Supplementary Section to

LC-MS based untargeted metabolomics reveals benzoic acid as a predictive biomarker for embryo implantation potential

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Table S1 Precision of selected mebolites ananlysis with different volume of methanol used for extraction.

MeOH/sample (v/v)		Gly	Ser	Asp	Asn	Arg	Gln	Glu
3:1	Average area	5.32E7	1.39E8	2.06E8	3.08E8	1.09E7	2.38E8	1.75E8
	RSD (%)	13.3	5.01	7.45	6.99	4.82	7.01	3.83
4:1	Average area	5.60E7	1.51E8	1.95E8	3.25E8	1.19E7	2.52E8	1.82E8
	RSD (%)	7.95	10.4	9.90	10.4	14.3	8.27	10.9
9:1	Average area	6.88E7	1.69E8	2.07E8	3.61E8	1.23E7	2.82E8	1.93E8
	RSD (%)	3.83	2.75	3.08	2.90	4.44	2.83	3.48

Table S2. Demographic and clinical characteristics of the patients undergoing day 3 embryo transfer and spent embryo culture medium collecting for Vitrolife and Cook Medical.

	Vitrolife			Cook Medical		
	s-implanting (n=12)	f-implanting (n=40)	*p* value	s-implanting (n=10)	f-implanting (n=16)	*p* value
Maternal age (years)	34.25±3.62	36.50±5.28	0.174	33.60±2.50	32.50±4.93	0.521
Paternal age (years)	35.00±4.13	38.90±6.76	*0.021*	33.70±2.87	34.81±5.91	0.586
Maternal BMI (kg/m^2)[a]	22.32±2.18	22.68±3.67	0.761	23.62±3.61	26.14±4.72	0.166
Infertility duration (years)	1.88±1.21	3.68±2.88	*0.003*	4.00±2.67	3.46±2.68	0.619
AMH (ng/ml)	2.73±2.14	2.24±1.99	0.498	2.76±1.86	2.88±3.41	0.922
Ovary stimulation duration (days)	9.25±2.42	8.85±1.98	0.563	9.60±0.97	9.75±1.61	0.793
Antral follicles (numbers)	10.00±7.20	7.55±5.26	0.201	10.10±4.33	10.38±8.75	0.927
Retrieved oocytes (numbers)	8.58±6.49	5.38±3.66	0.125	7.40±3.92	6.63±3.67	0.614
Endometrial thickness (mm)[b]	11.13±2.30	8.86±2.16	*0.003*	10.13±2.34	9.42±1.86	0.403
Primary infertility, n (%)	8(66.7)	17(42.5)	0.142	5 (50)	7 (46.7)	0.870

Embryos transferred on day 3 (numbers)	s-implanting (n=17)	f-implanting (n=52)		s-implanting (n=14)	f-implanting (n=23)	
7–10 cell embryo rate, n (%) [c]	16(94.1)	49(94.2)	0.986	14 (100)	22 (95.7)	0.429
Morphological grade I embryo rate, n (%) [d]	12(70.6)	28(53.8)	0.225	7 (50)	9 (39.1)	0.517

a BMI is the abbreviation of body mass index.

b Endometrial thickness: the thickness of the endometrium on the LH surge day.

c Proportion of embryos with 7 – 10 blastomeres on day 3.

d Proportion of morphological grade I embryos.

Italic *p*-value indicates significantly different.

Table S3 Differential ions between s-implantation and f-implantation groups identified through LC-MS based untargeted metabolomics.

Groups	Total	Number	Updown (S-F)	Observed m/z	Retention Time (min)
C1/ C2/ V2/ V3	1	1	DOWN	121.0290	5.5
C2/ V1/ V3	2	2	DOWN	146.9644	2.4
		3	DOWN	115.0750	6.0
C1/ C2/ V1	1	4	DOWN	330.0760	6.4
V1/ V2	1	5	DOWN	68.9947	4.7
C1/ V1	1	6	DOWN	113.9892	4.7
C2/ V1	2	7	DOWN	225.1098	4.1
		8	DOWN	138.9692	8.7
V2/ V3	1	9	DOWN	492.9896	12.9
C1/ V2	2	10	DOWN	122.0333	5.6
		11	DOWN	117.0197	7.3
C1/ V3	1	12	DOWN	239.0709	3.0
C2/ V3	54	13	DOWN	91.9780	0.7
		14	DOWN	165.0395	3.3
		15	DOWN	239.1282	3.3
		16	DOWN	211.7547	3.4
		17	DOWN	255.6583	3.4
		18	DOWN	437.2394	3.4
		19	DOWN	112.8578	3.5
		20	DOWN	113.1095	3.5
		21	DOWN	142.9290	4.1
		22	DOWN	518.3584	4.1
		23	DOWN	143.2851	4.8
		24	DOWN	212.3967	5.2
		25	DOWN	135.0437	5.8
		26	DOWN	244.6955	6.6
		27	DOWN	245.4933	6.6
		28	DOWN	164.0338	7.4
		29	DOWN	88.9345	7.6
		30	DOWN	89.1105	7.6
		31	DOWN	112.0151	7.9
		32	DOWN	118.9503	11.5
		33	DOWN	352.9450	13.2
		34	UP	449.1304	3.3
		35	UP	170.9430	3.5
		36	UP	227.9808	3.5
		37	UP	274.8896	3.5
		38	UP	276.8866	3.5
		39	UP	150.0005	3.8

	40	UP	160.8956	3.8
	41	UP	162.8926	4.2
	42	UP	112.9841	5.4
	43	UP	113.9874	5.4
	44	UP	258.9207	6.9
	45	UP	276.9311	6.9
	46	UP	330.9183	6.9
	47	UP	126.9345	7.3
	48	UP	128.9315	7.3
	49	UP	84.9414	7.4
	50	UP	256.8164	7.5
	51	UP	174.9362	8.5
	52	UP	162.8927	9.3
	53	UP	160.8955	11.3
	54	UP	222.8960	11.3
	55	UP	224.8938	11.3
	56	UP	74.9603	11.5
	57	UP	119.9456	11.5
	58	UP	172.9567	11.5
	59	UP	378.9557	11.6
	60	UP	198.9745	11.7
	61	UP	92.9265	13.0
	62	UP	94.9235	13.3
	63	UP	488.9084	13.5
	64	UP	280.9780	13.6
	65	UP	444.9844	13.6
	66	UP	698.4987	13.6

Table S4 Differential ions' HMDB searching results through LC-MS based untargeted metabolomics. *

Query mass	HMDB ID	Compound name	Formula	Adduct m/z	Delta (ppm)
121.0290	0244156	1,3-Benzodioxole	C7H6O2	121.0295	4
	0259299	Tropolone	C7H6O2	121.0295	4
	0034170	2-Hydroxybenzaldehyde	C7H6O2	121.0295	4
	0032918	3-(2-Furanyl)-2-propenal	C7H6O2	121.0295	4
	0001870	Benzoic acid	C7H6O2	121.0295	4
	0011718	4-Hydroxybenzaldehyde	C7H6O2	121.0295	4
115.0750	0249469	Butoxyacetaldehyde	C6H12O2	115.0765	13
	0245333	2,2-Dimethylbutyric acid	C6H12O2	115.0765	13
	0258840	Tert-butyl acetate	C6H12O2	115.0765	13
	0244061	1,2-Cyclohexanediol	C6H12O2	115.0765	13
	0258198	Sec-butyl acetate	C6H12O2	115.0765	13
	0040327	Pentyl formate	C6H12O2	115.0765	13
	0031221	2-Ethylbutanoic acid	C6H12O2	115.0765	13
	0030056	Isopropyl propionate	C6H12O2	115.0765	13
	0031246	2-Methylpropyl acetate	C6H12O2	115.0765	13
	0031325	n-Butyl acetate	C6H12O2	115.0765	13
	0031248	Ethyl 2-methylpropanoate	C6H12O2	115.0765	13
	0033889	Ethyl butyrate	C6H12O2	115.0765	13
	0031207	Methyl pentanoate	C6H12O2	115.0765	13
	0030059	Propyl propionate	C6H12O2	115.0765	13
	0040279	2,4,5-Trimethyl-1,3-dioxolane	C6H12O2	115.0765	13
	0033774	3-Methylpentanoic acid	C6H12O2	115.0765	13
	0034163	3-Methylbutyl formate	C6H12O2	115.0765	13
	0031511	Diacetone alcohol	C6H12O2	115.0765	13
	0031580	()-2-Methylpentanoic acid	C6H12O2	115.0765	13
	0030027	Methyl 3-methylbutanoate	C6H12O2	115.0765	13
	0037266	2,2,4-Trimethyl-1,3-dioxolane	C6H12O2	115.0765	13
	0029762	Methyl (S)-2-Methylbutanoate	C6H12O2	115.0765	13
	0000535	Caproic acid	C6H12O2	115.0765	13
	0000689	Isocaproic acid	C6H12O2	115.0765	13
330.0760	0252676	(2S,3S)-2-(2,4-Difluorophenyl)-3-methylsulfonyl-1-(1,2,4-triazol-1-yl)butan-2-ol	C13H15F2N3O3S	330.0729	9
	0250136	Chlorprothixene sulfoxide	C18H18ClNOS	330.0725	11
	0247240	7-Chloro-N-[3-(2-nitroimidazol-1-yl)propyl]quinolin-4-amine	C15H14ClN5O2	330.0763	1
	0260049	zotepine	C18H18ClNOS	330.0725	11

	0029367	Sanguinarine	C20H14NO4	330.0777	5
138.9692	0258590	Sulfoacetic acid	C2H4O5S	138.9707	11
	0029716	Liquid thiophthene	C6H4S2	138.9682	7
492.9896	0252943	Phosphatidylinositol 4,5-diphosphate	C9H21O17P3	492.9919	5
117.0197	0031204	4-Hydroxy-2-oxobutanoic acid	C4H6O4	117.0193	3
	0039324	xi-3-Hydroxy-2-oxobutanoic acid	C4H6O4	117.0193	3
	0000940	Threonolactone	C4H6O4	117.0193	3
	0000349	Erythrono-1,4-lactone	C4H6O4	117.0193	3
	0000202	Methylmalonic acid	C4H6O4	117.0193	3
	0000254	Succinic acid	C4H6O4	117.0193	3
239.0709	0341262	3,2'-Dihydroxychalcone	C15H12O3	239.0714	2
	0246634	4'-Hydroxyflavanone	C15H12O3	239.0714	2
	0246098	3'-Hydroxyflavanone	C15H12O3	239.0714	2
	0250218	Chrysophanol-9-anthrone	C15H12O3	239.0714	2
	0251315	2,3-Dihydroflavon-3-ol	C15H12O3	239.0714	2
	0036738	Lettucenin A	C15H12O3	239.0714	2
	0039612	2,4-Dihydroxychalcone	C15H12O3	239.0714	2
	0032129	2',5'-Dihydroxychalcone	C15H12O3	239.0714	2
	0032559	()-2-(1-Methylpropyl)-4,6-dinitrophenol	C10H12N2O5	239.0673	15
	0032130	3',4'-Dihydroxychalcone	C15H12O3	239.0714	2
	0256416	Idronoxil	C15H12O3	239.0714	2
165.0395	0242183	L-Xylonate	C5H9O6	165.0405	6
	0242170	D-Xylonate	C5H9O6	165.0405	6
	0059750	D-Xylonic acid	C5H10O6	165.0405	6
	0060255	L-Lyxonic acid	C5H10O6	165.0405	6
	0301774	Xylan	C5H10O6	165.0405	6
	0060256	L-Xylonic acid	C5H10O6	165.0405	6
	0059716	9-Methylxanthine	C6H6N4O2	165.0418	14
	0034394	S-Propyl 1-propanesulfinothioate	C6H14OS2	165.0413	11
	0010738	1-Methylxanthine	C6H6N4O2	165.0418	14
	0001991	7-Methylxanthine	C6H6N4O2	165.0418	14
	0001886	3-Methylxanthine	C6H6N4O2	165.0418	14
	0000867	Ribonic acid	C5H10O6	165.0405	6
	0000539	Arabinonic acid	C5H10O6	165.0405	6
239.1282	0249426	Budralazine	C14H16N4	239.1302	8
	0014862	Imiquimod	C14H16N4	239.1302	8
437.2394	0257521	N-[4-[2-(6-Cyano-3,4-dihydro-1H-isoquinolin-2-	C28H30N4O	437.2347	11

		yl)ethyl]cyclohexyl]quinoline-4-carboxamide			
	0034184	Licorisoflavan A	C27H34O5	437.2333	14
113.1095	0250652	Cyclohexanediamine	C6H14N2	113.1084	10
	0243564	(1S,2S)-(+)-1,2-Diaminocyclohexane	C6H14N2	113.1084	10
135.0437	0244196	1,4-Benzodioxane	C8H8O2	135.0452	11
	0245150	2-Hydroxyacetophenone	C8H8O2	135.0452	11
	0062810	M-toluic Acid	C8H8O2	135.0452	11
	0002340	2-Methylbenzoic acid	C8H8O2	135.0452	11
	0031459	3-Methoxybenzaldehyde	C8H8O2	135.0452	11
	0032400	3-(5-Methyl-2-furyl)prop-2-enal	C8H8O2	135.0452	11
	0032603	2-Hydroxy-4-methylbenzaldehyde	C8H8O2	135.0452	11
	0029686	4-Methoxybenzaldehyde	C8H8O2	135.0452	11
	0032568	2'-Hydroxyacetophenone	C8H8O2	135.0452	11
	0040638	3-(3-Furanyl)-2-methyl-2-propenal	C8H8O2	135.0452	11
	0033766	2-Methoxybenzaldehyde	C8H8O2	135.0452	11
	0033128	4-(2-Furanyl)-3-buten-2-one	C8H8O2	135.0452	11
	0029635	4-Methylbenzoic acid	C8H8O2	135.0452	11
	0033968	Methyl benzoate	C8H8O2	135.0452	11
	0038176	alpha-Methyl-2-furanacrolein	C8H8O2	135.0452	11
	0040733	Phenyl acetate	C8H8O2	135.0452	11
	0003767	4-Hydroxyphenylacetaldehyde	C8H8O2	135.0452	11
	0000209	Phenylacetic acid	C8H8O2	135.0452	11
	0041485	Benzyl formate	C8H8O2	135.0452	11
164.0338	0256052	4'-Nitroacetophenone	C8H7NO3	164.0353	9
	0256499	Phthalamic acid	C8H7NO3	164.0353	9
	0013030	Noradrenochrome	C8H7NO3	164.0353	9
	0036582	6-Methoxy-2(3H)-benzoxazolone	C8H7NO3	164.0353	9
	0038318	(R)-2-Hydroxy-2H-1,4-benzoxazin-3(4H)-one	C8H7NO3	164.0353	9
	0004291	5-Pyridoxolactone	C8H7NO3	164.0353	9
	0004089	Formylanthranilic acid	C8H7NO3	164.0353	9
	0003454	4-Pyridoxolactone	C8H7NO3	164.0353	9
112.0151	0245943	3-Nitro-1H-pyrazole	C3H3N3O2	112.0153	1
	0244186	1,3,5-Triazine-2,4(1H,3H)-dione	C3H3N3O2	112.0153	1
	0246535	4-Nitroimidazole	C3H3N3O2	112.0153	1
	0247036	6-Azauracil	C3H3N3O2	112.0153	1
	0245259	2-Nitroimidazole	C3H3N3O2	112.0153	1

449.1304	0240313	4-Androsten-3beta,17beta-diol disulfate	C19H30O8S2	449.1309	1
150.0005	0247072	6-Hydroxybenzothiazole	C7H5NOS	150.0019	9
	0249030	2(3H)-Benzothiazolone	C7H5NOS	150.0019	9
	0034413	1,2-Benzisothiazol-3(2H)-one	C7H5NOS	150.0019	9
	0040578	4-Thiocyanatophenol	C7H5NOS	150.0019	9
160.8956	0042048	Trichloroacetic acid	C2HCl3O2	160.8969	8
112.9841	0014118	Trifluoroacetic acid	C2HF3O2	112.9856	13
113.9874	0245050	2-Chloro-1,3,5-triazine	C3H2ClN3	113.9864	8
126.9345	0250119	Chloromethyl chloroformate	C2H2Cl2O2	126.9359	11
128.9315	0034906	Zinc dithionite	H2O4S2	128.9322	5
160.8955	0042048	Trichloroacetic acid	C2HCl3O2	160.8969	9
280.9780	0240482	Bergaptol sulfate	C11H6O7S	280.9761	7

* Adduct was set as $[M-H]^-$, m/z tolerance for database search was set as 15 ppm.

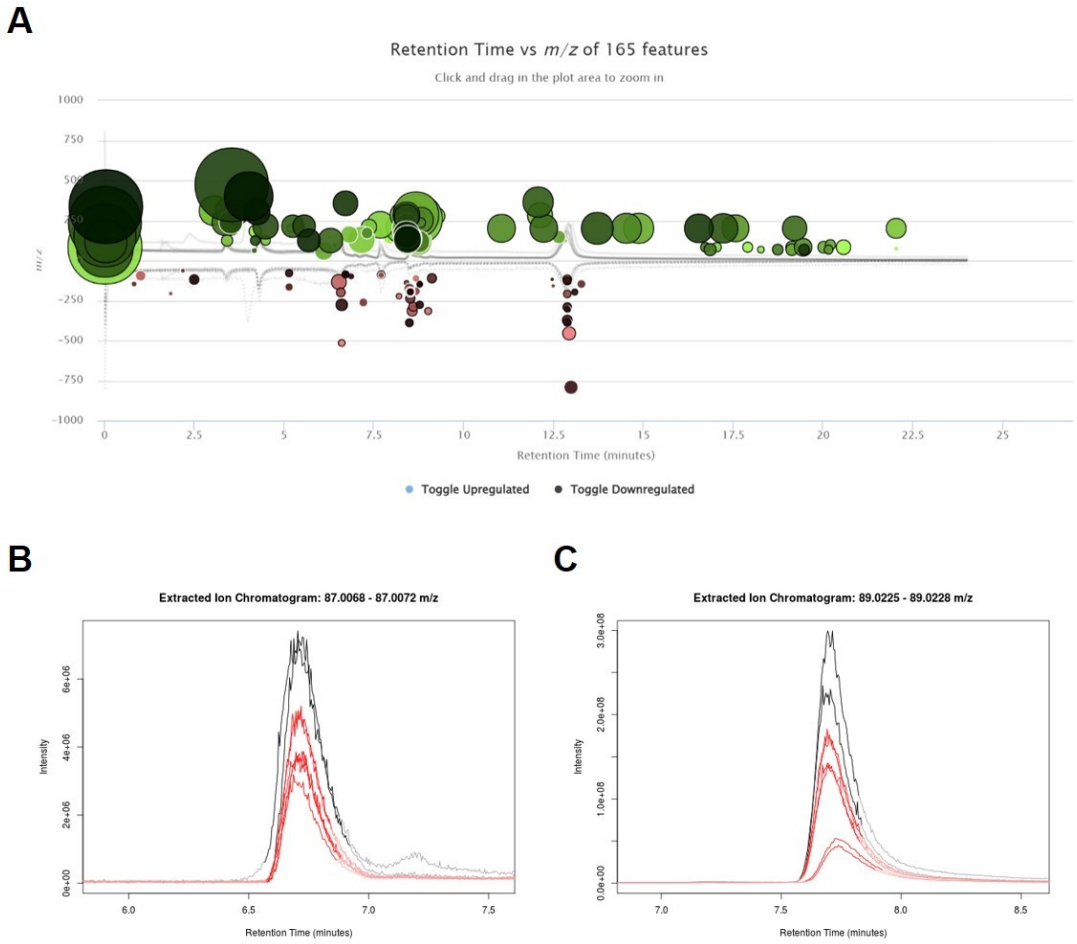

Figure S1 Contrast of SECM and fresh medium without embryo culture. (A) Metabolite analysis of SECM and fresh medium without embryo culture. XCMS parameters were set as following. *p*-value: 0-0.05; fold change: >=1.5; m/z range of 60-800; retention range of 1-23; max intensity: >1000000; radius scale: fold change. Green bubbles represented the upregulated features (s/f), red bubbles represented the downregulated features (s/f). *P*-value was represented by how dark or light the color. Fold change is represented by the radius of each feature. Retention time is represented by position on the x-axis. Mass-to-Charge ratio is represented by position on y-axis. (B) Extracted ion chromatogram of pyruvic acid. (C) Extracted ion chromatogram of lactic acid. The black solid lines represent fresh medium without embryo culture and red solid lines represent SECM.

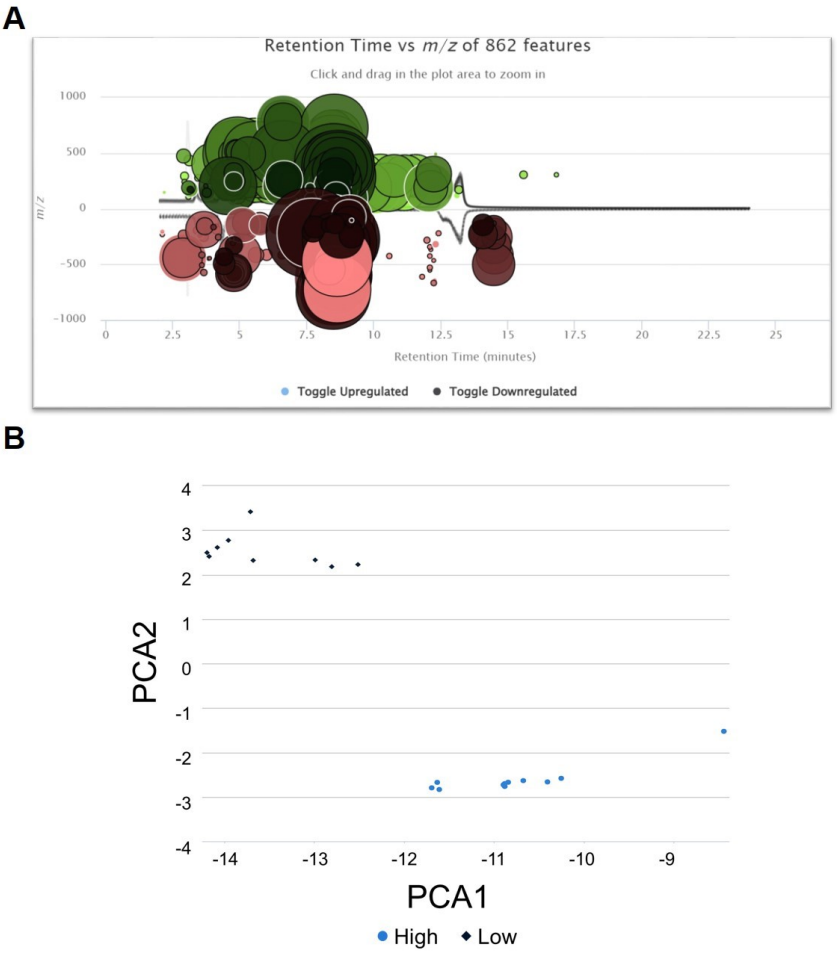

Figure S2 Distinguishing unknown brand of SECM samples according to chemical component by LC-MS. (A) cloud plot for differential metabolites , (B) principal component analysis results. *P*-value was represented by how dark or light the color. Fold change is represented by the radius of each feature. Retention time is represented by position on the x-axis. Mass-to-Charge ratio is represented by position on y-axis. Green and bubbles in (A), light and dark blue dots in (B) represented the classification of unknown brand of culture medium, which were proved to be distinguished as either Vitrolife or Cook Medical.

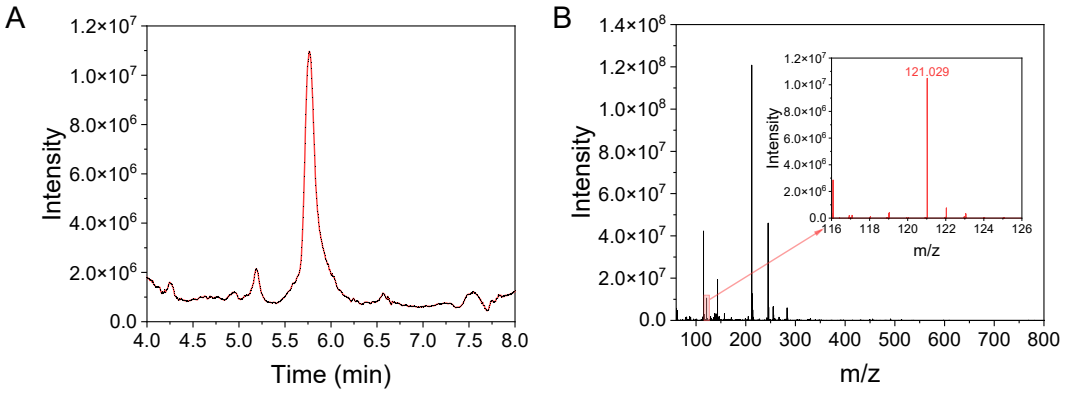

Figure S3. Chromatographic separation and parent ion of m/z 121.029. (A) Extracted ion chromatogram of the m/z 121.029 ion in SECM. (B) MS of parent ions in SECM at the peak of the m/z 121.029 ion.