Supplementary Information

Quantitative profiling of lipid mediators in sperm cells through on-line dilution on-line polymer matrix based solid phase extraction liquid chromatography with mass spectrometry detection

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LC-methods

Method 1

Time	Pump 1 (LPG/SPE)				Pump 2 (SPE)	Pump 3 + 4 (binary gradient / analytical)			Trap-Valve
[min]	A [%]	B [%]	C [%]			A [%]	B [%]		Position
	0.1 % HCOOH	MeOH	Isopro-panol / HCOOH (100/0.2)	Flow [mL/min]	0.1 % HCOOH Flow [mL/min]	MeCN / Water / HCOOH (10/90/0.1)	MeCN / Water (95/5)	Flow [mL/min]	
0	60	40	0	0.5	2	90	10	0.4	Trap-load
1.9					2				
2	60	40	0	0.5	0.001	90	10		
2.1									Trap-inject
2.15				0.5					
3	10	45	45	0.3					
5									
11				0.3		50	50	0.4	
12				1					Trap-load
15						0	100	0.4	
16	10	45	45	1					
16.1	60	40	0	0.5					
17						0	100	0.4	
18					0.001	90	10	0.4	
18.5					2				
24	60	40	0	0.5		90	10	0.4	

Table I: Method 1 was used for the determination of the following analytes: 19(*R*)-Hydroxy-prostaglandin E_1 (19(*R*)-HO-PGE_1), 19(*R*)-Hydroxy-prostaglandin E_2 (19(*R*)-HO-PGE_2), Prostaglandin E_1 (PGE_1), Prostaglandin E_2 (PGE_2), Prostaglandin D_2 (PGD_2), Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), Thromboxane B_2 (TXB_2)

Method 2

Time	Pump 1 (LPG/SPE)				Pump 2 (SPE)	Pump 3 + 4 (binary gradient / analytical)			Trap-Valve
[min]	A [%]	B [%]	C [%]			A [%]	B [%]		Position
	0.1 % HCOOH	MeOH	Isopro-panol / HCOOH (100/0.2)	Flow [mL/min]	0.1 % HCOOH Flow [mL/min]	MeCN / Water / HCOOH (10/90/0.1)	MeCN / Water (95/5)	Flow [mL/min]	
0	40	60	0	0.5	2	50	50	0.4	Trap-load
1.9					2				
2	40	60	0	0.5	0.001	50	50		
2.1									Trap-inject
2.15				0.5					
3	10	45	45	0.3					
9				0.3		0	100	0.4	
10				1					Trap-load
11						0	100	0.4	
13	10	45	45	1					
13.1	40	60	0	1					
14					0.001	50	50	0.4	
14.5				1	2				
15				0.5					
20	40	60	0	0.5		50	50	0.4	

Table II: Method 2 was used for the determination of the following analytes: 15-Hydroxyeicosatetraenoic acid (15(*S*)-HETE), Arachidonic acid-d_{11} (AA-d_{11}), Arachidonoylethanolamide (AA-EA), 2-Arachidonoylglycerol (2-AG), 2-Arachidonoylglycerol-d_5 (2-AG-d_5)

ISTD-Solution

Substanz	Mass [µg]
PGE_1-d_4	2
PGE_2-d_9	2
PGD_2-d_4	2
$PGF_{2\alpha}$-d_4	2
2-AG-d_8	4
AA-d_8	4
AA-EA-d_8	4
15(*S*)-HETE-d_8	4
TXB_2-d_4	2

Table III: ISTD-Analytes in 500 mL MeCN:MeOH (50/50, v/v)

Investigations into the properties of the TurboFlow Cyclone™ SPE column

Elution behaviour of the eicosanoids on the SPE column

To investigate the elution behaviour of the analytes on the trap column, mobile phase A (acetonitrile / ultrapure water / formic acid (10/90/0.1, v/v/v)) and mobile phase B (acetonitrile / ultrapure water (95/5, v/v)) were mixed in the respective compositions between 10 % and 90 % B. The test solution of the analytes was prepared in 400 µL HTF buffer with 1200 µL ISTD solution at a concentration of 1.25 µg/mL. In each case, 10 µL of this calibration standard was injected. The LC-MS system shown in Figure 1 was used for the investigations.

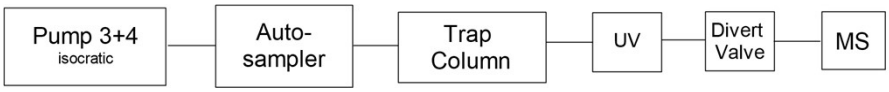

Figure 1: LC-MS system for investigating the elution behaviour of analytes on the SPE column

Figure 2 shows that 19-(*R*)-HO-PGE$_2$ is eluted quickly and entirely at a mobile phase B concentration of 20%. To maintain an adequate stacking effect on the analytical column, the mobile phase composition was set to 90% A and 10% B as the starting point for the analytical gradient. To ensure that all prostaglandins are completely backflushed from the solid-phase extraction (SPE) column to the analytical column, a backflush time of 9 minutes was selected.

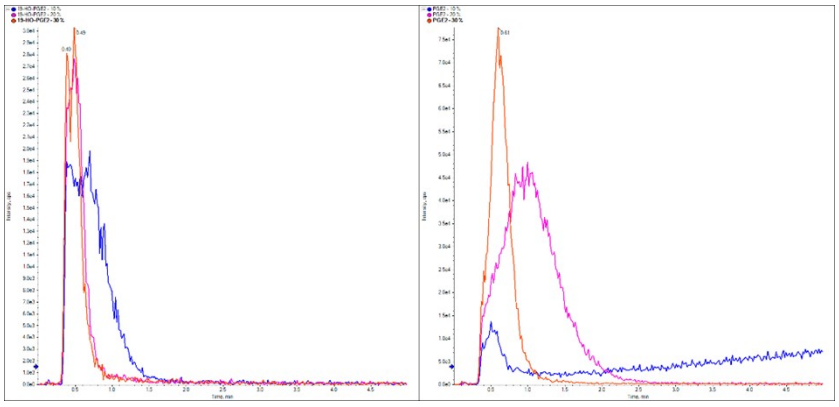

Figure 2: The elution profile of the analytes 19-(*R*)-HO-PGE$_2$ and PGE$_2$ on a TurboFlow™ Cyclone column during isocratic elution with 10% (orange), 20% (pink) and 30% (blue) mobile phase B

Figure 3 shows the elution behaviour for the representative analytes 15-HETE and 2-AG. From this, a composition of the mobile phase of the analytical gradient of 50% B can be derived. Due to the increasing proportion of B in backflush mode, all analytes are completely flushed onto the analytical column.

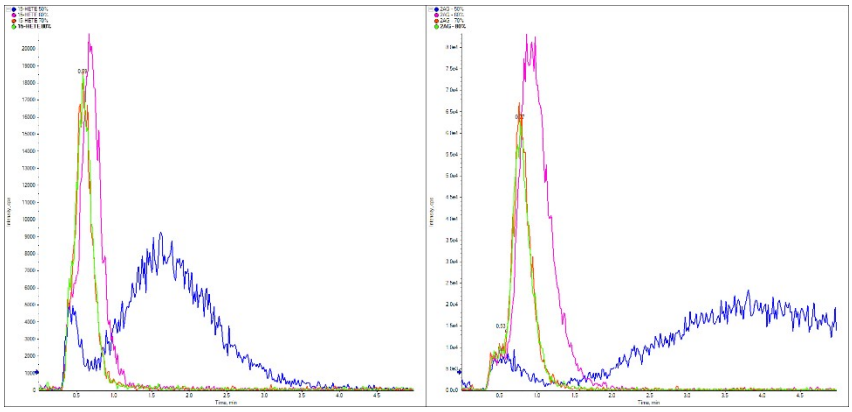

Figure 3: The elution profile of the analytes 15-HETE and 2-AG on a TurboFlow™ Cyclone column during isocratic elution with 50% (blue), 60 % (pink), 70 % (orange) and 80 % (green) mobile phase B.

Investigation of the retention and recovery behaviour of representative analytes on the SPE column

The chromatographic LC-MS system shown in Figure 4 was used to investigate the retention and recovery behaviour of representative analytes as a function of the composition of the mobile phase of loading pump 2 on the TurboFlow Cyclone™ trap column. The methods were adapted so that the methanol content of the mobile phase of loading pump 1 varied between 20% and 80%. The remaining chromatographic conditions were kept the same in accordance with standard methods 1 and 2. No internal standard was used.

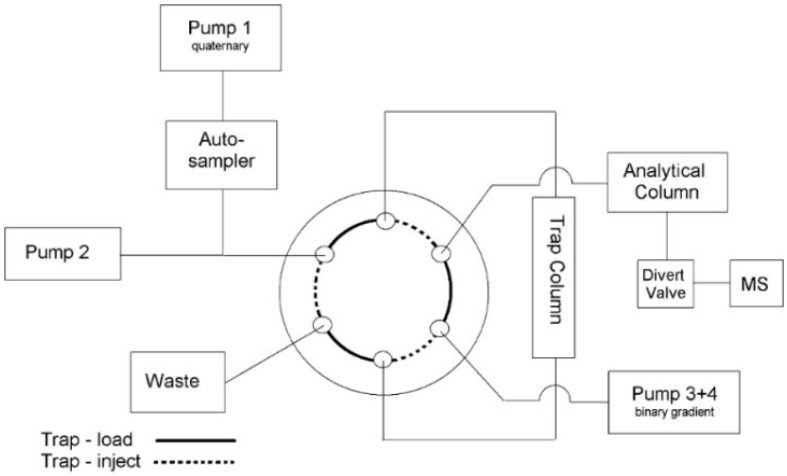

Figure 4: Chromatographic LC-MS system for investigations of retention and recovery behaviour

Figure 5 shows the results for the representative prostaglandins 19-(*R*)-HO-PGE_2 and PGE_2, and Figure 6 shows the results for 15-HETE, 2-AG and AA-D_{11}.
As theoretically expected, the technical recovery increases slightly with decreasing methanol content in the mobile phase. However, since a higher carry-over effect was observed with more lipophilic analytes as the methanol content decreased, a methanol content of 40% was found to be most suitable for the prostaglandins (method 1) and 60% for the other analytes (method 2) proved to be the most suitable.

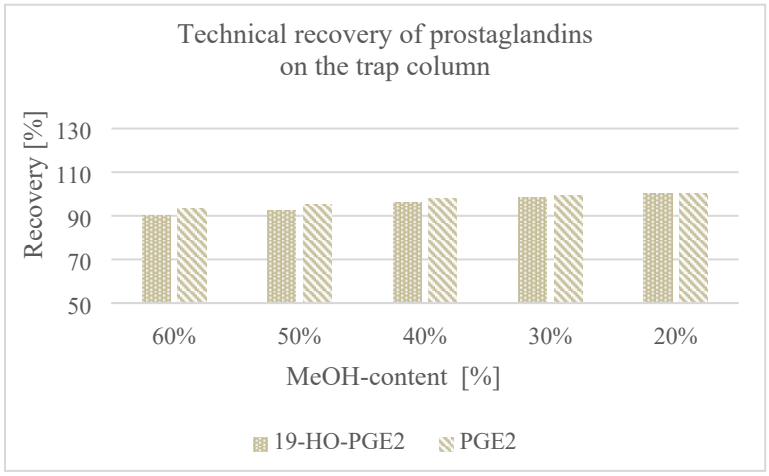

Figure 5: The technical recovery of 19-(*R*)-HO-PGE_2 and PGE_2 on the trap column as a function of the methanol content of the mobile phase of pump 1 is shown. The recovery of the representative prostaglandins increases slightly as the methanol content in the mobile phase of loading pump 1 decreases. The area of the 20% methanol content was set as relative 100% recovery.

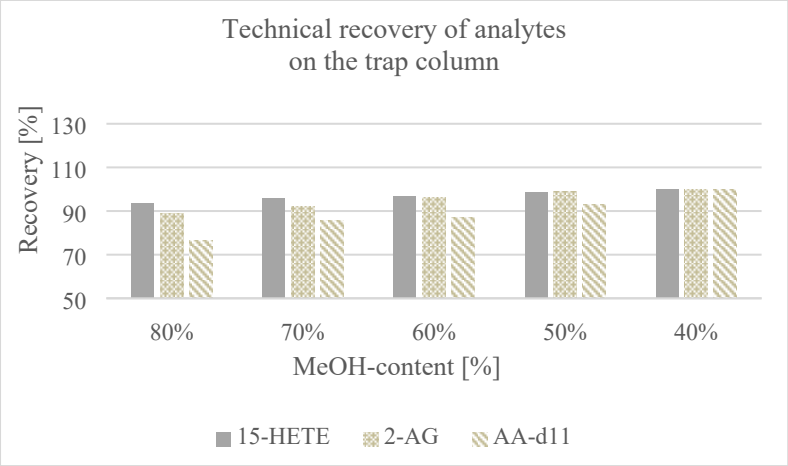

Figure 6: The technical recovery of 15-HETE, 2-AG and AA-d_{11} on the trap column as a function of the methanol content of the mobile phase of pump 1 is shown. The recovery of the representative analytes increases slightly as the methanol content in the mobile phase of loading pump 1 decreases. The area of the 20% methanol content was set as relative 100% recovery.

Investigation of the injection linearity of the trap column

An analyte solution of 19-(*R*)-HO-PGE_2, PGE_2, 15-HETE, 2-AG and AA-d_{11} with a concentration of 12.5 ng/mL (ULOQ) was prepared in 400 µl HTF buffer and 1200 µl MeCN:MeOH (50:50, v/v). Volumes of 50, 100, 150 and 200 µl of the analyte solution were injected and analysed using methods 1 and 2. The results were evaluated based on the area.
The result showed an approximately linear relationship with a respective r^2 value of 0.997 to 0.999. Only the residue distribution indicates a slight deviation from linearity. The precision was very high at the same injection volume.

Accuracy and precision

To determine accuracy and precision, quality control samples were prepared by spiking sperm matrix at four concentration levels (n=5 each) in three different batches that covered the entire calibration range: 0.02 ng (2 x LLOQ- QC), 0.05 ng (low QC), 2 ng (medium QC), 5 ng (high QC).
The within-run accuracy was calculated as the percentage mean of the 5 QC samples of the respective concentration level. The between-run accuracy was calculated as the percentage mean of all 15 QC samples of the respective concentration level of three different batches.
The within-run and between-run precision of the QC-samples is expressed as percent coefficient of variance (CV %).

Analyte / ISTD	pg/10^6 sperm cells		With-in run batch 1	(n=5 each) 2	3	Between-run batch 1-3	(n=15)
19(*R*)-HO-PGE_1 / -	20	Mean [%]	158.2	51.2	70.0	MW [%]	100.3
		CV [%]	13.2	21.5	74.2	CV [%]	58.2
	50	Mean [%]	132.9	69.8	91.7	MW [%]	100.3
		CV [%]	11.1	29.0	16.4	CV [%]	31.6
	2000	Mean [%]	102.3	97.0	103.2	MW [%]	100.9
		CV [%]	0.8	4.4	2.9	CV [%]	3.8
	5000	Mean [%]	94.6	99.8	102.3	MW [%]	98.4
		CV [%]	1.4	4.7	1.6	CV [%]	4.4
19(*R*)-HO-PGE_2 / PGE_2-d_9	20	Mean [%]	113.4	96.1	95.6	MW [%]	102.4
		CV [%]	1.5	2.9	8.6	CV [%]	9.7
	50	Mean [%]	108.7	97.5	103.3	MW [%]	103.5
		CV [%]	1.4	1.1	3.5	CV [%]	5.1
	2000	Mean [%]	102.0	93.9	91.7	MW [%]	96.3
		CV [%]	0.8	1.4	4.3	CV [%]	5.4
	5000	Mean [%]	96.5	93.9	94.0	MW [%]	95.0
		CV [%]	1.3	2.5	3.8	CV [%]	2.7
PGD_2 / PGD_2-d_4	20	Mean [%]	104.4	98.2	100.0	MW [%]	101.1
		CV [%]	1.3	2.5	2.2	CV [%]	3.3
	50	Mean [%]	103.0	100.6	103.7	MW [%]	102.5
		CV [%]	1.8	1.6	1.9	CV [%]	2.1
	2000	Mean [%]	99.3	95.3	99.7	MW [%]	98.2
		CV [%]	1.0	2.7	1.0	CV [%]	2.6
	5000	Mean [%]	90.0	91.4	94.6	MW [%]	91.7
		CV [%]	1.7	2.1	3.2	CV [%]	3.0

Analyte / ISTD	pg/10^6 sperm cells		With-in run batch 1	batch (n=5 each) 2	3	Between-run batch 1-3	(n=15)
PGE_1 /	20	Mean [%]	103.1	94.5	95.3	MW [%]	98.0
PGE_1-d_4		CV [%]	3.9	7.0	5.5	CV [%]	6.4
	50	Mean [%]	103.8	95.7	99.6	MW [%]	99.9
		CV [%]	1.5	2.8	3.1	CV [%]	4.2
	2000	Mean [%]	101.9	93.3	99.3	MW [%]	98.4
		CV [%]	1.5	1.7	2.0	CV [%]	4.1
	5000	Mean [%]	97.3	95.2	95.5	MW [%]	96.1
		CV [%]	1.0	2.1	3.8	CV [%]	2.4
PGE_2 /	20	Mean [%]	106.0	97.6	97.9	MW [%]	100.9
PGE_2–d_9		CV [%]	3.5	1.6	1.4	CV [%]	4.7
	50	Mean [%]	104.3	98.2	102.7	MW [%]	101.9
		CV [%]	1.0	1.2	2.3	CV [%]	3.0
	2000	Mean [%]	102.7	94.6	99.0	MW [%]	99.0
		CV [%]	1.7	2.8	2.4	CV [%]	4.1
	5000	Mean [%]	97.1	94.6	95.9	MW [%]	96.0
		CV [%]	1.2	1.9	1.6	CV [%]	1.9
$PGF_2\alpha$ /	20	Mean [%]	105.2	97.7	93.9	MW [%]	99.3
$PGF_2\alpha$-d_4		CV [%]	2.9	4.2	3.2	CV [%]	5.9
	50	Mean [%]	102.3	96.3	98.0	MW [%]	99.1
		CV [%]	3.8	2.9	2.5	CV [%]	4.0
	2000	Mean [%]	101.9	93.2	96.3	MW [%]	97.4
		CV [%]	1.2	1.9	0.9	CV [%]	4.1
	5000	Mean [%]	97.7	94.8	94.1	MW [%]	95.8
		CV [%]	0.7	1.2	2.0	CV [%]	2.1
TXB_2 /	20	Mean [%]	116.7	100.6	101.5	MW [%]	106.9
TXB_2-d_4		CV [%]	0.8	2.3	1.3	CV [%]	7.4
	50	Mean [%]	108.2	100.4	99.9	MW [%]	103.2
		CV [%]	1.6	1.8	10.8	CV [%]	6.8
	2000	Mean [%]	99.6	94.9	100.1	MW [%]	98.3
		CV [%]	0.3	1.3	0.6	CV [%]	2.5
	5000	Mean [%]	95.4	96.1	95.8	MW [%]	95.7
		CV [%]	0.4	0.6	6.2	CV [%]	3.2

Analyte / ISTD	pg/10^6 sperm cells		With-in run batch 1	(n=5 each) 2	3	Between-run batch 1-3	(n=15)
15-HETE /	20	Mean [%]	110.1	95.0	99.6	MW [%]	101.6
15-HETE-d_4		CV [%]	1.2	1.2	3.0	CV [%]	6.7
	50	Mean [%]	106.7	95.2	95.5	MW [%]	99.1
		CV [%]	1.8	1.4	1.3	CV [%]	5.7
	2000	Mean [%]	104.5	95.1	96.9	MW [%]	98.8
		CV [%]	0.7	0.7	0.3	CV [%]	4.3
	5000	Mean [%]	103.6	100.9	101.8	MW [%]	102.1
		CV [%]	0.6	0.5	0.6	CV [%]	1.3
AA-EA /	20	Mean [%]	99.8	98.5	101.0	MW [%]	99.8
AA-EA-d_8		CV [%]	4.4	5.3	5.5	CV [%]	4.9
	50	Mean [%]	101.1	97.2	98.1	MW [%]	98.8
		CV [%]	3.9	2.8	6.8	CV [%]	4.8
	2000	Mean [%]	101.1	93.6	98.5	MW [%]	97.8
		CV [%]	2.2	1.0	1.3	CV [%]	3.6
	5000	Mean [%]	98.8	97.3	101.6	MW [%]	99.2
		CV [%]	1.7	2.4	1.2	CV [%]	2.5
2-AG /	20	Mean [%]	108.2	90.6	95.1	MW [%]	98.0
2-AG-d_8		CV [%]	2.4	6.5	4.8	CV [%]	8.9
	50	Mean [%]	104.0	96.8	101.5	MW [%]	100.8
		CV [%]	2.6	2.6	2.2	CV [%]	3.8
	2000	Mean [%]	97.9	94.0	105.9	MW [%]	99.3
		CV [%]	1.6	1.3	0.6	CV [%]	5.2
	5000	Mean [%]	97.9	98.6	110.9	MW [%]	102.5
		CV [%]	1.3	0.5	1.0	CV [%]	6.1
2-AG-d_5 /	20	Mean [%]	110.6	90.9	95.1	MW [%]	98.9
2-AG-d_8		CV [%]	2.7	6.6	3.7	CV [%]	9.7
	50	Mean [%]	104.5	94.2	100.7	MW [%]	99.8
		CV [%]	2.9	1.7	1.8	CV [%]	5.0
	2000	Mean [%]	98.7	91.6	103.4	MW [%]	97.5
		CV [%]	1.3	1.3	0.7	CV [%]	5.2
	5000	Mean [%]	98.4	95.8	109.3	MW [%]	101.2
		CV [%]	1.1	0.6	0.4	CV [%]	6.0

Analyte / ISTD	pg/10^6 sperm cells		With-in run batch 1	batch (n=5 each) 2	3	Between-run batch 1-3	(n=15)
AA-d_{11} / AA-d_8	20	Mean [%]	115.5	102.7	118.1	MW [%]	112.1
		CV [%]	5.4	11.4	11.7	CV [%]	11.1
	50	Mean [%]	106.6	110.2	111.1	MW [%]	109.3
		CV [%]	5.9	8.2	4.1	CV [%]	6.2
	2000	Mean [%]	101.2	95.7	98.0	MW [%]	98.3
		CV [%]	0.6	1.4	1.0	CV [%]	2.5
	5000	Mean [%]	99.7	100.7	101.5	MW [%]	100.6
		CV [%]	1.1	0.8	1.2	CV [%]	1.3

Table IV

Linearity

Standard curves were prepared for all analytes with 9 calibration points ranging from 0.01 to 5 ng / $1x10^6$ sperm. For PGD_2, the calibration range was only covered up to 2 ng / $1x10^6$ sperm. A 1 /x weighted linear regression analysis of the area ratios (peak area analyte/peak area of the internal deuterated standard) was performed. The calibration for 19(*R*)-Hydroxy-PGE_1 was performed without considering of an ISTD. The lower limit of quantification (LLOQ) was set at the lowest concentration of the calibration standards. The signal-to-noise ratio of the matrix standards at the LLOQ gives a hint of a detection limit.

Analyte/ISTD		Matrix matched-STD	Matrix-STD	Lösemittel-STD	S/N at LLOQ Matrix-STD
19(*R*)-HO-PGE_1/ -	Calibration curve	y = 507.782 x +192250 r = 0.99986	y = 496.59 x +195803 r = 0.9996	y = 342.76 x + 12940 r = 0.9984	228
19(*R*)-HO-PGE_2/ PGE_2-d_9	Calibration curve	y = 0.00102 x + 0.02306 r = 0.99970	y = 0.00103 x + 0.02157 r = 0.99990	y = 0.000644 x + 0.000897 r = 0.9999	600
PGD_2 / PGD_2-d_4	Calibration curve	y = 0.00189 x + 0.00616 r = 0.99981	y = 0.00191 x + 0.00202 r = 0.9999	y = 0.00183 x + 0.00405 r = 0.9996	32
PGE_1 / PGE_1-d_4	Calibration curve	y = 0.00160 x + 0.01193 r = 0.9997	y = 0.00163 x + 0.00978 r = 0.9997	y = 0.00162 x + 0.00355 r = 0.9994	74
PGE_2 / PGE_2-d_9	Calibration curve	y = 0.00165 x + 0.01439 r = 0.9995	y = 0.00167 x + 0.01134 r = 0.9998	y = 0.00172 x + 0.00249 r = 0.9997	65
$PGF_{2\alpha}$ / $PGF_{2\alpha}$-d_4	Calibration curve	y = 0.00129 x + 0.00202 r = 0.9998	y = 0.00131 x + 0.000586 r = 0.9999	y = 0.00135 x + 0.000421 r = 0.9990	49
TXB_2- / TXB_2-d_4	Calibration curve	y = 0.00152 x + 0.00321 r = 0.99986	y = 0.00157 x + 0.0009798 r = 0.9990	y = 0.00158 x + 0.00222 r = 0.9989	250
15-HETE / 15-HETE-d_4	Calibration curve	y = 0.00094596 x + 0.00605 r = 0.9999	y = 0.0009573 x + 0.00421 r = 0.9991	y = 0.000971692 x + 0.00151 r = 0.9987	250
AA-EA / AA-EA-d_8	Calibration curve	y = 0.00101 x + 0.00637 r = 0.99959	y = 0.00103 x + 0.00505 r = 0.9994	y = 0.00106 x + 0.00481 r = 0.9990	57
2-AG / 2-AG-d_8	Calibration curve	y = 0.00845 x + 0.07496 r = 0.9997	y = 0.00886 x + 0.03984 r = 0.9996	y = 0.00856 x + 0.02363 r = 0.9988	97
2-AG-d_5 / 2-AG-d_8	Calibration curve	y = 0.00449 x + 0.01594 r = 0.9999	y = 0.00464 x + 0.00562 r = 0.99953	y = 0.00460 x + 0.01247 r = 0.9989	125
AA-d_{11} / AA-d_8	Calibration curve	y = 0.0001656 x + 0.0006362 r = 0.9999	y = 0.0001682 x - 0.00003338 r = 0.9993	y = 0.00017186 x + 0.0002886 r = 0.9993	28

Table V

Individual MRM transitions with their measurement parameters

ID	Q 1 Mass	Q3 Mass	RT-method 1	RT-method 2	Polarity	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
19-HO-PGE_2	367.11	331.20	5.60		neg.	-65.00	-10.00	-20.00	-17.00
19-HO-PGE_2	367.11	287.20	5.60		neg.	-65.00	-10.00	-26.00	-15.00
19-HO-PGE_1	369.08	333.10	6.00		neg.	-60.00	-10.00	-22.00	-19.00
19-HO-PGE_1	369.08	235.00	6.00		neg.	-60.00	-10.00	-24.00	-13.00
TXB_2	369.14	195.10	8.90		neg.	-40.00	-10.00	-18.00	-11.00
TXB_2	369.14	169.00	8.90		neg.	-40.00	-10.00	-22.00	-15.00
TXB_2-d_4	373.12	173.10	8.90		neg.	-50.00	-10.00	-24.00	-9.00
$PGF_{2\alpha}$	353.13	309.10	8.90		neg.	-65.00	-10.00	-26.00	-12.00
$PGF_{2\alpha}$	353.13	193.00	8.90		neg.	-65.00	-10.00	-34.00	-12.00
$PGF_{2\alpha}$ -d_4	357.12	313.30	8.90		neg.	-65.00	-10.00	-26.00	-15.00
PGE_2	351.12	271.10	9.20		neg.	-35.00	-10.00	-22.00	-19.00
PGE_2	351.12	315.00	9.20		neg.	-35.00	-10.00	-16.00	-17.00
PGE_2-d_9	360.18	280.20	9.20		neg.	-65.00	-10.00	-24.00	-15.00
PGE_1	353.14	235.00	9.40		neg.	-55.00	-10.00	-20.00	-25.00
PGE_1	353.14	317.00	9.40		neg.	-55.00	-10.00	-20.00	-17.00
PGE_1-d_4	357.15	239.10	9.40		neg.	-40.00	-10.00	-22.00	-11.00
PGD_2	351.12	271.20	9.60		neg.	-30.00	-10.00	-24.00	-23.00
PGD_2	351.12	315.20	9.60		neg.	-30.00	-10.00	-16.00	-15.00
PGD_2-d_4	355.10	275.10	9.60		neg.	-40.00	-10.00	-24.00	-15.00
15-HETE	319.12	219.00	14.10	6.30	neg.	-15.00	-10.00	-18.00	-17.00
15-HETE	319.12	257.30	14.10	6.30	neg.	-15.00	-10.00	-20.00	-13.00
15-HETE-d_8	327.18	226.10	14.10	6.30	neg.	-35.00	-10.00	-18.00	-9.00
AA-EA	348.28	287.30	15.00	7.50	pos.	46.00	10.00	15.00	20.00
AA-EA	348.28	203.20	15.00	7.50	pos.	46.00	10.00	19.00	12.00
AA-EA-d_8	356.20	294.10	15.00	7.50	pos.	86.00	10.00	27.00	34.00
2-AG	379.25	287.30	15.43	8.10	pos.	50.00	10.00	19.00	20.00
2-AG	379.25	269.30	15.43	8.10	pos.	50.00	10.00	21.00	16.00
2-AG-d_5	384.35	287.30	15.40	8.10	pos.	71.00	10.00	21.00	18.00
2-AG-d_5	384.35	269.40	15.40	8.10	pos.	71.00	10.00	23.00	18.00
2-AG d_8	387.03	294.10	15.43	8.10	pos.	36.00	10.00	17.00	18.00
AA	303.10	259.20	16.00	9.10	neg.	-70.00	-10.00	-18.00	-13.00
AA	303.10	205.00	16.00	9.10	neg.	-70.00	-10.00	-20.00	-9.00
AA-d_{11}	314.19	270.30	16.00	9.10	neg.	-30.00	-10.00	-18.00	-15.00
AA-d_{11}	314.19	216.20	16.00	9.10	neg.	-30.00	-10.00	-20.00	-13.00
AA-d_8	311.17	267.30	16.00	9.10	neg.	-40.00	-10.00	-18.00	-13.00

Table VI