Supplementary information

Evaluation and Analysis of Environmentally Sustainable Methodologies for Extraction of Betulin from Birch Bark with Focus on Industrial Feasibility

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Detailed reflux boiling (RB) conditions

Reflux boiling time. All reflux boiling experiments were performed using a 250 mL straight-type Allihn condenser. In the first RB experiment 20.0 g of bark was added to a round bottom flask together with 200 ml of solvent containing internal standard (IS). Samples were taken every 5 min; the first one when the solvent was boiling to evaluate suitable extraction time.

Reflux boiling with precipitation. In the second RB experiment 20.0 g of bark was added to a round bottom flask and 200 ml of solvent was added and boiled for 10 min. The bark was filtered away from the hot process fluid using a juice strainer followed by a coffee filter. An approximately equal volume of water (MilliQ quality) was added, resulting in a cloudy liquid. The solution was allowed to rest in the refrigerator (+4°C) for at least 4 h before it was suction filtrated and washed with 300 mL of water over a Grade 00H Munktell filter (Falun, Sweden). After drying, part of the precipitate was dissolved in methanol. Both the filtrate prior to precipitation (n) and the dissolved precipitate (2) were analyzed, respectively, for evaluating losses during the precipitation step.

Reflux boiling with pre-boiling of the bark. The effect of pre-boiling the bark in water (MilliQ) for 10 min prior to the extraction was investigated. After the pre-boiling the water was filtrated using a juice strainer and the bark was allowed to dry for two days at room temperature. The extraction then proceeded as described above. The pre-boiling was evaluated both by itself and in combination with precipitation, these extracts will be referred to as (3) if only pre-boiling was performed and (b) if both pre-boiling and precipitation was performed.

Reflux boiling with filtration: In addition to the juice strainer followed by a coffee filter filtration used in all reflux boiling filtration trough a 102 Double Ring filter paper was used after the reflux boiling to improve purity.

Detailed pressurized liquid extraction (PLE) conditions

1.0 g bark was mixed with 3 mm diameter glass beads (to fill out the void) in a 11 ml stainless steel extraction cell. Extractions were carried out at 50 bar and 120 °C. 1.053 ml internal standard (IS, 14.24 mg/ml) was added to each fraction which were then diluted to 25 ml with extraction solvent, resulting in a final concentration of 0.6 mg/ml IS. During the extraction about 10 % of the solvent was lost based on measuring the amount of solvent used for extraction and amount of solvent collected after extraction.

Detailed supercritical fluid extraction (SFE) conditions

1.0 g bark was mixed with glass beads (3mm diameter) and added to a 10 ml extractor vessel. Extraction was then done at 350 bar, 55°C using a 10/90 (v/v) mixture of 95% ethanol/CO_2 with a total flow rate of 1 ml/min for 60 min. Ultrapure CO_2 was provided by Air Products (Amsterdam, Netherlands). Circulating water at 5°C was used to cool the syringe pump. 1 ml ethanol was used as solvent trap. Experiments were carried out using an ISCO system (ISCO.INC. Lincoln, Nebraska, U.S.A.). The equipment consisted of a syringe pump (model ISCO 260 D, Teledyne ISCO, Thousand Oaks, CA), pump controller (D-series), extractor (SFX 2-10), restrictor and temperature controller. Extraction was done in dynamic mode using ethanol as co-solvent. The extracts were evaporated under a stream of nitrogen and stored at -18°C until analysis. The SFE method was not optimized.

Gravimetric purity

For the RB extracts (2) and (b), gravimetric purity was determined by dissolving parts of the precipitate in methanol (*approx.* 1 ml/mg precipitate) followed by dilution in methanol approximately 12 times. The concentration as determined by the diode array detector (DAD) using a calibration curve was then used to calculate the mass of betulin in the precipitate. Finally the mass of betulin was compared to the total mass of the dissolved precipitate to obtain the gravimetric purity (in %). For SFE, the gravimetric purity was determined in a similar way; part of the extract was dissolved in methanol and diluted (up to 13 times) to obtain a response within the DAD calibration curve. The mass of betulin in the undiluted sample was calculated and compared to the mass of dissolved extract.

As the final product after PLE was a liquid, 1 ml of one extract from each cycle was weighed and dried using a Thermo Scientific Savant ISS110 Integrated SpeedVac System (Waltham, MA). After drying, the mass of solid residue from one replicate from each cycle was weighed, and the obtained ratios of mg solid/mg (liquid) extract were used for all three replicates of the same cycle and gravimetric purity was calculated using the estimated amount of solid residue. This procedure was also applied to RB extracts (n) and (3) for each solvent.

The extracted amount was determined by relating the total amount of betulin extracted, calculated as for the gravimetric purity determination, to the total amount of bark used in the extraction.

Fourier transform infrared spectroscopy (FTIR) experiments

A Cary 630 FTIR (Agilent Technology, Hannover, Germany) was used. Solid birch bark extracts from the RB process, betulin and birch bark was analyzed. The samples from the birch bark RB was compared against betulin standard and ATR demo library included in the FTIR software.

Comments on SFE

The SFE method resulted in lower purity (39.6±2.4 % DAD) compared to both RB and PLE as well as lower recovery (15.4±6.8 mg betulin/g bark)

Figures and Tables

Tables S1 and S2 contain data from DAD and charged aerosol detector (CAD) respectively, while Tables S3-S5 show mass spectrometric (MS) data for the RB, PLE and SFE extracts. MS/MS data from betulin, betulinic acid and some of the major impurities are shown in Table S6, where a tentative identification is made for most of them. A summary of the compounds observed in all extracts can be found in Table S7 with data from all detectors. Figure S1 shows ion chromatograms of RB extracts with the total ion chromatogram (TIC) in a) and extracted ion chromatograms (XIC's) of *m/z* 425 (corresponding to the loss of H_2O from betulin) and 439 (corresponding to the loss of H_2O from betulinic acid) in b) and c) respectively. TIC's and XIC's from PLE and SFE can be found in Figure S2 and S3 respectively. In Figure S4 solid samples from the RB experiments are photographically presented. Figure S5 shows chromatograms (DAD) of a PLE extract before and after precipitation (performed as described for RB extracts).

Table S1. Signals seen with the DAD (210 nm), with retention time and peak assignment; RB (reflux boiling) signals are from extracts without pre-boiling or precipitation (n), Internal standard (progesterone) is marked by IS.

Peak nr	Retention time (min)	In extract(s)
2	10.0	RB
3	10.2	RB[3]
4	10.7	RB[3], PLE
5	10.8	RB[3], PLE
7	12.0	RB[3], PLE, SFE
8/9[?1]	12.3	RB
10	13.0	RB[3], PLE, SFE
11	13.4	RB[3], PLE, SFE
12	13.5	RB[3], PLE, SFE
13	14.2	RB[3], SFE
14	14.3	RB[3], PLE, SFE
16	14.6	RB[3]
17	15.0	RB[3], PLE, SFE
18	15.5	RB[2 3], PLE, SFE
19	16.1	RB[2,3], SFE
20	16.2	RB[2,3], SFE
21	16.4	RB[3], SFE
22	16.9	RB[3], SFE
23	17.5	RB[2,3], PLE, SFE
24	17.6	PLE
25	17.6	SFE
31	20.3	RB[2,3], SFE
35	20.9	RB[2,3], SFE
37	21.8	RB[3]
38	22.6	RB, SFE
40	23.4	RB[2,3]
41	24.0	RB, SFE
42	25.1	RB[2,3], PLE, SFE
45	26.6	RB[2,3], SFE
IS	26.6	PLE
46	27.8	RB[2,3], SFE
51	30.1	RB, PLE, SFE
56[ba]	33.1	RB[2,3], PLE, SFE
57[bet]	34.2	RB[2,3], PLE, SFE
58	34.5	RB, SFE
60	35.1	RB[3], SFE
61	35.5	RB[2,3], SFE
62	36.0	RB[2,3], SFE
64	36.7	RB[3], SFE
65	37.4	RB[3], SFE
67	37.8	RB[2,3], SFE
68	38.0	RB[3]
69/71[?2]	38.1	RB
73	39.2	RB[2,3], SFE
76	40.2	RB[2,3], SFE
77	40.6	RB[2,3], SFE
78	41.2	RB[2,3], SFE

[ba]betulinic acid
[bet]betulin
[2]also seen after precipitation (2)
[3]also seen after pre-boiling (3)
[?1]uncertain peak assignment, could be either of the suggested peaks, based on MS data (Table S4) peak 8 seems more likely
[?2]uncertain peak assignment, could be either peak 69 or 71

Table S2. List of peaks seen in the CAD, an X indicates that the peak was observed. Internal standard (progesterone) is marked by IS. For RB samples, the character in parenthesis indicates what purification step was performed; (n): no purification, (2): precipitation, (3): pre-boiling and (b): both pre-boiling and precipitation.

Peak	Retention time (min)	RB (n)	RB (2)	RB (3)	RB (b)	PLE			SFE
						Cycle 1	Cycle 2	Cycle 3	
7	12.1	X	X*	X		X	X	X	X
11	13.4	X				X	X	X	X*
13	14.2					X			
14	14.4					X			
16	14.7					X	X	X	
17	15.1	X	X	X	X*	X	X	X	X***
19	16.0	X	X	X	X*	X	X	X	X***
21	16.4	X	X	X	X*	X	X	X	
22	17.0								X*
23	17.7	X							
24	17.8					X	X	X	
25	17.8								X***
30	19.7								X**
31	20.4					X**	X	X*	X**
IS	26.7					X	X	X	
45	26.8	X	X	X	X				X**
46	27.8								X*
47	28.1								X*
48	28.6								X**
49	29.0								X*
50	29.3								X*
53	31.1								X***
54	32.1								X***
55	32.3								X**
56[ba]	33.3[a]	X	X	X	X	X	X	X	X
57[bet]	34.2[b]	X	X	X	X	X	X	X	X
60	35.0	X**	X	X**	X	X	X**	X*	X***
61	35.4								X***
62	35.9	X	X	X	X	X	X	X	X
63/64[?]	36.5	X	X	X	X	X	X	X	X
65	37.3								X***
66	37.9					X	X	X	
67	37.9	X	X	X	X				X
71	38.5								X***
73	39.2	X	X	X	X	X	X	X	X
75	39.9	X	X	X	X	X	X	X	X
76	40.3								X*
78	41.0								X***

[ba]betulinic acid
[bet]betulin
*only seen in one replicate
**seen in two replicates
***seen in three replicates
[?]uncertain peak assignment, could be either of the suggested peaks

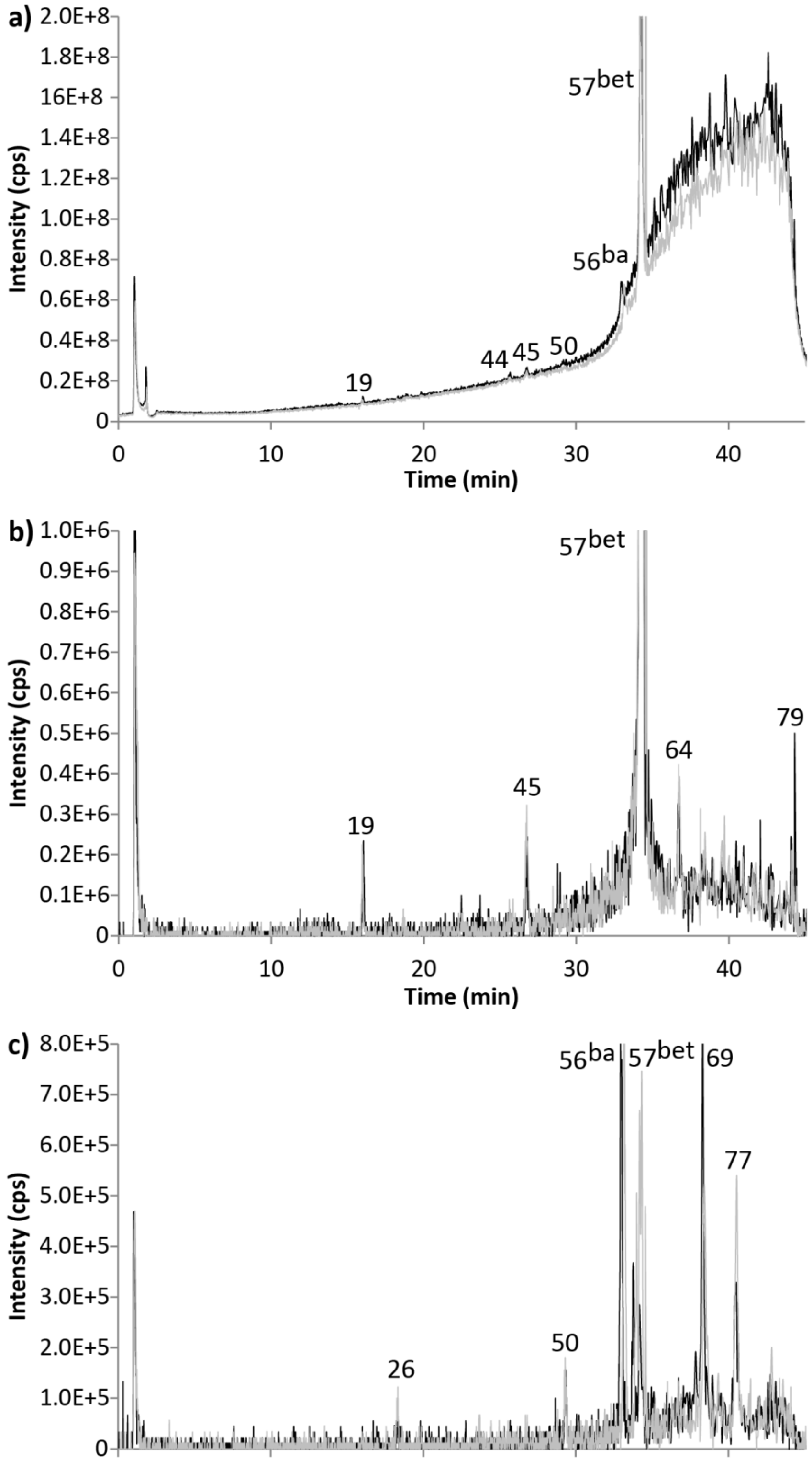

Figure S1. Comparison of RB extracts; (2) (black) and (b) (grey); a) TIC, b) XIC, *m/z* 425 and c) XIC, *m/z* 439**.** Betulin is marked as ^{bet} (peak 57) and betulinic acid is marked as ^{ba} (peak 56).

Table S3. MS data (EMS) for RB extracts purified by precipitation (2), purified by pre-boiling in water (3), and purified by both pre-boiling in water and precipitation (b). The base peak is written in bold.

Peak	Retention time (min)	In extract(s)	Major *m/z*
8	12.3	(3)	**107**, 147, 299, 329, 478
12	13.5	(3)	405, 423, **441**, 459
14	14.4	(3)	407, 425, **443**
17	14.8	(3)	**107**, 133, 437, 455
18	15.2	(3)	**107**, 133, 297, 315
19	16.0	(2), (3), (b)	407, 425, **443**, 461
21	16.4	(3)	107, 433, **612**
22	17.0	(3)	365, 405, 423, 441, **459**
23	17.5	(3)	421, **439**, 457
26	18.4	(2), (3)	421, **439**
30	19.8	(3)	419, 437, 455, **473**, 490
44	25.7	(2), (3), (b)	391, **409**, 427
45	26.8	(2), (3), (b)	407, **425**
50	29.2	(2), (3), (b)	**407**, 421, 439, 457
56[ba]	33.0	(2), (3), (b)	189, **393**, 203, 411, 439
57[bet]	34.3	(2), (3), (b)	189, 203, 217, **407**, 425
64	36.7	(2), (3), (b)	395, 407, **425**
67	37.8	(3)	393, **439**
68	38.0	(3)	407, **425**
69	38.1	(2), (3), (b)	393, **439**
75	39.9	(3)	403, **421**, 439
77	40.5	(2), (3), (b)	421, **439**, 536
79	44.3	(2)[X], (3), (b)[X]	**407**, 425

[ba]betulinic acid
[bet]betulin
[X]only seen in one replicate

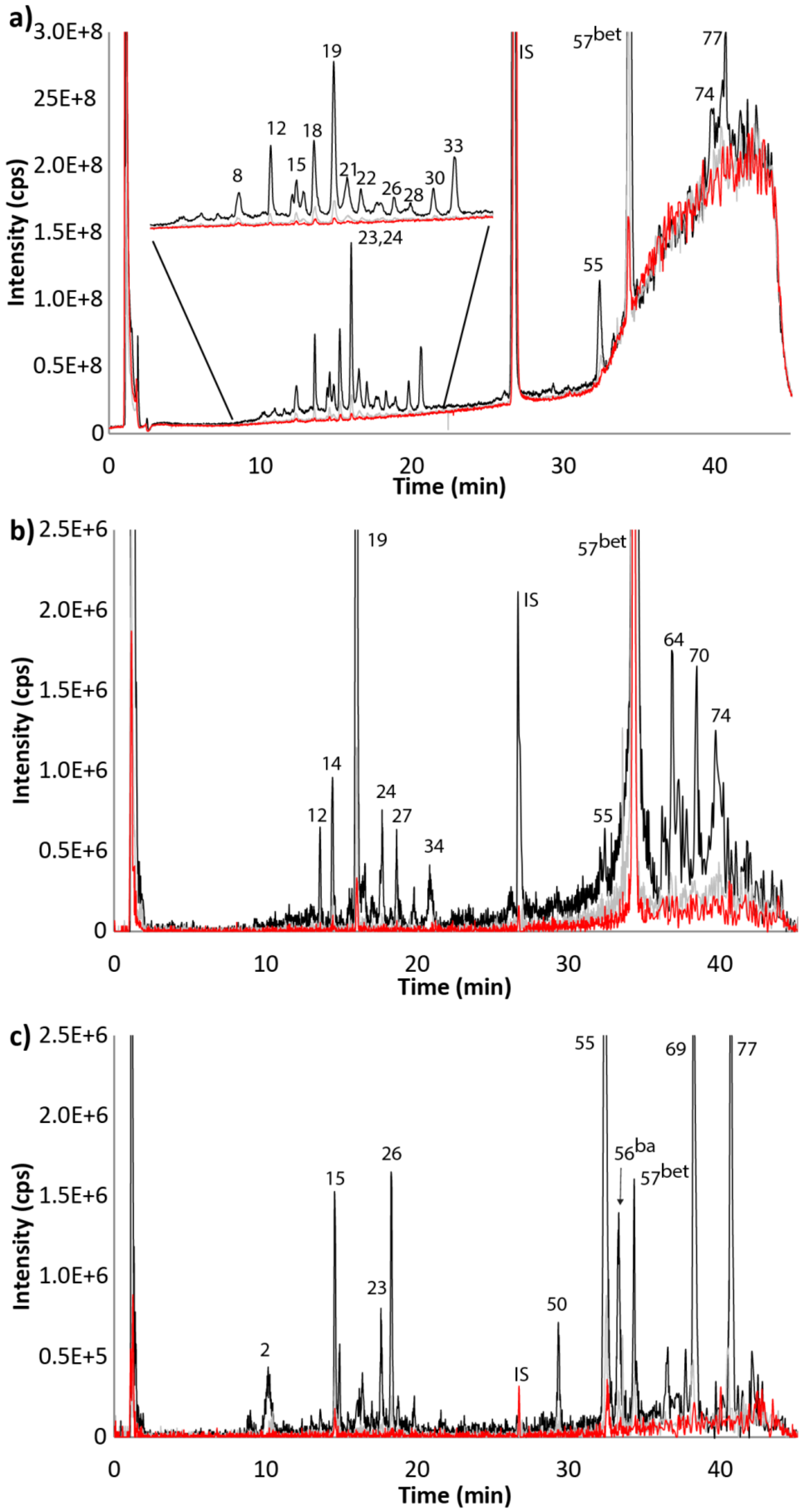

Figure S2. a) TIC, b) XIC of *m/z* 425, c) XIC of *m/z* 439, from each cycle of one of the PLE replicates, with internal standard marked by IS, betulin by [bet] (peak 57) and betulinic acid by [ba] (peak 56). Cycle 1 (black), cycle 2 (grey) and cycle 3 (red).

Table S4. MS data (EMS) for the three cycles of the PLE extracts. Numbers indicate for which cycle the peak was found, 1 is the first cycle *etc*. The base peak is written in bold.

Peak	Retention time (min)	In cycle(s)	Major *m/z*
1	8.8	1**	**439**
2	10.1	1	121, 139, **439**
6	11.5	1, 2, 3*	405, 423, **441**, 459, 467
8	12.3	1, 2, 3	**107**, 147, 299, 329, 478
12	13.5	1, 2, 3	405, **423**, 441, 459, 467
14	14.4	1, 2, 3	407, 425, **443**, 469
15	14.5	1, 2, 3**	391, 403, 421, 439, **457**
17	14.8	1, 2, 3*	107, 133, **437**, 455, 685*
18	15.2	1, 2, 3	107, 133, 297, 315, **423**, 437*
19	16.0	1, 2, 3	389, 407, 425, **443**, 518
21	16.4	1, 2, 3	**107**, 147, 433, 612
22	17.0	1, 2, 3**	365, 405, **423**, 441, 459
23	17.5	1, 2, 3*	421, **439**, 457, 483
24	17.6	1	407, **425**
26	18.3	1, 2, 3**	403, 421, **439**, 457, 475
27	18.6	1, 2, 3**	407, 425, **445**
28	18.9	1, 2, 3*	395, 423, 437, **453**
30	19.8	1, 2, 3	409, 419, **437**, 455, 473
32	20.4	1	407, **425**, 453, 471
33	20.6	1*	239, 257, **275**
34	20.8	1**, 2**, 3*	407, **425**, 453, 471
39	22.8	1*	309, 337, 407, **425**
43	25.5	1*	407, **425**, 453, 467, 513
IS	26.7	1, 2, 3	279, 297, **315**
50	29.3	1, 2, 3*	407, 421, 439, **457**
55	32.3	1, 2, 3	393, 411, **439**
56ba	33.2	1, 2, 3	189**, 203**, 393, 411, **439**
57bet	34.3	1, 2, 3	189, 203, 217, **407**, 425
58	34.5	1**, 2**, 3**	189, **203**, 217
63	36.3	1, 2**, 3**	405, **423**, 441
64	36.7	1, 2*	203, 395, 407, **425**
66	37.7	1, 2*	407, **425**
69	38.1	1, 2**, 3*	393, **439**
70	38.3	1, 2*	407, **425**
72	38.9	1, 2*	**409**, 427
74	39.6	1, 2, 3	201, 215, 409, **423**, 536
77	40.6	1, 2, 3	**421**, 439

IS internal standard

[ba]betulinic acid

[bet]betulin

*only seen in one replicate

**seen in two replicates

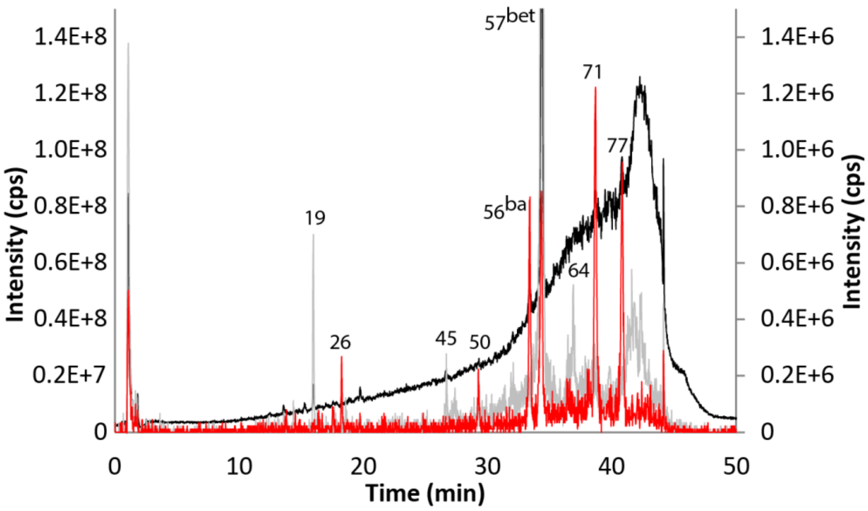

Figure S3. MS data for one of the SFE extracts, TIC (black, left side y-axis)), XIC's (right side y-axis) of *m/z* 425 (grey) and 439 (red). Betulin is marked by $^{\text{bet}}$ (peak 57) and betulinic acid by $^{\text{ba}}$ (peak 56).

Table S5. MS data (EMS) for the SFE extracts. Key to samples: the number indicates in which replicate(s) the peak was found. The base peak is written in bold.

Peak	Retention time (min)	In replicate(s)	Major *m/z*
7	12.1	1, 2, 3, 4	**459**, 476, 520^{+}
9	12.4	1, 2, 3, 4	**520**, 558^{+}
12	13.6	1, 2, 3, 4	405, **423**, 441, 459, 467
14	14.4	4	425, **443**
17	14.8	2, 3, 4	**437**, 455^{+}
18	15.3	3, 4	**107**, 315, 423, 437, 455^{+}
19	16.0	1, 2, 3, 4	407, 425, **443**$^{+++}$, 461^{+++}, 469^{+++}
22	17.0	1, 2, 3, 4	423, **441**$^{+++}$, 459
25	17.6	1, 2, 3, 4	421^{+++}, **439**, 457^{+++}
26	18.2	1, 2, 3, 4	403^{+}, 409^{+++}, 421, **439**, 457^{+++}, 475
28	18.9	1, 2, 3, 4	395, 423^{+}, 435^{+}, **453**
29	19.5	1, 2, 3, 4	**385**, 401^{+++}, 429, 445^{+++}
30	19.8	1, 2, 3, 4	419, **437**, 455, 473, 490^{+++}, 499^{+}, 517^{+}
36	21.7	1, 2, 3, 4	433+++, **451**, 469^{+++}
45	26.7	1, 2, 3, 4	**407**$^{+++}$, 425^{+++}, 452^{+}
50	29.2	1, 2, 3, 4	407^{+++}, 421, 439, **457**
52	30.5	1, 2, 3, 4	**437**
54	31.9	1, 2, 3, 4	**407**, 425^{+++}, 453, 467, 513
56$^{\text{ba}}$	33.4	1, 2, 3, 4	189, 203, **393**, 409^{++}, 411^{++}, 439
57$^{\text{bet}}$	34.3	1, 2, 3, 4	189, 203, 217, **407**, 425
59	34.7	1, 2, 3, 4	**437**
61	35.4	1, 2, 3, 4	**409**, 437, 455^{++}
63	36.3	1, 2, 3, 4	405, **423**, 441
64	36.9	1, 2, 3, 4	**407**, 425
71	38.6	1, 2, 3, 4	393, **439**
77	40.8	1, 2, 3, 4	**421**, 439

$^{\text{ba}}$betulinic acid
$^{\text{bet}}$betulin
$^{+}$only seen in one replicate, $^{++}$seen in two replicates, $^{+++}$seen in three replicates

Table S6. MS/MS data (EPI) for the betulin and betulinic acid standards and some impurities.

Peak	*m/z*, major signals (MS)	*m/z*, parent ion 1	*m/z*, major signals (parent ion 1)	*m/z*, parent ion 2	*m/z*, major signals (parent ion 2)	Possible identity
56	189, 203, 393, 411, 439	439	123, 177, 189, 203, 213, 243, 259, 393, 439	393	145, 161, 171, 185, 203, 215, 215, 393	betulinic acid
57	189, 203, 217, 407, 425	425	123, 137, 149, 161, 189, 203, 217, 407, 425	407	137, 159, 189, 201, 215, 255, 269, 407	betulin
12	405, 423, 441, 459, 467	441	161, 175, 187, 201, 215, 405, 423, 441	423	145, 161, 175, 189, 203, 217, 405, 423	betulinic aldehyde + 26 Da, possibly also with an H_2O adduct
14	407, 425, 443, 469	443	135, 147, 161, 177, 201, 351, 407, 425, 443	425	145, 161, 175, 189, 201, 367, 407, 425	betulin + 337 Da
17	107, 133, 437, 455, 685	455	161, 211, 361, 337, 381, 437, 455	437	175, 187, 361, 419, 437	betulonic acid + 230 Da
19	407, 425, 443, 461, 518	443	147, 173, 201, 367, 407, 425, 443	425	135, 147, 159, 189, 201, 407, 425	betulin + 275 Da, possibly also with an H_2O adduct
26	421, 439, 457, 475	439	147, 161, 201, 381, 403, 421, 439	421	119, 173, 187, 201, 215, 335, 421	
44	391, 409, 427	427	135, 147, 187, 217, 409, 427	409	135, 191, 149, 203, 217, 391, 409	lupeol or β-amerin
45	407, 425	425	135, 147, 163, 177, 191, 203, 217, 245, 425	407	133, 149, 189, 201, 227, 269, 351, 407	
63	405, 423, 441,	423	121, 147, 189, 203, 217, 405, 423	405	149, 161, 171, 203, 405	betulinic aldehyde
64	395, 407, 425	425	149, 177, 191, 203, 217, 229, 243, 407, 425	407	185, 191, 201, 269, 283, 407	
69	393, 439	439	123, 135, 149, 191, 203, 217, 249, 393, 439	393	159, 189, 213, 227, 393	
77	421, 439, 536	439	119, 179, 187, 203, 233, 421, 439	n.d.	n.d.	betulinic acid + 79 Da

n.d. not detected

Table S7. Complete list of peaks seen with the different detectors with indication of in which extracts the peaks were seen. Major signals seen by mass spectrometry (MS) included in column *m/z* with the base peak in bold, multiple bold signals for one peak means that different extraction methods resulted in different base peaks. For more detailed information, see supporting information Table S2 (DAD), Table S3 (CAD), Table S4 (MS, RB), Table S5 (MS, PLE) and Table S5 (MS, SFE**).**

Peak	Retention time (min)	DAD			CAD			MS			*m/z*
		RB	PLE	SFE	RB	PLE	SFE	RB	PLE	SFE	
1	8.8								X		**439**
2	10.1	X	X						X		121, 139, **439**
3	10.3	X									
4	10.7	X	X								
5	10.8	X	X								
6	11.5								X		405, 423, **441**, 459, 467
7	12.1	X	X	X	X	X	X			X	**459**, 476, 520
8	12.3	X[?1]						X	X		**107**, 147, 299, 329, 478
9	12.4	X[?1]								X	**520**, 558
10	13.0	X	X	X							
11	13.4	X	X	X	X	X	X				
12	13.5	X	X	X				X	X	X	405, **423**, **441**, 459, 467
13	14.2	X		X		X					
14	14.4	X	X	X		X		X	X	X	407, 425, **443**, 469
15	14.5								X		391, 403, 421, 439, **457**
16	14.6	X									
17	14.8	X	X	X		X		X	X	X	**107**, 133, **437**, 455, 626
18	15.3	X			X	X	X	X	X	X	**107**, 133, 297, 315, **423**, 437, 455
19	16.0	X		X	X	X	X	X	X	X	389, 407, 425, **443**, 461, 469
20	16.2	X		X							
21	16.4	X		X	X	X		X	X		**107**, 147, 433, **612**
22	17.0	X		X			X	X	X	X	365, 405, **423**, **441**, **459**
23	17.5	X	X	X	X			X	X		421, **439**, 457, 483
24	17.6		X			X			X		407, **425**
25	17.6			X			X			X	421, **439**, 457
26	18.3							X	X	X	403, 409, 421, **439**, 457, 475
27	18.6								X		407, 425, **445**
28	18.9			X					X	X	395, 423, 435, 437, **453**
29	19.5									X	**385**, 401, 429, 445
30	19.8						X	X	X	X	409, 419, **437**, 455, **473**, 490, 499, 517
31	20.3	X		X		X	X				

32	20.4								X		407, **425**, 453, 471
33	20.6								X		239, 257, **275**
34	20.8								X		407, **425**, 453, 471
35	20.9	X		X							
36	21.7									X	433, **451**, 469
37	21.8	X									
38	22.6	X		X							
39	22.8								X		309, 337, 407, **425**
40	23.4	X									
41	24.0	X		X							
42	25.1	X	X	X							
43	25.5								X		**407**, 425, 453, 467, 513
44	25.7							X			391, **409**, 427
45	26.7	X		X	X		X	X		X	**407**, **425**, 452
IS	26.7		X			X			X		279, 297, **315**
46	27.8		X				X				
47	28.1						X				
48	28.6						X				
49	29.0						X				
50	29.2						X	X	X	X	**407**, 421, 439, **457**
51	30.1	X	X	X							
52	30.5									X	**437**
53	31.1						X				
54	31.9						X			X	**407**, 425, 453, 467, 513
55	32.3						X		X		189, 203, 393, 411, **439**
56[ba]	33.2	X	X	X	X	X	X	X	X	X	189, 203, **393**, 411, **439**
57[bet]	34.4	X	X	X	X	X	X	X	X	XS	189, 203, 217, **407**, 425
58	34.5								X		189, **203**, 217
59	34.7	X		X						X	**437**
60	35.1	X		X	X	X	X				
61	35.4	X		X			X			X	**409**, 437, 455
62	36.0	X		X	X	X	X				
63	36.3								X	X	405, **423**, 441
63/64[?]	36.5				X	X	X				
64	36.7	X		X				X	X	X	203, 395, **407**, **425**
65	37.4	X		X			X				
66	37.7					X			X		407, **425**
67	37.8	X		X	X		X	X			393, **439**
68	37.9	X						X			407, **425**

69	38.1	X[?2]						X	X		393, **439**
70	38.3								X		407, **425**
71	38.6	X[?2]		X			X			X	393, **439**
72	38.9								X		**409**, 427
73	39.2	X		X	X	X	X				
74	39.6		X						X		201, 215, 409, **423**, 536
75	39.9				X	X	X	X			403, **421**, 439
76	40.2	X		X			X				
77	40.6	X		X				X	X	X	**421**, **439**, 536
78	41.0	X		X	X		X				
79	44.3	X						X			**407**, 425

[ba]betulinic acid
[bet]betulin
[?]uncertain peak assignment, could be either of the suggested peaks
[?1]assignment not certain, the signal could be either peak 8 or peak 9, considering the MS data (Table S4) peak 8 seems more likely
[?2]assignment not certain, the signal could be either peak 69 or peak 71

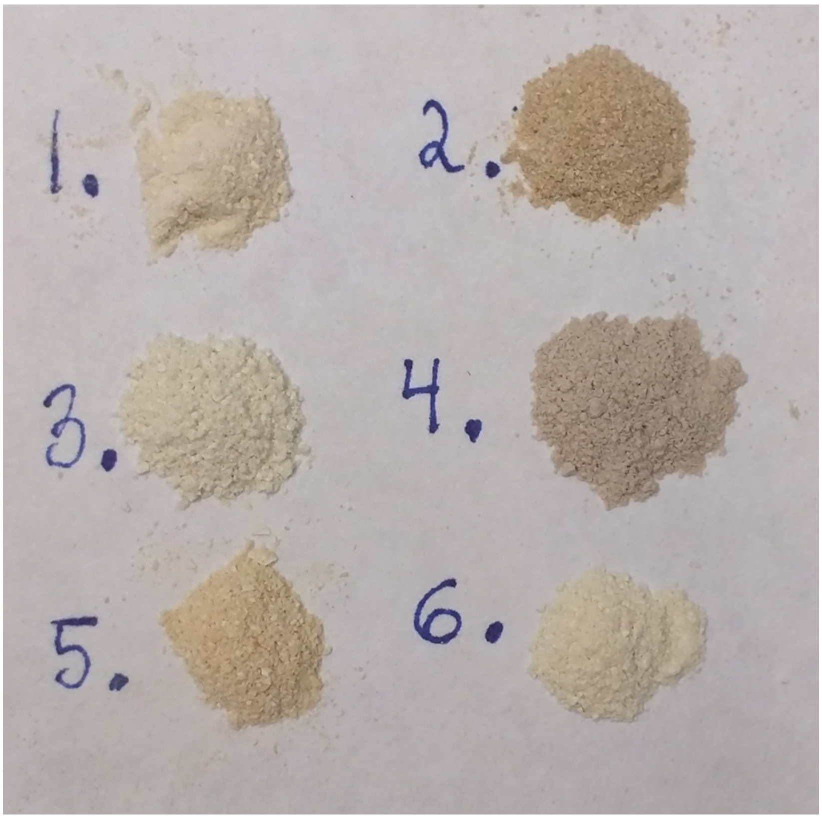

Figure S4. The picture shows dried process fluids and precipitations in the reflux boiling process.
(1): Process fluid after reflux boiling using pre-boiled birch bark
(2) Process fluid after reflux boiling
(3) Reflux boiling using pre-boiled birch bark and precipitated
(4) Reflux boiling and precipitated
(5) Filtrated process fluid after reflux boiling
(6) Precipitated filtrated process fluid after reflux boiling

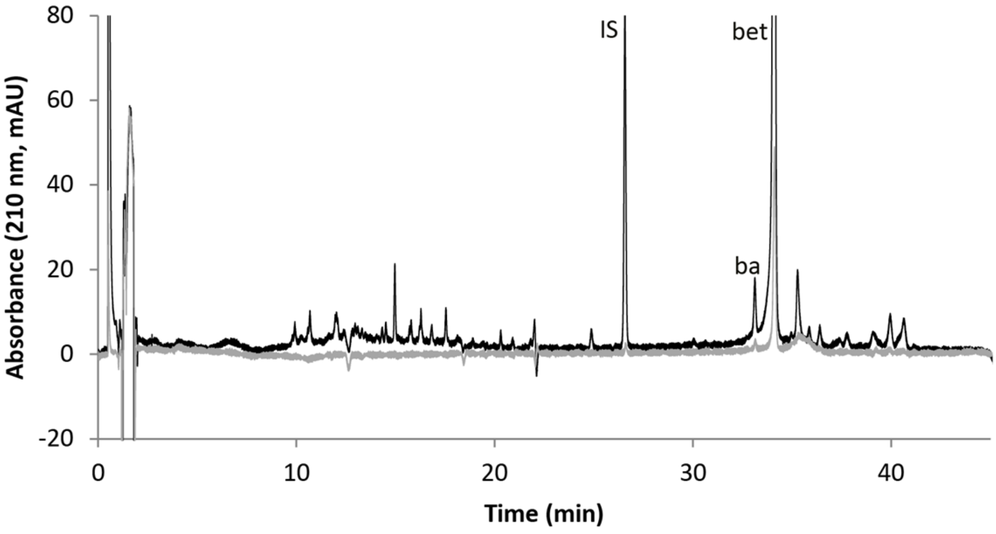

Figure S5. Chromatograms of extracts obtained by PLE recorded with DAD 210 nm using no purification (black, chromatographic purity 69.0%) and precipitation (grey, chromatographic purity 80.0%). Betulin is labeled bet, betulinic acid ba and internal standard (progesterone) IS.