-Cyano [UC-CN] column. The chromatogram from the simulation (Figure 7; panel-A [*bottom*]) looks similar to the separation using the generic screening gradient [*top*]. A longer delay before the start of elution is also notable, but generally peak shapes have not degraded. Alternatively, on the 2-ethylpyridine [2EP] column, shown in Figure 7; panel-B, when using the generic gradient [*top*], peak shapes were good (except for CLNB [green]). However, the on-line simulation (*bottom*), shows a distinct deterioration in the quality of the chromatography, exemplifying poor plug-retention. Some of the analytes have migrated down the column before analysis could begin. The high baseline noise in multiple MRMs, indicate the compounds have been shmeared across the entire chromatogram. This migration (e.g., non-retention of the plug) clearly degrades the analysis and some of the peaks have disappeared completely.

SFE-simulation results

Plug-retention test results summary. Supplemental Figure S_Figure 7 shows the results of the extraction simulation for all 15 screened columns. Some columns that gave the best resolution between MS-critical compounds (in the SFC-only column screening) were ruled out as candidates for further development due to failed plug-retention potential.

For example, phases that showed little capability for retaining an on-line extraction-plug, such as the diol (S_Figure 7; F), amino [NH2] (S_Figure 7; G), 2-ethylpyridine [2EP] (S_Figure 7; H), UC-HyP (S_Figure 7; J) and the UC-PBr (S_Figure 7; M) columns, all had high migration of all

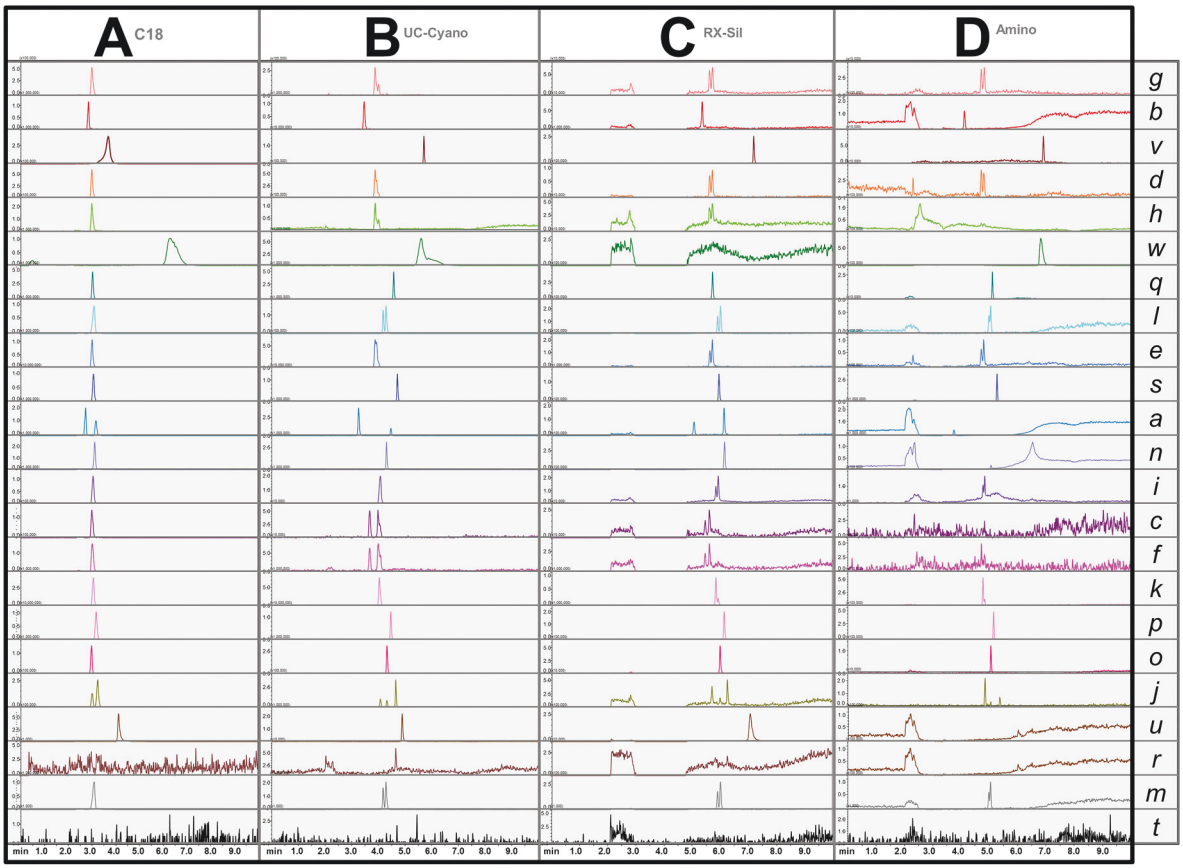

Figure 8. "Plug-retention test" stacked SFC-MS chromatograms for targeted anabolic agents produced by the SFE-simulation method across a polarity range of traditional SFC stationary phases, in order of increasing phase polarity from left to right: (A) C18 column; (B) UC-CN column; (C) RX-Sil column; and (D) NH2 column. Conditions: Separations of 1.0 μL liquid injections of the steroid mixture [AAS-mix]; using the SFE-simulation method (0–2 m, *extraction-simulation*; 3–8 m, *SFE-simulation gradient* [5–40% MeOH]), with column temperature of 50 °C; and system outlet pressure of 150 bar (*post-column*, BPR_A). *Stacked MRM-TIC chromatograms displayed on normalized intensity scale. Analyte ID key*: [*g*] 1DHEA (*pink*); [*b*] 1STEN (*red*); [*v*] ADAR (*maroon*); [*d*] ADEN (*orange*); [*w*] CLNB (*dark green*); [*q*] DNZL (*turquoise*); [*l*] EPIT (*cyan*); [*e*] ETIO (*blue*); [*s*] GSTN (*cobalt*); [*a*] KETO (*teal*); [*k*] MTHY (*light pink*); [*c*] MSAL (*magenta*); [*f*] MSEL (*rose*); [*i*] MIBL (*purple*); [*n*] METD (*lilac*); [*o*] OXAN (*hot pink*), [*p*] MTRB (*coral*); [*j*] PRST (*gold*); [*u*] STNZ (*mocha*); [*r*] TRNB (*brown*); [*m*] TSTO (*gray*); and [*t*] ZRNL (*black*).

compounds down the column before analysis was started, resulting in highly degraded chromatographic performance. High baseline noise was observed across the majority of the MRMs and ultimately resulted in the loss of most peaks. These columns were considered to have failed the plug-retention test and were ruled out for further development.

Furthermore, although the UC-Choles (S_Figure 7; K), UC-Triazole (S_Figure 7; L), PolarX (S_Figure 7; I), and both of the silica columns (S_Figure 7; D and E) all showed better potential (with improved peak shapes on the earlier eluting compounds). Analyte plug-retention was still inadequate, due to (although less significant than observed above) substantial migration of the analytes, resulting in significantly decreased peak areas for early-eluting compounds. Therefore, these phases were also ruled out for further development, due to poor plug-retention potential. On the other hand, best overall potential for analyte plug-retention was seen using the C18 (S_Figure 7; A) and both the cyano columns (S_Figure 7; B and C).

Plug-retention predictions: across a range of traditional SFC phase polarities. Stacked MRM-TIC chromatograms for each analyte are shown in Figure 8 using four columns ranging from a non-polar, C18 phase (*left*), to a more polar, amino phase (*right*). Stacking the chromatograms allows an overview of the compound-specific effect of the simulation. The high signal noise shows the deterioration in plug-retention of later eluting (more polar) compounds, especially on the more polar SPs. This is to be expected as a general trend, but since the effect is compound specific, this provides the user with vital information on the compound specific trends that should be expected across a range of phase polarities for the set of target analytes. This demonstrates the effectiveness of the simulation as an important diagnostic screening tool. Having knowledge early in the MD process of trends across phase polarities for plug-retention capabilities adds to the ability to choose a legitimate column candidate for further on-line SFE-method development.

Plug-retention predictions: nontraditional SFC phases. Predicting where column performance may lay (i.e., "plug"-retention capability) within a range of column polarities for some nontraditional phases could be difficult, especially if the phase is proprietary (as in the PolarX column). The usefulness of the simulation is especially well exemplified with the screening of the nontraditional phases. Many of the nontraditional phases produced better resolution between MS-critical groups in the SFC-only column scouting, but

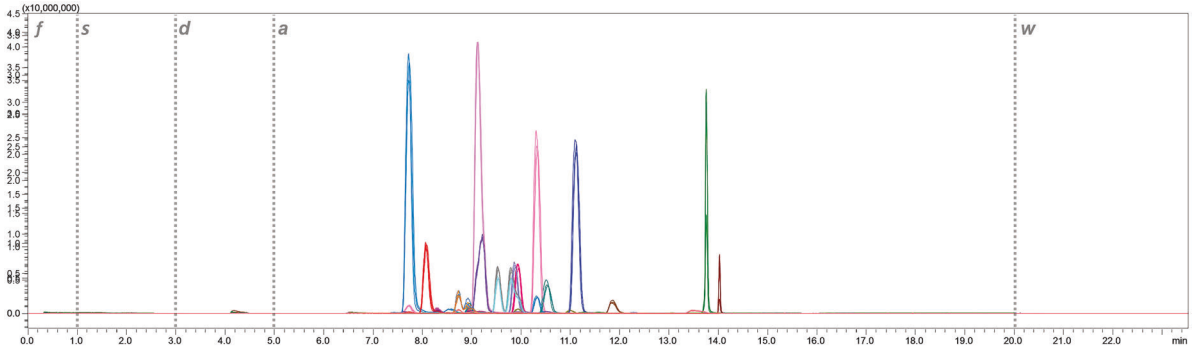

Figure 9. Overlaid SFE-SFC-MS chromatograms for "proof-of-concept" on-line extractions of replicate AAS-cores using the un-optimized hyphenated method with the UC-cyano [UC-CN] column. Showing the extraction method steps: (*f*) vessel filling; (*s*) static extraction; (*d*) dynamic extraction; (*a*) analysis ["SFE-SFC-MS gradient"]; (*w*) extraction-loop wash. *AAS-core:* One microliter of the steroid mixture ([AAS-mix] 1.0-µL/spot) applied to cellulose-based sample collection cards, dried, cored (6-mm punch) and placed inside 0.2-mL extraction vessels and extracted on-line. *Displaying overlaid MRM-TIC chromatograms for triplicate vessels overlaid on a normalized intensity scale.*

these same columns performed poorly in the plug-retention test. Normally, without the SFE-simulation, these phases would have been chosen as promising candidates and taken through further method development, and much time could have been wasted before ultimately being thrown out due to poor "plug" retentivity. Both PolarX and UC-HyP, being good examples, produced promising resolution with the generic screening gradient, but showed poor plug-retention during the simulation.

Summary of best performing phases

In the SFC-only screening, for the traditional SFC phases, improved retention was observed for all compounds on the higher polarity phases. For the nontraditional phases PolarX and UC-Trizole showed good potential for overall resolution. Specifically for the resolution of MS-critical groups: cyano, diol, UC-HyP, UC-Choles and UC-PBr were considered to be the most promising phases for the potential separation of group 1; The RX-Sil, NH2, and PolarX columns gave the best potential resolution for group 2; and UC-CN, diol, 2EP, and UC-HyP for group 3.

In the plug-retention test, diol, NH2, 2EP, UC-HyP and the UC-PBr columns failed to retain a plug of analytes and were ruled out. Although showing improved plug retention for late and mid-eluting compounds, UC-Choles, UC-Trizole, PolarX, and both of the silica columns (RX-Sil and HIL-Sil) produced significantly decreased peak areas for early-eluting compounds, and were also ruled out, due to poor plug-retention potential. Out of the best performing phases in the plug-retention test, the C18 phase could be quickly ruled out for further development, due to low overall analyte retention in the SFC-only screening. Out of this group, ultimately UC-cyano [UC-CN], was chosen as the best performing phase, which exhibited high potential not only for the resolution of the analytes but also high plug-retention potential, as well as the best peak shapes for late eluting compounds, and was chosen for further development.

Proof-of-concept on-line SFE-extractions

To provide a "proof-of-concept" for the validity of the column selected via the "plug-retention test," triplicate on-line extractions were performed using the UC-cyano column with a minimally optimized (non-fully developed) on-line SFE-SFC-MS method. For on-line extractions, the instrument was operated in SFE-SFC-MS mode (S_Figure 3; C), where a single AAS-core (1.0-µL AAS-mix/spot) was extracted and analyzed per run via the on-line extraction-loop. On-line SFE-SFC-MS chromatograms are compared (overlaid) for three replicate vessels in Figure 9. The UC-cyano column successfully trapped the extract-plug for on-line SFC-MS analysis, producing good peak shapes and peak resolution that were similar throughout all three extractions. Resulting retention time and peak area reproducibility are presented for each targeted analyte in Table 5. Retention times were reproducible (SD $\leq$ ±0.02 min) for all anabolic agents, except ZRNL (Rt = 11.1 ± 0.4 min). The signal for ZRNL was much lower than the other targeted compounds due to lower initial concentrations, and the high expense of the standard (ultimately limited the ability to fully investigate ZRNL at this stage of the investigation). Peak area reproducibilities ranged from 4% to 20% (av. peak area RSD = 7%), which were considered within reason for on-line extraction, especially when considering the un-optimized nature of the hyphenated method utilized. The exception was the latest eluting compounds CLNB and ADAR, both having > 45% RSD. Considering again that the extraction method has not been fully optimized, poor extraction performance, especially for later eluting (more polar) compounds (e.g., CLNB, ADAR and ZRNL), should be expected.

For extraction, the same steroid mixture (used in SFC-injections, [AAS-mix]) was spotted (1.0 µL/spot) onto collection cards. A 6-mm core [AAS-core] was then punched from the card for on-line extraction. Using the same volume of the same solution used for SFC-injections allows for a rapid comparison of the results. These extractions were performed in split-less mode (meaning all effluent from the extraction [i.e., the entire extract] is directed on-column). This allows for direct comparison of average extracted peak

Table 5. Proof-of-concept on-line extraction, average area and retention times for anabolic agents.

Elution order	Analyte ID	Peak ID	Retention time [Rt] Average (min) ± SD ($n = 3$)	Peak area		
				Average ($n = 3$)	%RSD (area)	%SFC inj. area (%$Area_{SFC}$)
1	KETO	*a*	7.73 ± 0.01	2.0×10^7	13%	102 ± 7%
2	1STEN	*b*	8.08 ± 0.01	4.2×10^6	13%	42 ± 3%
3	MSAL	*c*	8.30 ± 0.02	1.2×10^5	7%	99 ± 4%
4	ADEN	*d*	8.74 ± 0.02	2.1×10^5	3%	78 ± 1%
5	ETIO	*e*	8.90 ± 0.03	6.9×10^5	12%	38 ± 2%
6	MSEL	*f*	8.99 ± 0.01	3.5×10^5	12%	81 ± 5%
7	1DHEA	*g*	9.03 ± 0.01	7.3×10^4	15%	23 ± 2%
8	MTHY	*i*	9.13 ± 0.01	1.2×10^7	19%	52 ± 5%
9	PRST	*j*	9.17 ± 0.02	6.2×10^4	8%	67 ± 3%
10	MIBL	*k*	9.22 ± 0.01	6.1×10^6	16%	44 ± 4%
11	EPIT	*l*	9.81 ± 0.01	1.8×10^6	10%	43 ± 2%
12	TSTO	*m*	9.80 ± 0.01	2.2×10^6	9%	46 ± 2%
13	METD	*n*	9.88 ± 0.01	7.6×10^5	4%	35 ± 1%
14	OXAN	*o*	9.95 ± 0.01	2.0×10^6	7%	35 ± 1%
16	DNZL	*q*	10.53 ± 0.01	2.4×10^5	2%	26 ± 1%
17	TRNB	*r*	11.23 ± 0.01	4.3×10^4	10%	8 ± 1%
18	GSTN	*s*	11.12 ± 0.02	3.4×10^6	5%	58 ± 1%
19	ZNRL	*t*	11.12 ± 0.38	1.7×10^3	10%	29 ± 1%
20	STNZ	*u*	11.85 ± 0.01	1.2×10^6	16%	26 ± 2%
21	CLNB	*v*	13.76 ± 0.01	1.3×10^7	45%	10 ± 2%
22	ADAR	*w*	14.03 ± 0.01	3.1×10^6	62%	4 ± 1%
–	ADON	*h*	–	–	–	–

areas to those obtained from SFC-injections. Percent of the SFC-injection areas were calculated (Table 5; %$Area_{SFC}$) and are shown in S_Figure 8 for each analyte. The %$Area_{SFC}$ was compound specific and ranged from 5% to 102% (av. = 52%). All compounds were above 25%, except CLNB (13%) and ADAR (5%). Lower extractability was expected, both being late eluting (more polar) compounds, using the un-optimized extraction method. Alternatively, KETO gave %$Area_{SFC}$ > 100% of the SFC injections. This could be an effect of pressure differences during plug loading onto the column changing peak shape/broadening of peaks, and or decreasing resolution between neighboring peaks, contributing co-eluting area which was not present in the SFC-only injections, but is most likely an effect of background matrix from the sampling materials (e.g., the cellulose-based collection card). Both background matrix and optimization of the extraction method will be required in further method development stages for future work.

To further extend the preliminary tests on the best performing column, it could also be envisioned that at this stage, an on-line extract test could be performed using spiked blank sample matrix (if available). Extending the test to include an early evaluation of the effect (if any) of the presence of the sample matrix, on the columns ability to retain a plug of analytes, would provide valuable information of potential matrix interferences. Columns that were unable to maintain good plug retention in the presence of the sample matrix could also be ruled out at an early stage. Therefore, the need for re-optimizations would only depend on the complexity of the separation required (e.g., demanded by the set of target analytes and the complexity of the sample matrix). Particularly difficult situations may still require further re-optimization at later stages. In most cases, much time is saved by providing the information needed to make well-informed SP selection choices at very early stages of SFE-SFC-MS MD process.

Conclusions

Thirteen stationary phases were screened as part of the second development step of a hyphenated SFE-SFC-MS method for the on-line extraction of anabolic agents in anti-doping testing. Columns were compared using a rapid screening approach, allowing for an expedited narrowing down of potential phases. This approach adapted the traditional hyphenated MD flow for the first time to include a "plug-retention test" to aid specifically in the column selection procedure for on-line extractions.

This "plug-retention test" evaluated each column via an SFE-simulation method which mimicked the on-line loading of an SFE-based extract-plug, by instead trapping an SFC-based injection-plug under instrument conditions similar to those that would be involved with an actual on-line extraction. This enabled a rapid evaluation of the potential "active trapping" of analytes at the head of the analytical column. Therefore, this novel approach provided a more informed SP screening process, which took into consideration not only the potential for the chromatographic resolution of targeted analytes but also gave an early evaluation of each phases ability to retain/'trap' a plug of analytes. Using the adapted SP scouting approach, the best performing column was determined to be the UC-cyano column, not only for its potential for chromatographic separation of MS-critical AAS groups, but also good potential for plug-retention during the SFE-simulation.

Proof-of-concept extractions were performed on-line using the selected column with a generic extraction method. These on-line extractions proved the successful selection of a stationary phase (capable of retaining a plug of the targeted analytes) on the first round of SFE-SFC-MS column selection. A unidirectional flow of the on-line MD process circumvents the need to restart MD at later stages. Demonstrating a successful first round SP selection procedure saves ample time and effort

normally spent on re-optimizations and effectively streamlines the hyphenated SFE-SFC-MS method development process.

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Disclosure statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: YF, WH, and MN are employed by Shimadzu Corporation or Shimadzu Scientific Instruments, Inc., the manufacturer of the instrumentation utilized in this work.

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