Application Note

Clinical Research

Quantitative Determination of a Panel of Endogenous Steroids in Human Serum by LC/MS/MS

Using an Agilent Supported Liquid Extraction (SLE) Chem Elut S Plate

Author

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Abstract

Agilent Chem Elut S supported liquid extraction (Chem Elut S) uses synthetic media to provide better consistency and higher water holding capacity than traditional diatomaceous earth. This study demonstrates the application of Chem Elut S 2 mL 96-well plates for the quantitative determination of a panel of 15 endogenous steroids from human serum by LC/MS/MS. Serum samples are prepared using supported liquid extraction (SLE) to extract target analytes and remove matrix interferences such as salts, proteins, and partial phospholipids. The entire sample treatment was conducted in the 96-well plate as a batch process with aqueous sample loading onto the SLE sorbent first, then gravity elution with a water immiscible solvent. The Chem Elut S workflow in-plate offers significant time and labor savings compared to traditional liquid-liquid extraction (LLE). With the excellent method analytes accuracy (80 to 120%) and reproducibility (RSD <15%), the established SLE method was verified for the broad calibration range of 5 to 10,000 pg/mL in serum for targeted steroids, except 10 to 10,000 pg/mL for estradiol and testosterone and 20 to 10,000 pg/mL for hydrocortisone, 17-hydroxypregnolone, and progesterone. The method selectivity and carryover were evaluated as well, and the raised limit of quantitation (LOQ) for five analytes were due to the selectivity in serum matrix. In addition, the synthetic SLE sorbent also provides better phospholipid depletion from biological fluid matrices compared to diatomaceous earth-based SLE and traditional LLE, given the sample solvent used for extraction.

Introduction

Traditional LLE has been widely used for endogenous steroid extraction from biological sample matrices such as serum and plasma for steroids analysis with or without derivatization.[1–3] In these protocols, biological samples such as serum or plasma were usually aliquoted to the individual microcentrifuge tubes followed by extraction solvent. After sample mixing and centrifuging, the upper organic layer was transferred to another set of tubes or a 96-well plate for the subsequent sample treatment. In practice, this procedure includes labor-intensive and time-consuming steps such as tube labeling, sample mixing, phase separation, and organic layer transfer, which are the rate-limiting steps for high-throughput sample preparation.

An alternative approach to LLE that overcomes these disadvantages is SLE. The sorbent of SLE cartridges provides a chemically inert surface for the aqueous sample to coat onto. After equilibrium, the target analytes are eluted with a water-immiscible solvent using gravity, gentle vacuum, or pressure, while the aqueous phase remains in the cartridge. Due to the large supported aqueous surface area provided by the SLE sorbent, target analytes are efficiently extracted into the organic solvent as it flows through the medium. Figure 1 shows the extraction process. With SLE, mixing steps are not needed, and emulsions can efficiently be prevented. In addition, the intimate contact between the aqueous and organic phases allows efficient partitioning, giving high analyte recoveries. Because of the loading and elution workflow, the SLE workflow is simple, with significantly reduced labor and time demands. Lastly, the 96-well plate SLE format is especially amenable to automation, which increases sample preparation throughput.

Traditionally, the sorbent used for SLE is highly purified diatomaceous earth. However, diatomaceous earth is a natural material, consisting of irregular fossilized micro-organisms, and it is difficult to control the batch-to-batch consistency of the sorbent particles. The sorbent variability adds difficulties to product manufacturing and quality control, as well as leading to product performance inconsistency. Furthermore, diatomaceous earth can give lower and inconsistent water-holding capacity compared to a synthetic medium.

The Chem Elut S synthetic sorbent greatly improves water-holding capacity, batch-to-batch consistency, and performance consistency. This Application Note demonstrates the application of Chem Elut S 96-well plates for the quantitative determination of a panel of 15 endogenous steroids (Table 1) in human serum.

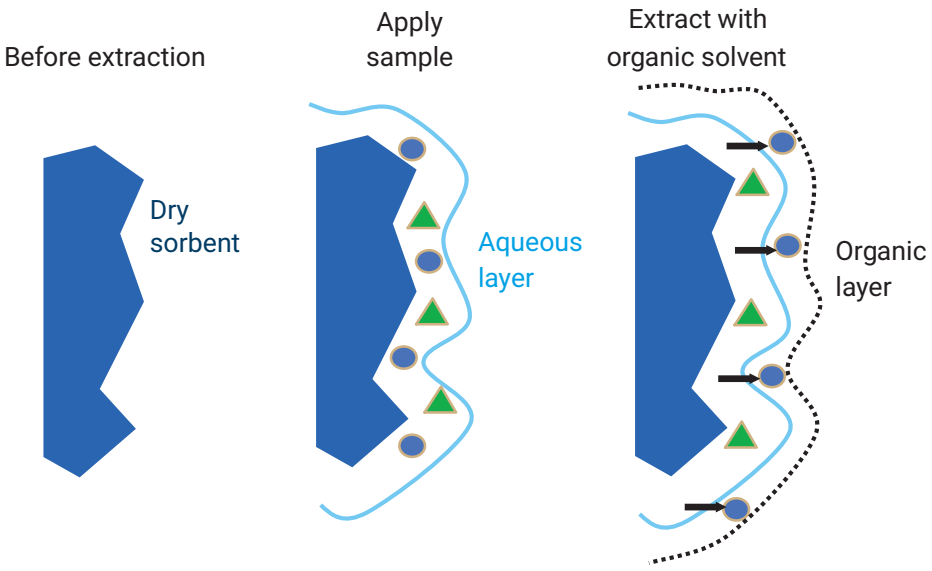
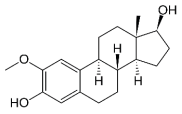
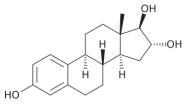
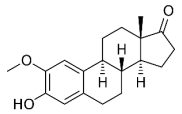
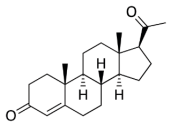
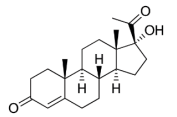
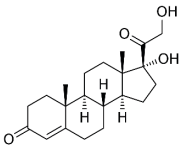
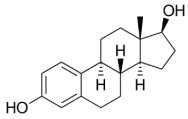
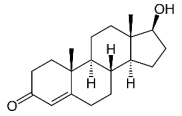
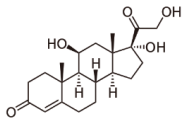
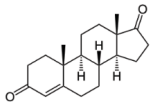
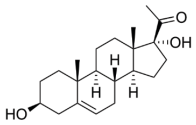
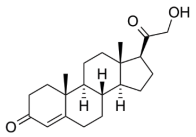
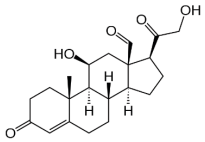
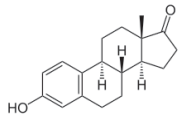
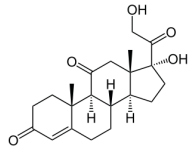


Figure 1. The process and analyte extraction mechanism of SLE.

Table 1. Molecular formulas and structures for 15 target steroids.

Name	Log P	Molecular Formula	Structure
2-Methoxyestradiol (2-MeE1)	4	$C_{19}H_{26}O_3$	
Estriol (E2)	2.5	$C_{18}H_{24}O_3$	
2-Methoxyestrone (2-MeE1)	3.1	$C_{19}H_{24}O_3$	
Progesterone (PGT)	3.9	$C_{21}H_{30}O_2$	
17-Hydroxyprogesterone (17-OH PGT)	3.2	$C_{21}H_{30}O_3$	
11-Deoxycortisol (11-DCTS)	2.5	$C_{21}H_{30}O_4$	
Estradiol (E2)	4	$C_{18}H_{24}O_2$	
Testosterone (TTS)	3.4	$C_{19}H_{28}O_2$	

Name	Log P	Molecular Formula	Structure
Hydrocortisone (HCTS)	1.6	$C_{21}H_{30}O_5$	
Androstenedione (ASD)	2.7	$C_{19}H_{26}O_2$	
17-α-Hydroxypregnenolone (17-OH PGN)	3.1	$C_{21}H_{32}O_3$	
11-Deoxycorticosterone (11-DCCS)	2.9	$C_{21}H_{30}O_3$	
Aldosterone	1.1	$C_{21}H_{28}O_5$	
Estrone (E1)	3.1	$C_{18}H_{22}O_2$	
Cortisone (CTS)	1.5	$C_{21}H_{28}O_5$	

Experimental

Reagent and chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA), methyl *t*-butyl ether (MTBE) was from VWR-BDH Chemicals (Radnor, PA, USA), and ethyl acetate (EtOAc) was from J.T. Baker (Center Valley, PA, USA). Ammonium fluoride, all steroid standards, and internal standard stock solutions, either 1 mg/mL in MeOH or 100 µg/mL in MeOH, were from Sigma-Aldrich (St. Louis, MO, USA). Human serum (DC Mass Spect Gold, MSG4000) was purchased from Golden West Biologicals, Inc. (Temecula, CA, USA). The serum was stored at –70 °C until use.

Standards and solutions

The standard spiking solution was 500 ng/mL in 1:1 MeOH/water, and the IS spiking solution was 50 ng/mL in 1:1 MeOH/water. Steroid compounds are sensitive to glass surfaces, especially at low levels. Therefore, it is important to use plastic vials for preparing and storing all spiking solutions. All spiking solutions were stored at –20 °C until use.

The 1 mM ammonium fluoride (NH_4F) solutions in water and MeOH were made by dissolving 37.04 mg of NH_4F into 1 L of Milli-Q water and MeOH. The 0.2 mM NH_4F in water (mobile phase A) solution and 0.2 mM NH_4F in MeOH (mobile phase B) were prepared by diluting 1 mM solutions with Milli-Q water and MeOH, respectively.

The extraction solvent, 1:1 MTBE/EtOAc, was freshly prepared by mixing 100 mL of MTBE and EtOAc, and used for the day. A 1:1 MeOH/water solution was used to reconstitute dried samples after SLE.

Equipment and materials

Equipment used for sample preparation included:

- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Multitube vortexer (VWR, PA, USA)
- Eppendorf pipettes and repeater
- SPE Dry 96 evaporator
- Agilent positive pressure manifold 96 processor (PPM-96, part number 5191-4116)
- ViaFlo 96 Liquid Handler (Integra, Hudson, NH, USA)
- Agilent Chem Elut S 2 mL 96 well plate, 200 µL (part number 5610-2003)
- Agilent square 96-well 2 mL collection plate (part number 5133009)
- Agilent square 96-well sealing caps (part number 5133005)

Instrument conditions

The samples were run on an Agilent 1290 Infinity LC consisting of an Agilent 1290 Infinity binary pump (G4220A), Agilent 1290 Infinity high performance autosampler (G4226A), and an Agilent 1290 Infinity thermostatted column compartment (G131C). The UHPLC system was coupled to an Agilent G6490 triple quadrupole LC/MS system equipped with an Agilent Jet Stream iFunnel electrospray ionization source. Agilent MassHunter workstation software was used for data acquisition and analysis.

See Table 2 for analyte parameters and Figure 2 for the LC/MS/MS chromatogram at 1 ng/mL of steroids in serum.

HPLC conditions	
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 100 × 2.1 mm, 1.8 µm (p/n 959758-902) Agilent ZORBAX RRHD C18 guard, 5 × 2.1 mm, 1.8 µm (p/n 821725-901)
Flow Rate	0.4 mL/min
Column Temperature	40 °C
Injection Volume	20 µL
Mobile Phase	A) 0.2 mM ammonium fluoride in water B) 0.2 mM ammonium fluoride in MeOH
Needle Wash	1:1:1:1 ACN/MeOH/IPA/H_2O w/ 0.2% FA
Gradient	Time (min) %B Flow rate (mL/min) 0 50 0.4 3.0 60 0.4 8.0 90 0.4 8.5 100 0.4
Stop Time	8.5 minutes
Post Time	2.5 minutes
MS Conditions	
Gas Temperature	180 °C
Gas Flow	11 L/min
Nebulizer	20 psi
Sheath Gas Heater	400 °C
Sheath Gas Flow	10 L/min
Capillary	3,500 V (positive) 3,000 V (negative)
iFunnel Parameters	High-pressure RF: 150 V (positive and negative) Low-pressure RF: 100 V (positive and negative)
Data Acquisition	dMRM

Table 2. Steroid analytes, IS used, retention time, and MRM conditions.

Analyte	Internal Standard Used	Retention Time (min)	Polarity	Precursor Ion (*m/z*)	Product Ion (*m/z*)			
					Quant Ion	CE (V)	Qual Ion	CE (V)
Estriol	IS 1	2.04	NEG	287.2	171.0	45	143.1	73
Aldosterone-D_4 (IS1)		2.31	POS	365.2	347.1	17	319.2	21
Aldosterone	IS 1	2.32	POS	361.2	343.2	17	91.1	80
Cortisone-D_8 (IS2)		2.71	POS	369.2	169.2	21	125.1	49
Cortisone	IS 2	2.75	POS	361.2	163.2	25	91.0	73
Hydrocortisone	IS 2	3.24	POS	363.2	121.1	25	91.1	73
17-Hydroxypregnenolone	IS 2	3.24	NEG	331.2	313.2	21	--	
11-Deoxycortisol-D_5 (IS3)		4.53	POS	352.3	100.0	37	113.0	45
11-Deoxycortisol	IS 3	4.58	POS	347.2	109.0	41	97.0	41
Androstenedione	IS 3	5.31	POS	287.2	97.0	25	109.0	29
Estradiol-D_5 (IS4)		5.39	NEG	276.2	147.0	45	187.0	49
Estrone-$^{13}C_3$ (IS5)		5.43	NEG	272.3	148.0	41	146.0	61
Estrone	IS 5	5.42	NEG	269.1	145.0	49	143.1	73
Estradiol	IS 4	5.45	NEG	271.2	145.1	49	183.0	49
11-Deoxycorticosterone	IS 3	5.72	POS	331.0	97.1	25	109.1	33
2-Methoxyestrone	IS 5	5.85	NEG	299.2	284.1	25	--	--
Testosterone	IS 6	5.86	POS	289.3	97.1	23	109.1	25
2-Methoxyestradiol	IS 4	6.01	NEG	301.2	286.2	25	--	--
17-Hydroxyprogesterone-D_8 (IS6)		6.21	POS	339.3	100.1	45	113.2	37
17-Hydroxyprogesterone	IS 6	6.26	POS	331.2	109.1	33	97.1	29
Progesterone-D_9 (IS7)		7.39	POS	324.3	100.1	29	113.0	25
Progesterone	IS 7	7.44	POS	315.2	97.1	25	108.9	37

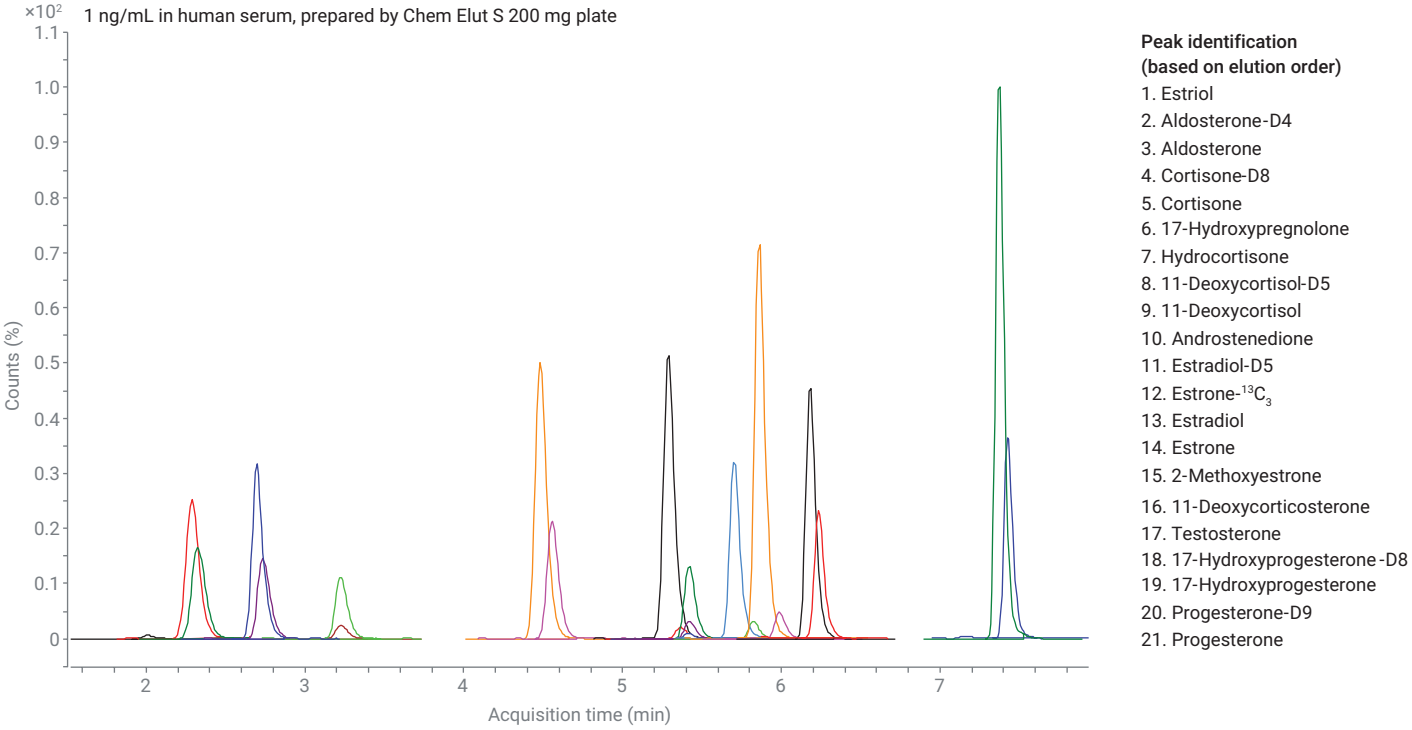


Figure 2. An LC/MS/MS chromatogram of 1 ng/mL steroids in serum prepared using the Agilent Chem Elut S supported liquid extraction method.

Calibration standards and QC sample preparation

An intermediate standard spiking solution of 50 ng/mL in serum was prepared using the 500 ng/mL standard spiking solution (in 1:1 MeOH/water). This intermediate standard spiking solution was then used to prepare calibration curve standards in human serum. The calibration standards, including 5, 10, 20, 50, 100, 500, 1000, 5000, and 10,000 pg/mL in serum, were prepared by spiking the appropriate volume of intermediate standard spiking solution into serum blank. Four levels of quality control (QC) samples were run for accuracy and precision method verification tests, including lowest limit of quantitation (LLOQ) of 5(10/20) pg/mL, mid QC of 100 pg/mL, high QC of 1,000 pg/mL, and highest limit of quantitation (HLOQ) 10,000 pg/mL. These QC samples were prepared by spiking the appropriate volume of intermediate serum sample as well. All the calibration standards and QCs were prepared in the 2 mL snap cap tubes or 5 mL plastic tubes. They were then aliquoted into the 96-well plate for extraction.

Sample extraction

The SLE extraction optimization was based on the consideration of analyte recovery and matrix cleanliness, including the selection of solvent and elution volume and times. Figure 3 describes the optimal sample preparation procedure in detail. The entire protocol includes four major sections, sample aliquoting on the plate, mixing with IS and transferring to the SLE plate, sample extraction on the SLE plate, and sample posttreatment on the collection plate. Serum samples were aliquoted into a 1 mL collection plate followed by the addition of IS spiking solution. After sample vortexing, the entire sample mixture was transferred to a Chem Elut S 2 mL plate with a 2 mL square collection plate beneath. Use caution when adding the extraction solvent to the SLE plate to avoid solvent splashing, which can result in cross-contamination. Gravity elution was used until the final sorbent drying step, where slowly initiating pressure or vacuum was used. Due to the use of the volatile solvents MTBE and EtOAc for extraction, it is important to conduct the extraction steps in a fume hood.

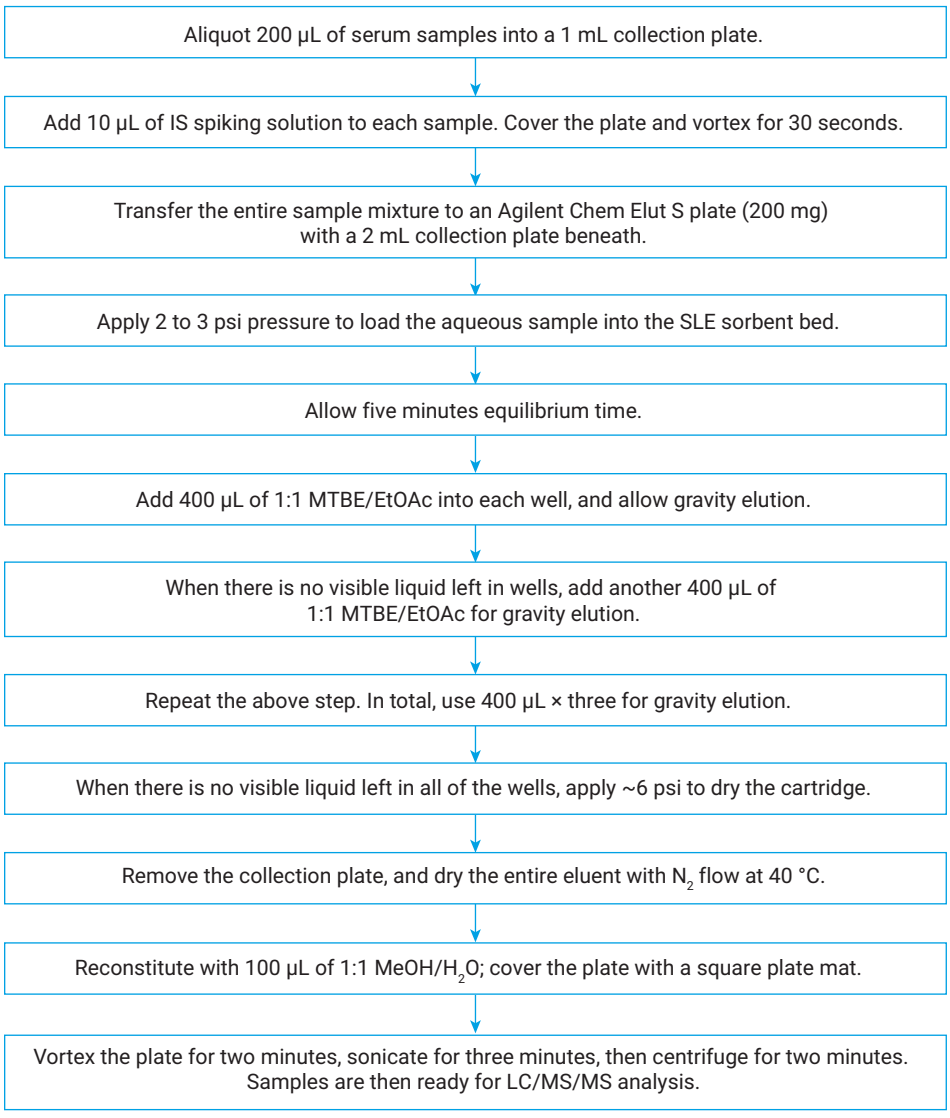


Figure 3. Sample preparation workflow scheme for steroids analysis in serum by Agilent Chem Elut S 2mL plate, 200 µL.

Method verification

Method verification applied a three-day accuracy and precision (A and P) run. As three different batches of SLE plates were used for each day's A and P run, the three-day A and P method verification was also used for SLE plate batch-to-batch reproducibility tests. Both calibration standards and QCs were prespiked appropriately. Samples were aliquoted into a collection plate with the following sequence: double matrix blank, matrix blank (spiked with IS), first set of calibration standards, two to three matrix blanks, LLOQs (n = 6), mid QCs (n = 6), high QC (n = 6), and HLOQ (n = 6), two to three carryover matrix blanks, double matrix blank, matrix blank, a second set of calibration standards, two to three matrix blanks.

Method and product comparison

The verified SLE method was also compared with LLE and diatomaceous earth-based SLE for analyte recoveries, reproducibility (RSDs), matrix effects, and calibration curve linearity.

Analyte absolute recoveries were studied by comparing the analytes' instrument responses (peak areas) between prespiked and postspiked QC samples at 1 ng/mL in serum. Prespiked QCs were spiked appropriately in serum directly and samples were prepared with the developed method. Postspiked QCs were spiked in the matrix blanks after extraction. In detail, postspiking happened during the sample reconstitution step using the appropriate neat standard solution to reconstitute dried matrix blank samples. Matrix effects were studied by comparing the analytes' instrument responses (peak areas) between postspiked QC samples and corresponding neat standards made in reagent blank solvent.

Matrix phospholipids depletion

Matrix cleanliness was investigated by monitoring the phospholipids profile. The serum sample extraction using the Chem Elut S plate demonstrated partial phospholipids depletion. The phospholipids profiles of matrix blanks were compared for both stripped serum and other kinds of plasma. The use of different extraction solvents on phospholipid depletion was further compared using the Chem Elut S plate and a competitor's corresponding diatomaceous earth-based SLE plate.

Results and discussion

This study focused on demonstrating the use of Chem Elut S plates for the quantitative determination of steroids in serum for clinical research applications.

Method development and optimization

Instrument method and special cautions: The ionization of five steroid analytes: estriol, estrogen, estradiol, 2-methoxyestrogen, and 2-methoxyestradiol, with an ESI or APCI source is very difficult. With regular conditions, it is hard to see these compounds' signals even at high concentrations. Derivatization of these compounds is an option, but it requires extra steps during sample preparation. It is also hard to perform a combined sample preparation method for steroids with and without derivatization. It has been reported that the use of ammonium fluoride buffer helps with negative ion mode ionization,[4] and improves the steroid analytical sensitivity dramatically.[5] Based on these learnings, a 1 mM ammonium fluoride buffer was used as mobile phase for those difficult steroid compounds, and provided a significant improvement in instrument analytical sensitivities. Further investigation of the buffer concentrations found that lower ammonium fluoride concentration (0.2 mM) provided better analytical sensitivity. In addition, the same salt concentration in both mobile phases A and B provided more consistent results. The optimized condition was then decided to be 0.2 mM ammonium fluoride in water and MeOH as mobile phase A and B.

As mentioned previously,[5] the use of ammonium fluoride buffer not only increased ionization of these difficult steroid compounds in negative mode, but also increased the ionization of other compounds in positive mode. An instrument method was established based on this condition for the entire panel of 15 steroid analytes. Figure 4 shows the E1 and E2 chromatograms at the level of 5 pg/mL in serum, the limit of quantitation (LOQ), as the demonstration of improved method analytical sensitivity.

An important point of information is that steroid compounds are very sensitive to glass surfaces, especially at low concentrations. The use of glass vials could result in significant variations and loss of steroid compounds, especially in sample medium containing high aqueous levels. As a result, glass vials and tubes should be avoided during standard and sample preparation and handling.

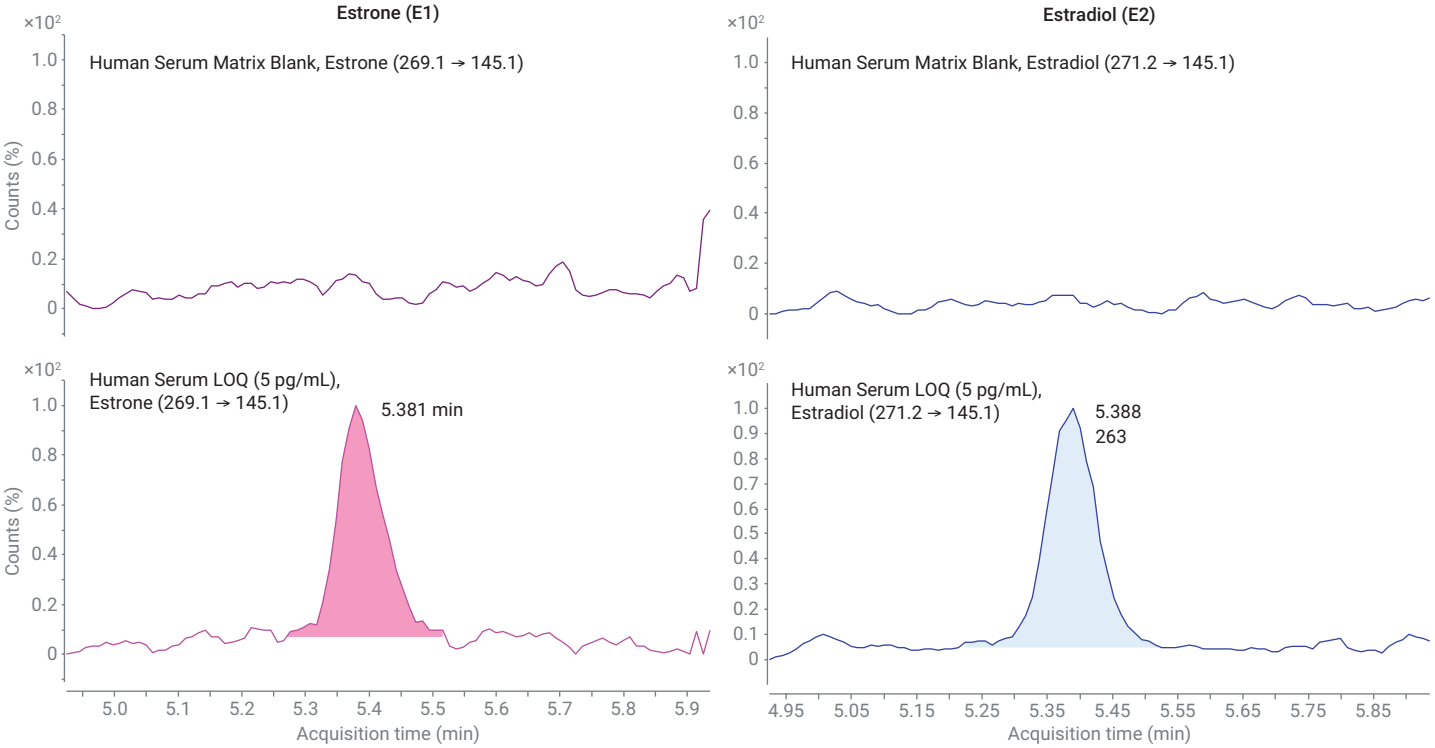


Figure 4. Estrogen (E1) and estradiol (E2) chromatogram for serum blank and LOQ at 5 pg/mL in serum.

SLE method optimization: The SLE method can be developed from an existing LLE method, or it can be developed directly on SLE cartridges or plates. In this study, the SLE method was optimized directly on SLE plates with the referencing of existing published LLE methods. SLE method optimization included solvent, solvent mixture, and sample elution optimization. The optimization was based on the combinatory assessment of average analyte recovery, RSD, and matrix effect, as shown in Figure 5.

The extraction solvent was investigated first with the evaluation of four commonly used LLE solvents/solvent mixtures: MTBE, diethyl ether (DEE), EtOAc, and DCM/MTBE (1:4). As shown in the first group comparison in Figure 5 (left) for solvent selection, MTBE extraction provides the best average matrix effect, indicating the cleanest matrix, but the average recovery is lower. EtOAc extraction provides the best extraction efficiency but also results in more matrix ion suppressions. Both DEE and DCM/MTBE mixture extraction have lower analyte recoveries, and DCM/MTBE extraction also causes poorer reproducibility. As a result, the mixture of MTBE/EtOAc was further studied to find the best balance between analytes recoveries and matrix effects.

In the second group comparison (mid) in Figure 5 for solvent mixture optimization, MTBE and EtOAc were mixed at the ratio of 1:1, 2:1, and 1:2 and used for sample extraction. Figure 5 shows that the 1:1 MTBE/EtOAc extraction mixture provided the best balance between analyte recovery and matrix effect, and therefore was selected as the optimal solvent for extraction. These studies were based on double extractions with 500 µL of solvent. The sample extraction was further investigated using 400 µL of the optimal solvent for triple extraction. The comparison results are shown as the third group comparison (right) in Figure 5. Using triple extraction with 400 µL elution, the average analyte recovery was further increased, without the sacrifice of matrix effect. As a result, the SLE method was optimized with triple extraction using 400 µL of 1:1 MTBE/EtOAc.

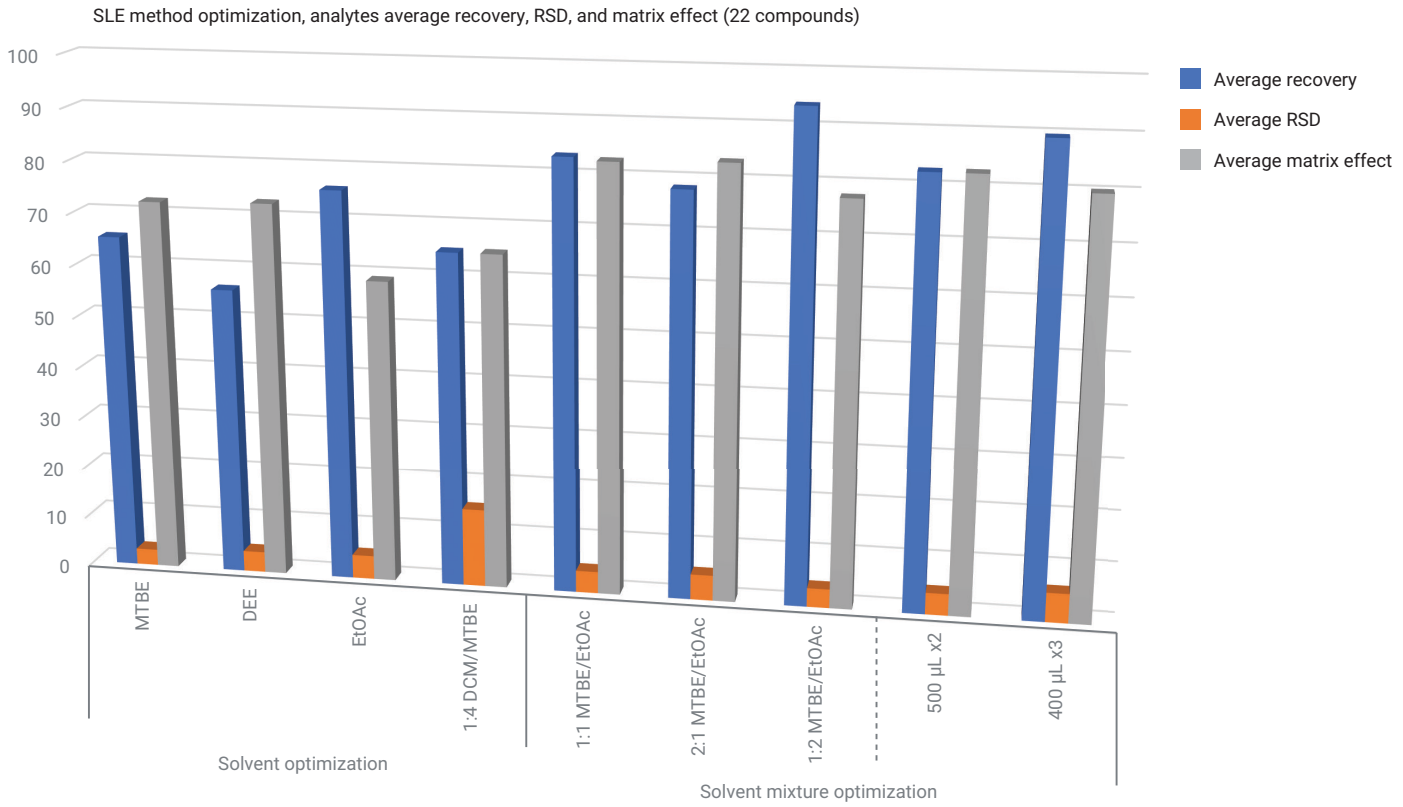


Figure 5. SLE method optimization for solvent selection and sample elution. The optimization was based on the combinatory assessment on average analyte recoveries, RSDs, and matrix effects.

Method verification

The optimized method was verified by three-day accuracy and precision runs to collect the complete quantitative results. The results shown in Table 3 include calibration curve data and three-day/batch accuracy and precision data. Calibration curves were generated using a linear regression with a weight of $1/x^2$ for all the analytes, with R^2 >0.99. An LOQ of 5 pg/mL in serum was established for most of the analytes, except 10 pg/mL for estradiol and testosterone, and 20 pg/mL for progesterone, 17-hydroxypregnolone, and hydrocortisone due to matrix interference or analytes occurring in the matrix blank. The three-day accuracy and precision runs not only demonstrate the method verification for reliable quantitation, but also the Chem Elut S plate batch-to-batch reproducibility as three manufacturing lots of SLE plates were used. Figure 6 shows the summary of SLE plates batch-to-batch reproducibility, based on analyte accuracy and precision.

Table 3. Method verification results for the quantitative determination of 15 steroid compounds in human serum.

Analyte	LOQ (pg/mL)	Calibration Range (pg/mL)	QC Spiking Concentration (pg/mL)	Accuracy and Precision						
				Day 1 (SLE Batch 1)		Day 2 (SLE Batch 2)		Day 3 (SLE Batch 3)		
				Accuracy% (n = 6)	RSD% (n = 6)	Accuracy% (n = 6)	RSD% (n = 6)	Accuracy% (n = 6)	RSD% (n = 6)	Interday/Batch RSD% (n = 18)
Estriol	5	5–10,000	5	95	18.0	97	6.9	103	11.6	12.8
			100	102	6.5	97	8.3	107	5.2	6.4
			1,000	98	2.8	92	1.2	92	4.6	3.1
			10,000	107	5.9	92	5.6	101	4.2	5.0
Aldosterone	5	5–10,000	5	106	4.7	103	12.4	100	9.4	9.2
			100	96	9.3	101	12.8	97	3.0	8.1
			1,000	111	2.5	103	4.2	98	1.9	3.3
			10,000	113	2.4	95	7.5	107	4.2	4.2
Cortisone	5	5–10,000	5	104	9.8	92	10.1	99	7.6	9.5
			100	93	7.0	93	9.2	97	7.6	7.6
			1,000	104	3.5	94	3.7	100	2.4	3.3
			10,000	106	5.6	95	7.8	106	5.5	6.2
Hydrocortisone	20	20–10,000	20	89	6.5	97	13.6	86	12.6	11.1
			100	96	18.2	90	6.7	96	9.5	11.3
			1,000	96	4.4	93	2.4	93	4.1	3.8
			10,000	95	6.3	92	4.1	97	6.8	5.6
17-Hydroxypregnenolone	20	20–10,000	20	109	1.8	99	18.9	91	8.1	9.8
			100	94	19.2	91	8.2	103	15.4	14.0
			1,000	94	6.0	91	2.1	100	5.2	4.6
			10,000	93	3.6	97	3.8	106	9.3	5.3
11-Deoxycortisol	5	5–10,000	5	99	8.3	95	10.7	95	16.2	11.9
			100	94	6.5	89	10.4	97	9.1	8.5
			1,000	105	1.5	87	3.8	99	1.7	2.5
			10,000	110	2.7	92	7.2	104	4.5	4.6
Androstenedione	5	5–10,000	5	99	11.4	102	9.5	110	11.2	10.9
			100	88	8.2	89	8.8	106	10.7	9.0
			1,000	104	2.1	90	3.7	91	1.6	2.7
			10,000	109	5.5	101	5.7	108	7.6	6.0
Estrone	5	5–10,000	5	95	8.7	92	11.3	102	8.9	9.8
			100	98	10.1	95	7.5	99	4.3	7.1
			1,000	104	2.8	95	2.8	96	3.0	3.1
			10,000	105	6.2	94	3.3	98	4.3	4.4

Analyte	LOQ (pg/mL)	Calibration Range (pg/mL)	QC Spiking Concentration (pg/mL)	Accuracy and Precision						
				Day 1 (SLE Batch 1)		Day 2 (SLE Batch 2)		Day 3 (SLE Batch 3)		
				Accuracy% (n = 6)	RSD% (n = 6)	Accuracy% (n = 6)	RSD% (n = 6)	Accuracy% (n = 6)	RSD% (n = 6)	Interday/Batch RSD% (n = 18)
Estradiol	10	10–10,000	10	105	10.2	101	19.3	100	9.7	13.2
			100	97	11.4	96	10.2	102	10.2	10.4
			1,000	99	3.0	102	5.1	95	2.4	3.7
			10,000	101	2.9	102	2.1	100	4.3	2.9
11-Deoxycorticosterone	5	5–10,000	5	108	12.3	101	13.5	113	6.5	11.0
			100	94	7.6	94	9.8	104	13.3	10.4
			1,000	108	4.8	105	5.2	103	4.7	4.7
			10,000	109	5.8	108	5.7	115	5.3	5.4
2-Methoxyestrone	5	5–10,000	5	109	9.0	111	4.8	100	7.8	7.4
			100	93	3.7	94	7.7	95	7.2	6.0
			1,000	106	2.9	93	4.1	91	4.7	4.1
			10,000	111	3.2	101	7.2	104	2.6	4.3
Testosterone	10	10–10,000	10	100	9.0	95	8.9	94	10.9	8.2
			100	94	3.7	91	6.6	100	8.5	8.2
			1,000	102	2.9	89	4.7	97	6.2	5.6
			10,000	104	3.2	106	6.3	102	5.0	5.7
2-Methoxyestradiol	5	5–10,000	5	92	9.1	109	7.4	94	10.1	9.1
			100	96	6.2	103	7.9	98	7.2	6.9
			1,000	99	1.9	94	1.9	93	4.5	2.9
			10,000	106	3.1	99	2.5	105	2.9	2.6
17-Hydroxyprogesterone	5	5–10,000	5	98	14.9	103	9.1	95	10.8	11.8
			100	89	5.2	93	9.2	98	7.2	7.0
			1,000	105	4.8	89	4.2	100	3.3	4.3
			10,000	106	4.5	103	4.9	100	2.2	3.7
Progesterone	20	20–10,000	20	108	4.8	97	11.0	89	4.0	6.8
			100	97	6.7	91	12.1	101	5.2	7.8
			1,000	119	6.7	91	3.3	95	2.5	4.3
			10,000	112	6.8	99	7.3	104	4.2	5.9

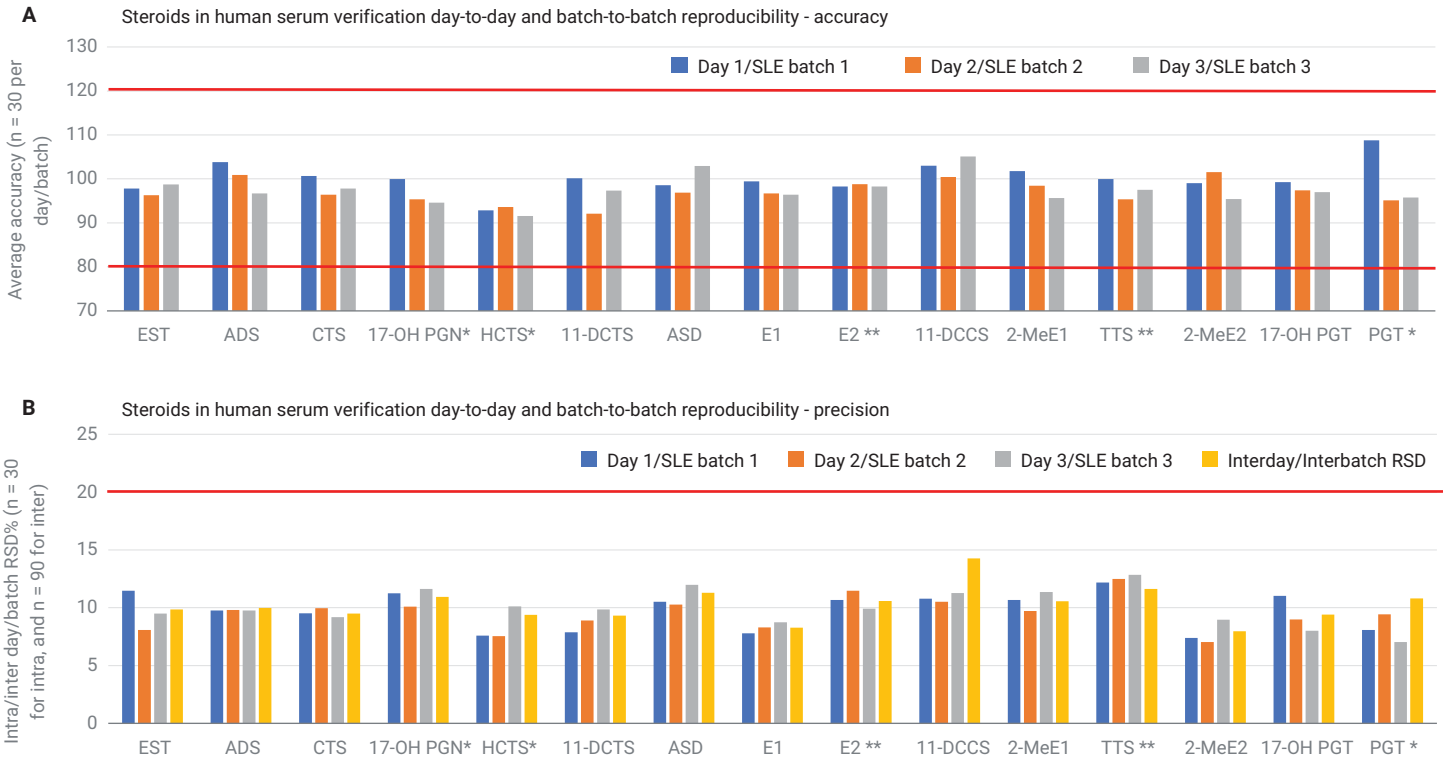


Figure 6. A day-to-day verification and plate batch-to-batch performance reproducibility summary of the Agilent Chem Elut S method based on accuracy and precision.

Method and product comparison

The developed Chem Elut S method was compared with traditional LLE and competitor's diatomaceous earth-based SLE for the method performance based on analyte recovery, reproducibility, matrix effect, and calibration curve linearity.

When comparing SLE with LLE, labor and time savings are apparent. The SLE workflow significantly improves sample preparation efficiency by saving labor and time on critical steps such as sample mixing, phase separation, and organic supernatant transferring. By obsoleting these steps, the SLE protocol can easily save 50% or more time depending on the number of samples.

In addition to time and labor savings, the SLE procedure prevents emulsion from happening and improves method reliability.

Figure 7 shows the method performance comparison based on analyte recovery (7A) and reproducibility (7B). The SLE protocol provides intimate contact between the aqueous and organic phases, allowing efficient partitioning, which can improve analyte recoveries. Using SLE methods, analyte recoveries increased 10 to 20% overall compared to recoveries by the LLE method. Higher analyte recoveries improve analyte responses, and result in better method analytical sensitivity.

Equivalent or slightly higher recoveries were achieved using diatomaceous earth-based SLE, however, significant well-to-well variations were observed, especially for more polar analytes. This can be attributed to the inconsistency of diatomaceous earth sorbent and water-holding capacity. Because of the improved well-to-well reproducibility and method analytical sensitivity provided by Chem Elut S plates, the calibration curve linearity was improved as well. All these improvements make the quantitation method more reliable and consistent.

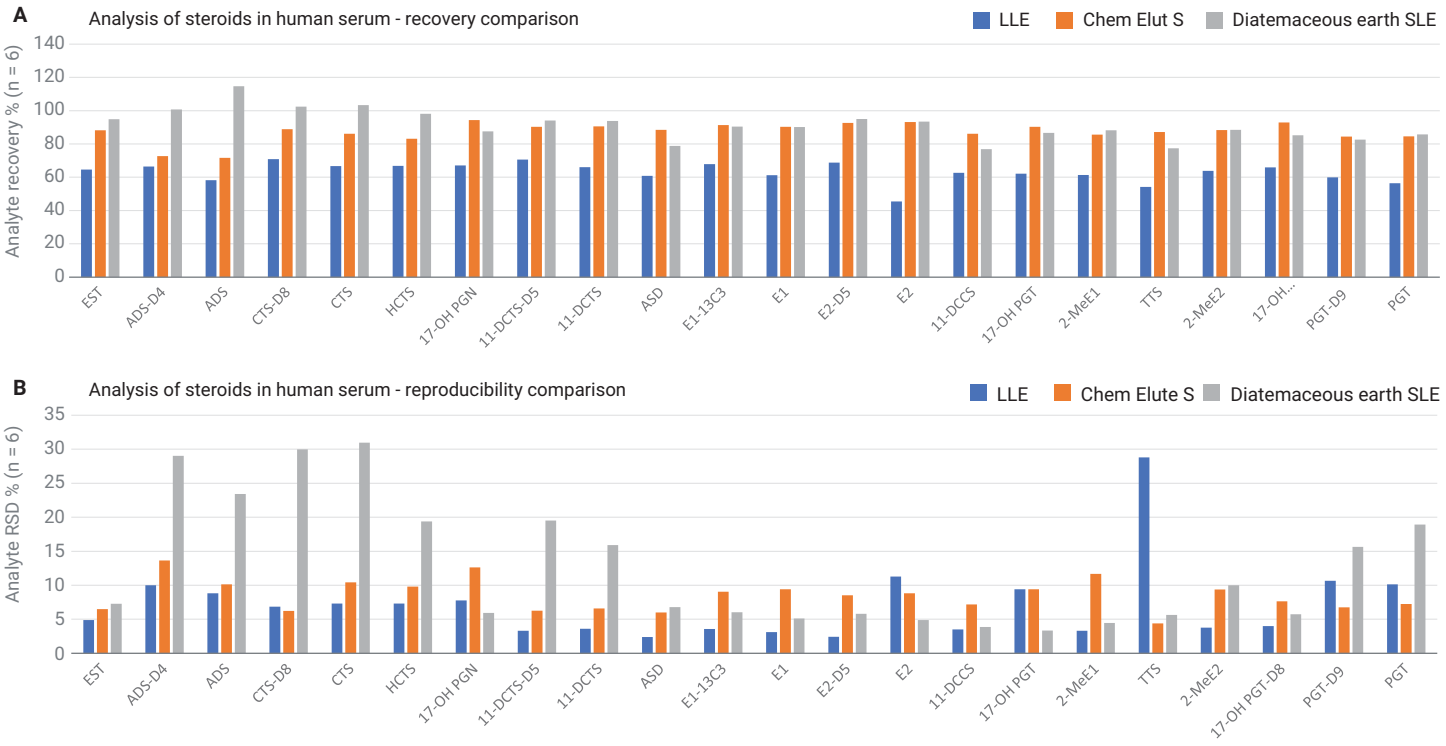


Figure 7. Method and product performance comparison for LLE versus Agilent Chem Elute S versus diatemaceous earth SLE.

Matrix phospholipids depletion

The use of Chem Elut S plates for biological fluids preparation also provides partial phospholipids depletion.

This study used hormone-stripped serum to achieve good matrix selectivity for extreme low quantitation limits for targeted steroids. However, the stripped serum was also stripped of phospholipids and may not be needed for other application tests. To investigate phospholipids depletion, nonstripped plasma was used for phospholipids profiling in the matrix using standard LLE, Chem Elut S, and a competitor's diatomaceous earth SLE method. The profile comparison in Figure 8A shows that the use of the Chem Elut S method using MTBE solvent provided 99% greater phospholipid depletion than the competitor's diatomaceous earth SLE and 30% greater phospholipid depletion than MTBE-based LLE.

Further comparison studies between Chem Elut S and diatomaceous earth SLE were conducted using different extraction solvents on different types of plasma, and the results are shown in Figure 8B. Phospholipids total responses were normalized based on the abundance. The findings included that:

- Different types of plasma may have variations in phospholipid abundance, with lipemic plasma containing most abundant phospholipids.
- Different solvents extract different levels of phospholipids, with EtOAc and DCM extracting more phospholipids than MTBE and DEE.
- Chem Elut S can provide more efficient phospholipids depletion than a competitor's diatemaceous earth SLE.

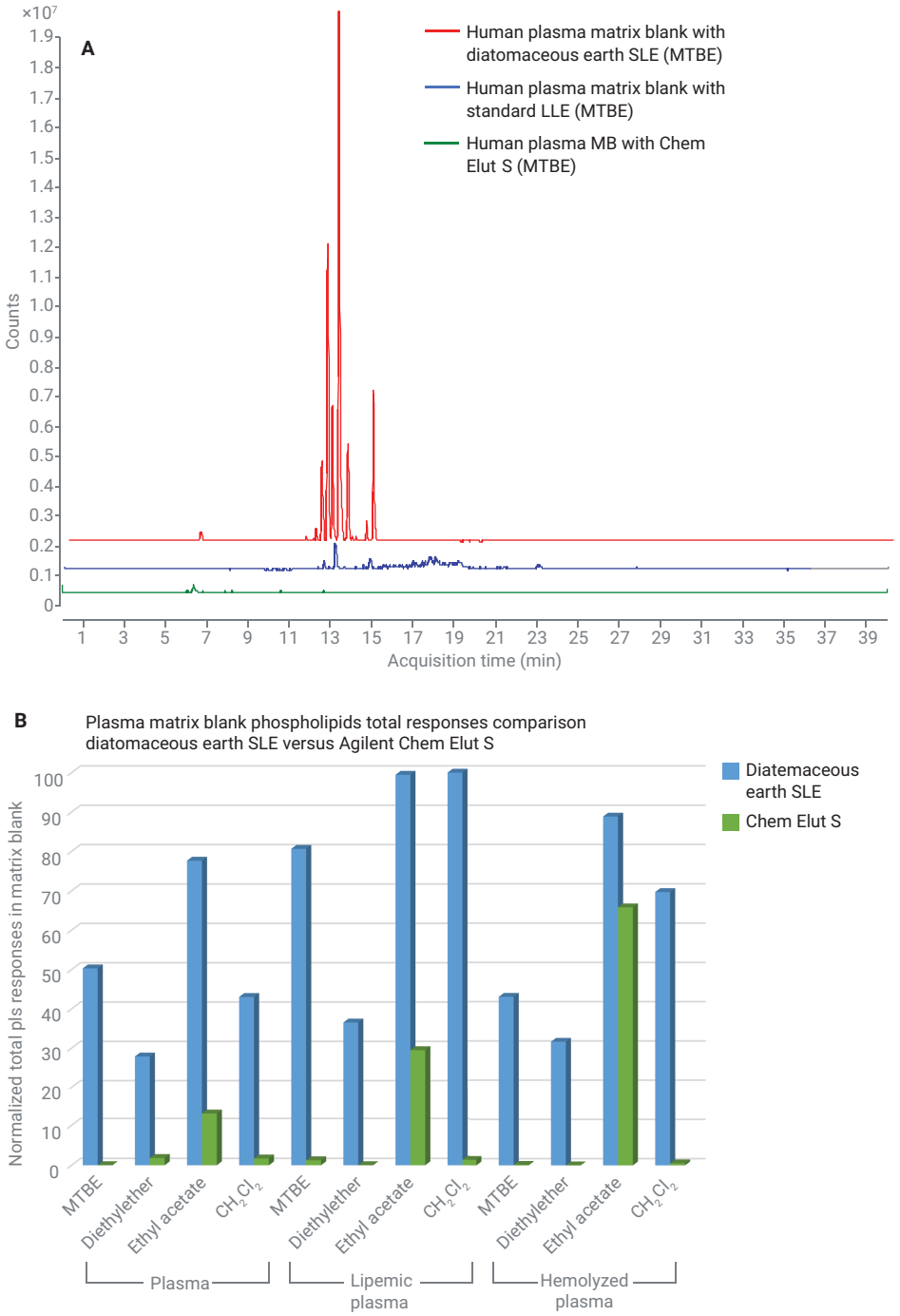


Figure 8. Phospholipids depletion comparison in plasma for Agilent Chem Elut S versus diatomaceous earth SLE versus LLE using different solvents.

Conclusion

A sample preparation method using Agilent Chem Elut S 2 mL plates was developed and verified for the quantitative determination of a panel of 15 steroid compounds in human serum. The SLE method was optimized for solvent use and sample elution based on analyte recovery, method reproducibility, and matrix effects. Three-day accuracy and precision runs verified that this method provided acceptable calibration curve linearity, exceptional intra- and interday accuracy and precision, excellent analyte recovery, and method producibility. When compared to standard LLE and diatomaceous earth SLE, the Chem Elut S method provided higher overall analyte recoveries than standard LLE and better sorbent consistency and well-to-well cartridge reproducibility than diatomaceous earth SLE. It also provides efficient phospholipid depletion for biological fluid matrices such as plasma and serum. The developed protocol on the 96-well plate format fits well for fast and automatable sample preparation needs in high-throughput labs. The convenient loading and elution procedure also simplifies the liquid extraction workflow with significant labor and time savings. Chem Elut S has a higher sample holding capacity than diatomaceous earth sorbents, delivering efficient sample adsorption and reducing the chance of sample break-through. The new plate design offers:

- Large headspace for samples and eluent
- A square upper frit that holds sample until pressure or vacuum is applied
- A full skirt for hardware compatibility
- Fast, consistent elution

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