Optimization of Baicalin Samples Characterization by MALDI-TOF MS

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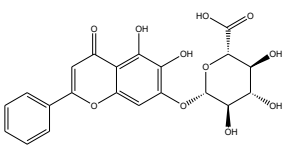
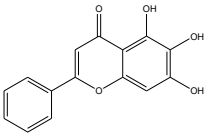
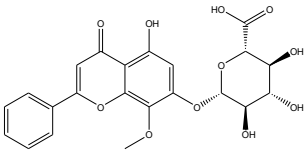


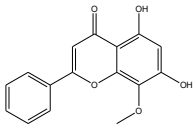
Baicalin (BG)
PM 446,36



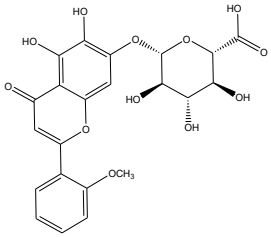
Beicalin (BE)
PM 270,20



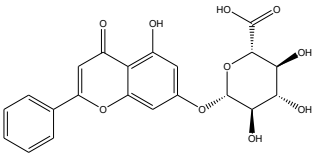
Wogonoside (WG)
PM 460,39



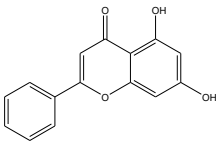
Wogonin (WO)
PM 284,20



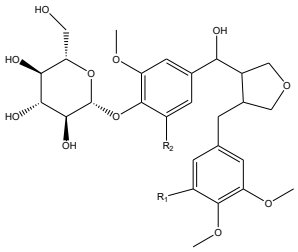
Lateriflorein 7-O-glucuronide (Later)
PM 476,2



Chrysin-7-glucuronide (Chr-G)
PM 430,47



Chrysin (Chr)
PM 254,20



Baicalensinoside-A (B-A)
R_1 = OMe; R_2 = Ome
PM 582.3

Chart SI1 – Structures of the most important bioactive molecules detected by MALDI-TOF MS analysis of the baicalin's samples.

1. Extraction and Purification procedure

1.1 Purification Procedure of commercial baicalin-30%

In preliminary trials, several solvents and reflux times were tested. The results are summarized in Table SI1. The procedure that gave the best result is described below.

3 g of baicalin-30% were treated with 45 ml of a 2-propanol/water mixture (70:30 v/v) at 60 °C and kept under reflux for 1.5 hours in a round bottom flask. Firstly, a yellow precipitate was observed together with a pale-yellow solution that gradually turned amber by the end of the heating time. Then, the solution was filtered, whereas the solid residue was dried overnight in a vacuum oven at 30 °C and both subsequently analysed by MALDI-TOF MS. The soluble fraction was distilled in a rotavapor under vacuum and the residue was dried at 30°C in a vacuum oven, obtaining 1.3 g of sample. It was treated with 20 ml of butanol at 60°C for 1h, the obtained solution was filtered and distilled under vacuum in a rotavapor system obtaining 0.36 g of solid sample. The insoluble fraction in butanol was further purified by the crystallization method. The insoluble solid (0.82 g) was treated with the minimum volume (20 ml) of water/ 2-propanol/acetic acid 4:3:1 ratio (v/v/v) ternary solvent mixture at 60 °C and kept at this temperature (T) for 30 min. Afterwards, the solution was slowly cooled to room temperature (RT) and stored overnight in a fridge. The partially crystallized solution was filtered and dried at 30°C overnight in a vacuum system, obtaining 0.46 g of sample, which was analysed by the MALDI-TOF MS and ^{1}H-NMR methods.

Table SI1 – Molar composition (%) and yield of the most important baicalin-fractions extracted at 60°C for different times from the commercial baicalin 30% sample.

Solvent	Time (h)	%mol[(a)] BG/BE/WG/WO	Yield[(b)] %
Isopropanol\water 70:30 v/v	1.5	86.0/4.4/6.3/3.1	13
Isopropanol\water 80:20 v/v	1.5	74.7/10.4/8.3/6.6	8
Isopropanol\water 60:40 v/v	2.0	82.5/6.8/6.3/4.4	10
Isopropanol	2.0	57.8/18.2/15.7/8.3	9
Ethanol	2.0	79.3/5.6/8.3/6.6	7

(a) Molar composition of the four bio-active compounds (BG, BE, WG, WO) calculated from 5 mass spectra of the crystallized purified/extracted fractions. The values were determined with a standard deviation of approximately ± 0.3 for those below 20% and approximately ± 0.8 for those above 50%.
(b) Yield of the final crystallized purified/extracted fractions.

1.2 Extraction and Purification procedure from root

The extraction-purification-crystallization procedure carried out to obtain high-purity samples of baicalin from the root of *Scutellaria Baicalensis* was similar to that used for baicalin 30%. 5 g of root were cut into small pieces and extracted with ethanol (or isopropanol) under reflux at 60 °C for different times (6 and 12 h). After that, the reaction mixture was filtered, and the filtrate was distilled using a rotary evaporator. The obtained dry residue (0.8 g) was treated with butanol (15 ml) and the insoluble residue was filtered and dried at 30 °C for 12 h in a vacuum oven. The purified extract (0.6 g) was further purified by the crystallization procedure, using a minimal volume of the ternary solvent mixture water/2-propanol/ acetic acid in a ratio of 4:3:1 (v/v/v), obtaining 0.35 g of crystallised sample. The results are summarized in Table SI2

Table SI2 – Molar composition (%) and yield of the purified/crystallized fractions extracted at 60°C for different times from the commercial *S. baicalensis* root

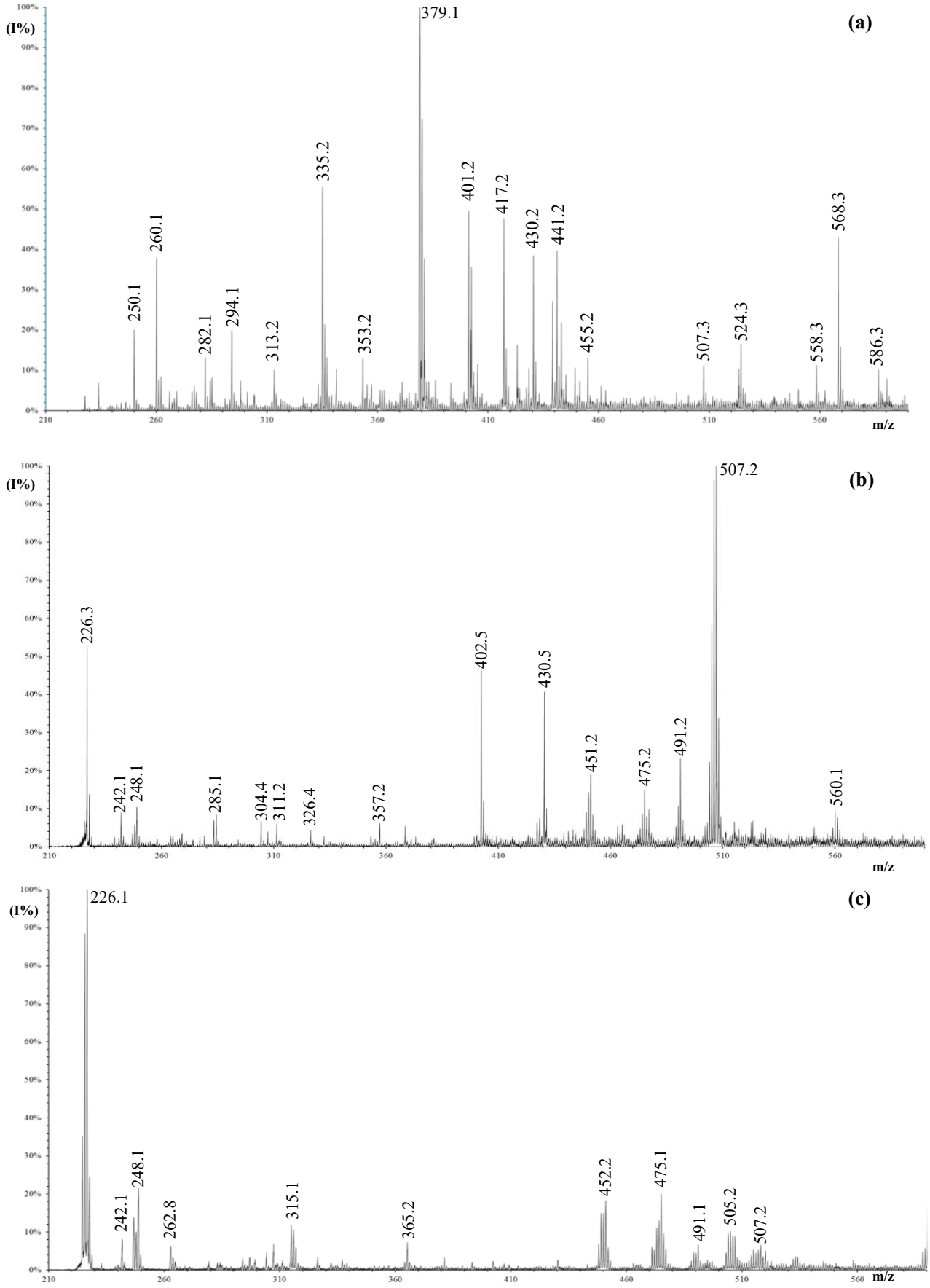
Solvent	**Time (h)**	***Yield*[(a)] %**	**% mol[(b)] BG/BE/WG/WO**
Ethanol	6	1.2	58.2/15.8/12.6/13.4
Ethanol	12	2.3	76.3/9.8/7.6/6.2
Isopropanol	6	1.4	50.3/10.9/14.5/9.7[(c)]
Isopropanol	12	0.5	44.2/14.7/14.4/14.1[(d)]

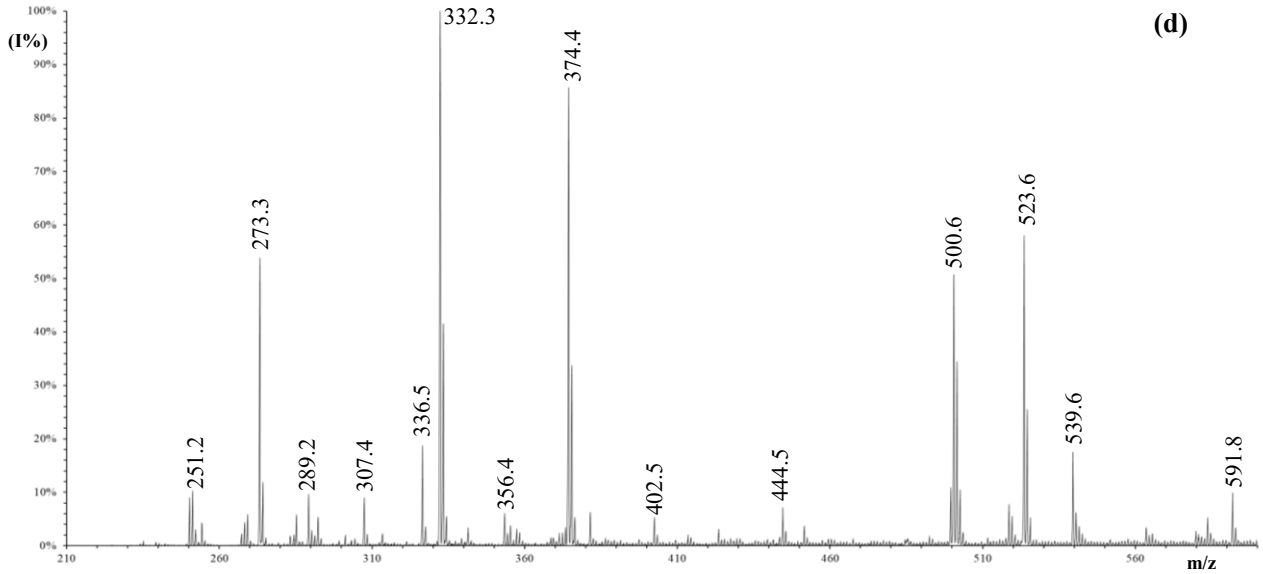
a) Yield of the final crystallized purified/extracted fractions.

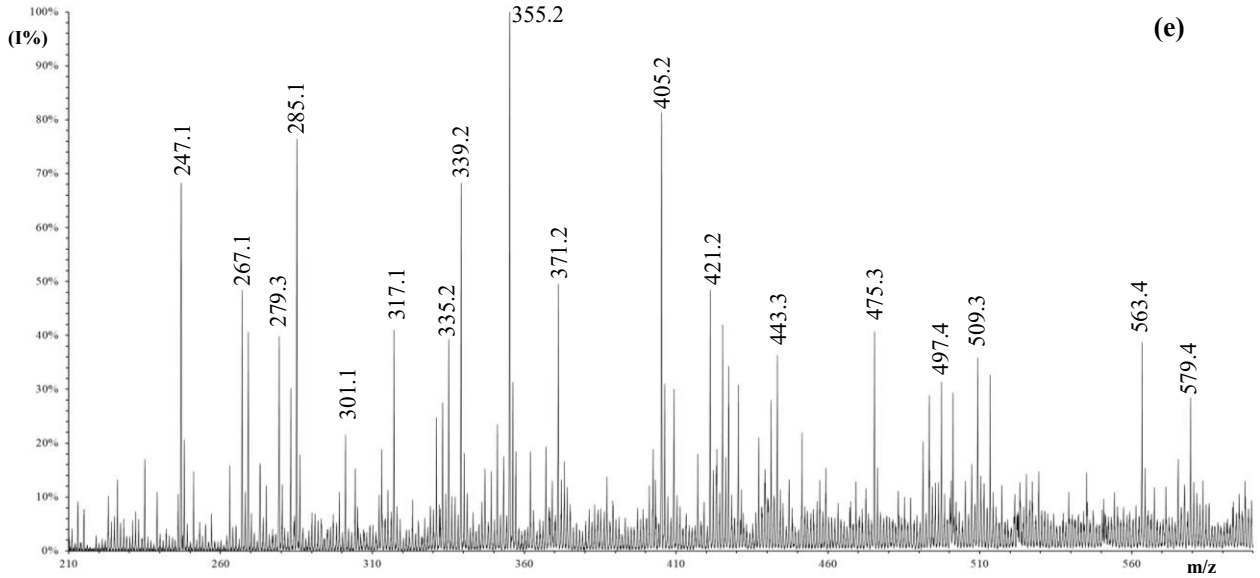
(b) Molar composition of the four bio-active compounds (BG, BE, WG, WO) calculated from 5 mass spectra of the crystallized purified/extracted fractions. The values were determined with a standard deviation of approximately ± 0.6 for those below 20% and approximately ± 1.2 for those above 50%.

(c) Molecules such as Later (12.1%mol) and another with MW of 522.2 (2.4%) were detected

(d) Molecules such as Later (9.6%mol) and another with MW of 522.2 (2.9%) were detected


(a)
(I%)
379.1
335.2
401.2
417.2
430.2
441.2
260.1
568.3
250.1
294.1
282.1
313.2
353.2
455.2
507.3
524.3
558.3
586.3
m/z
(b)
(I%)
507.2
226.3
402.5
430.5
451.2
491.2
475.2
242.1
248.1
285.1
304.4
311.2
326.4
357.2
560.1
m/z
(c)
(I%)
226.1
248.1
452.2
475.1
242.1
262.8
315.1
365.2
491.1
505.2
507.2
m/z


(d)
(I%)
332.3
374.4
273.3
523.6
500.6
539.6
591.8
251.2
289.2
307.4
336.5
356.4
402.5
444.5
m/z


(e)
(I%)
355.2
405.2
247.1
285.1
339.2
267.1
371.2
279.3
421.2
317.1
335.2
443.3
475.3
497.4
509.3
563.4
301.1
579.4
m/z

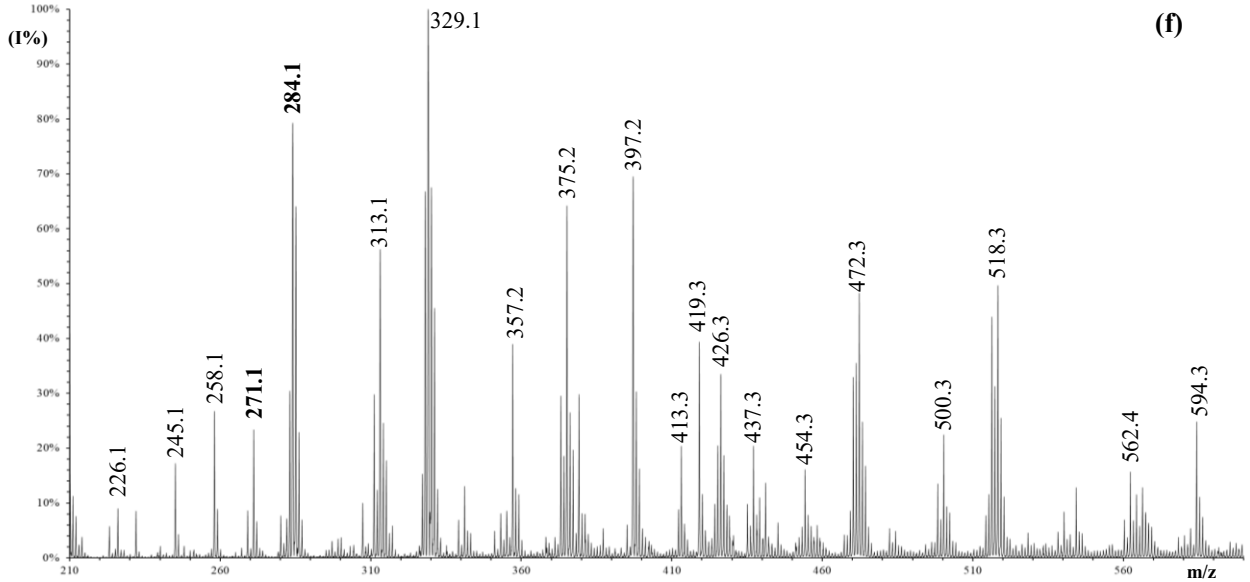

(f)
(I%)
329.1
284.1
375.2
397.2
313.1
357.2
419.3
426.3
472.3
518.3
258.1
271.1
245.1
413.3
437.3
454.3
500.3
562.4
594.3
226.1
m/z

Figure SI1 – MALDI-TOF mass spectra of some matrices investigated: a) α-Cyano-4-hydroxycinnamic acid (CHCA), b) 1,8,9-anthracenetriol (dithranol), c) dithranol doped with sodium trifluoroacetate, d) trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB), e) 2,5-dihydroxybenzoic acid (DHB), and f) trans-3-indoleacrylic acid (IAA).

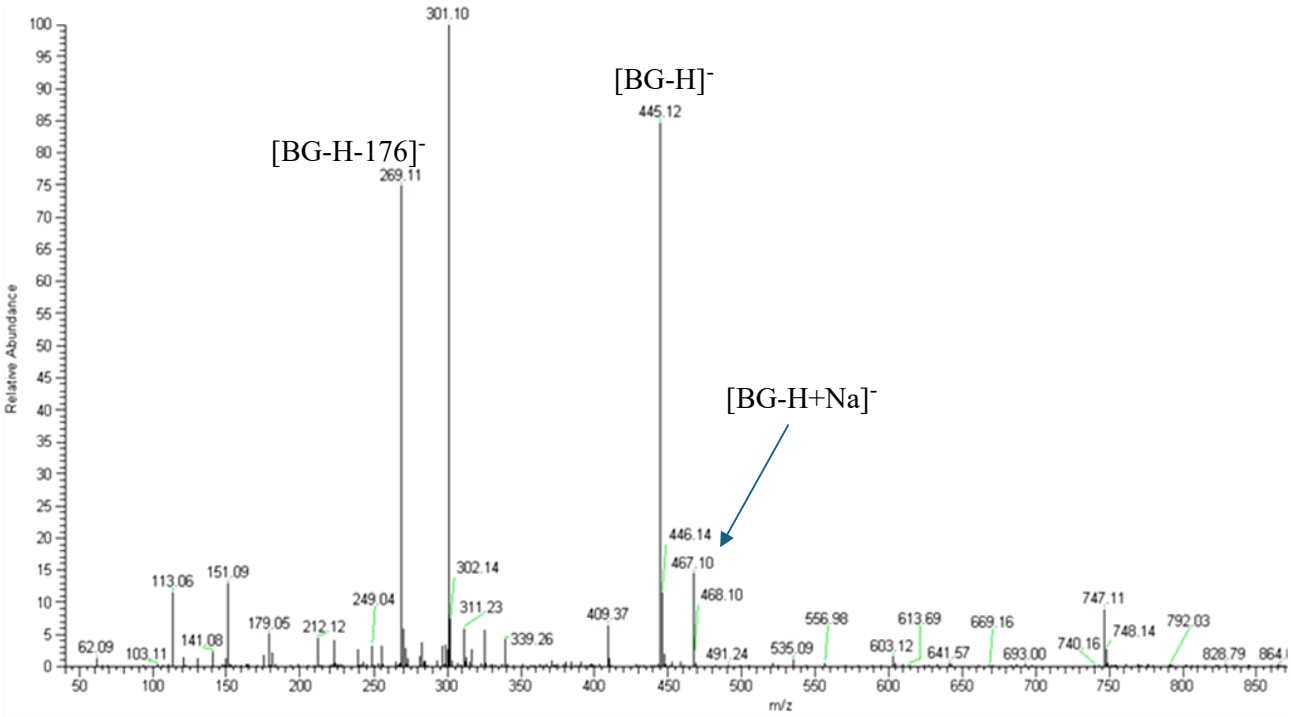


Figure SI2 – ESI mass spectrum of the baicalin 95%**,** recorded in negative ions mode by direct infusion.

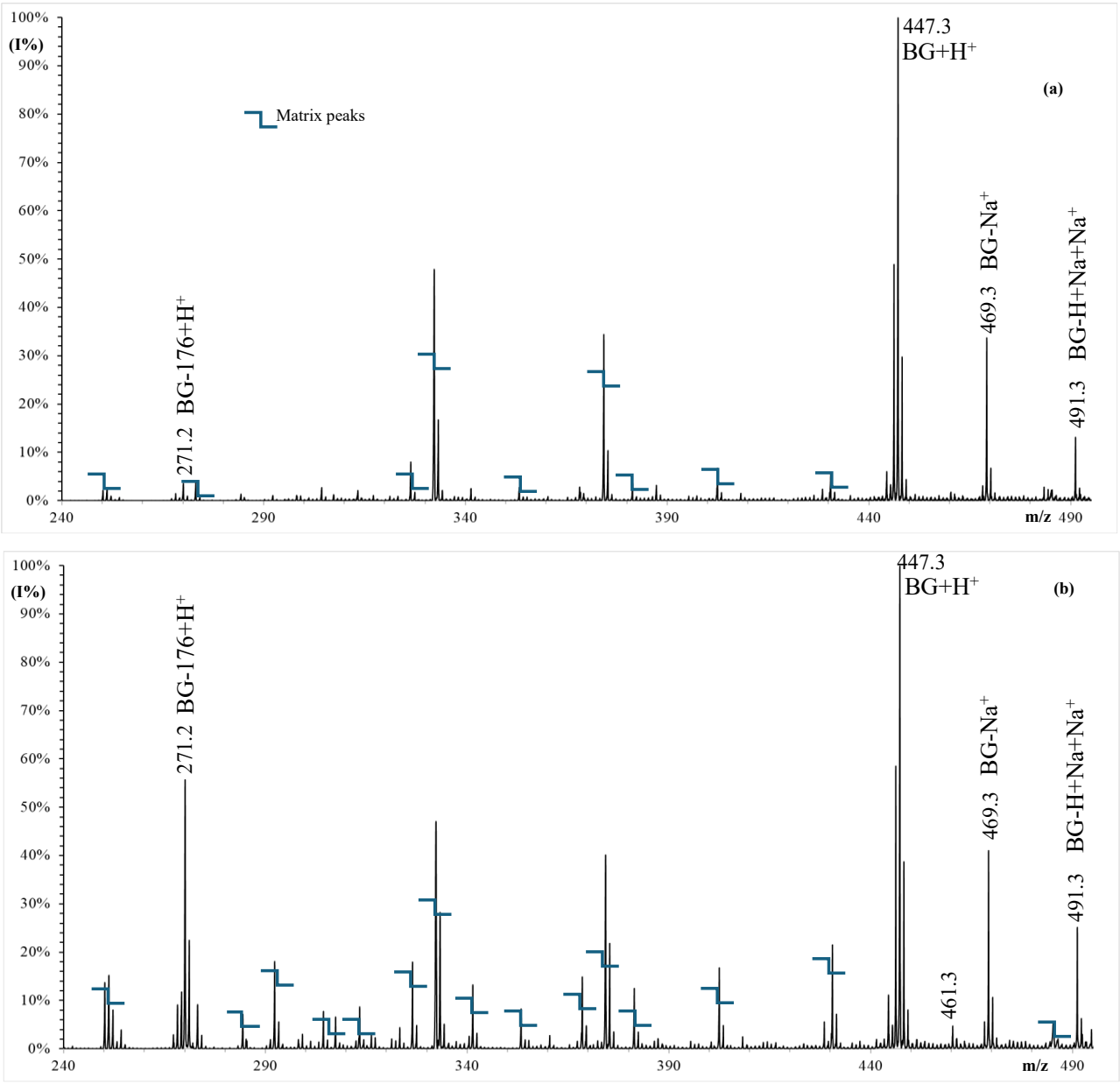


Figure SI3 – MALDI-TOF mass spectra of baicalin 95% acquired using DCTB as matrix at laser intensities corresponding to a) the ionization threshold and b) 10% above the threshold value.

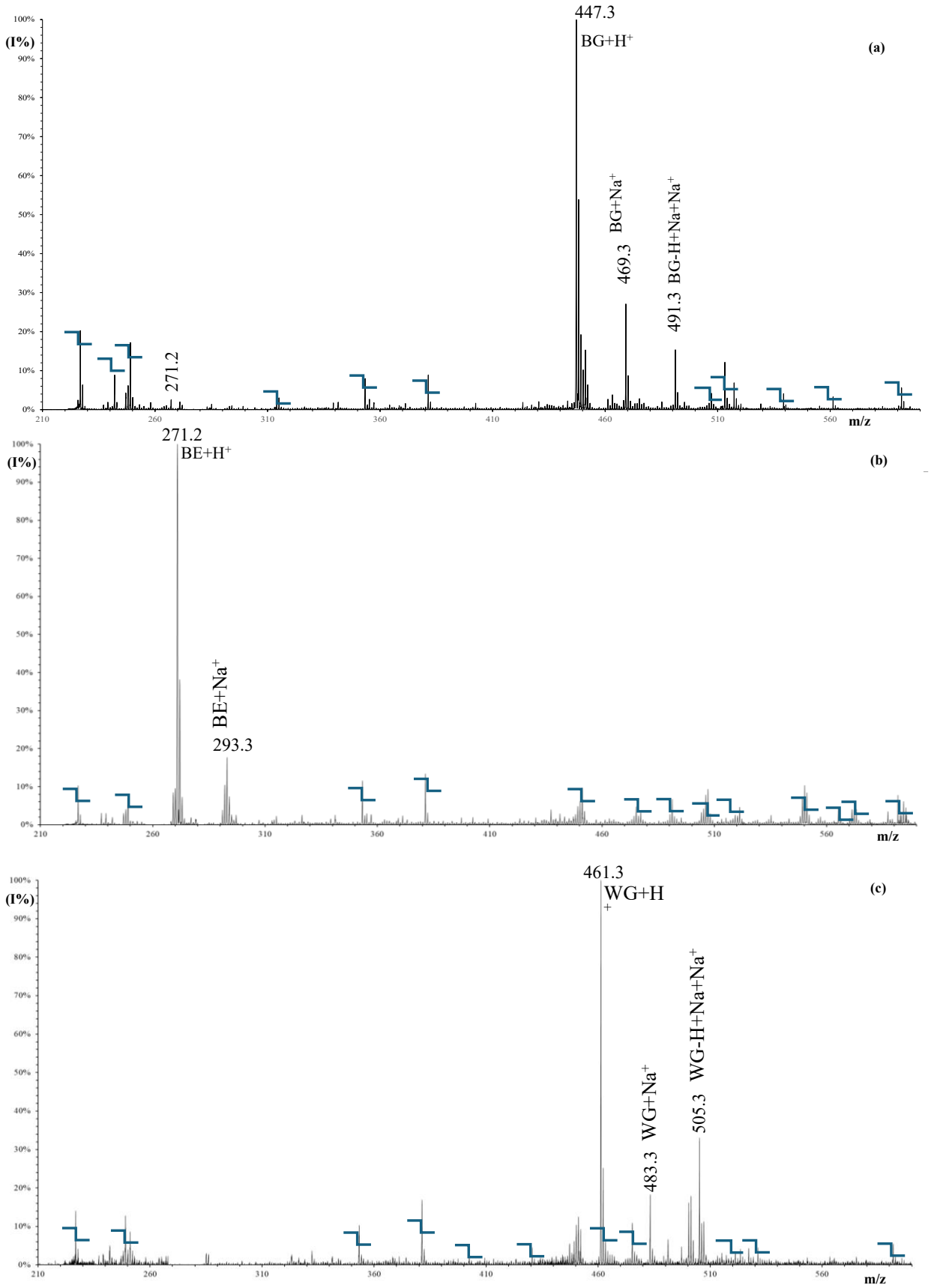


Figure SI4– Positive ions MALDI-TOF mass spectra of the standards: a) Baicalin (BG), b) Baicalein (BE) and c) wogonoside (WG), recorded using dithranol/CF_3COONa as matrix and applying a threshold laser intensity.

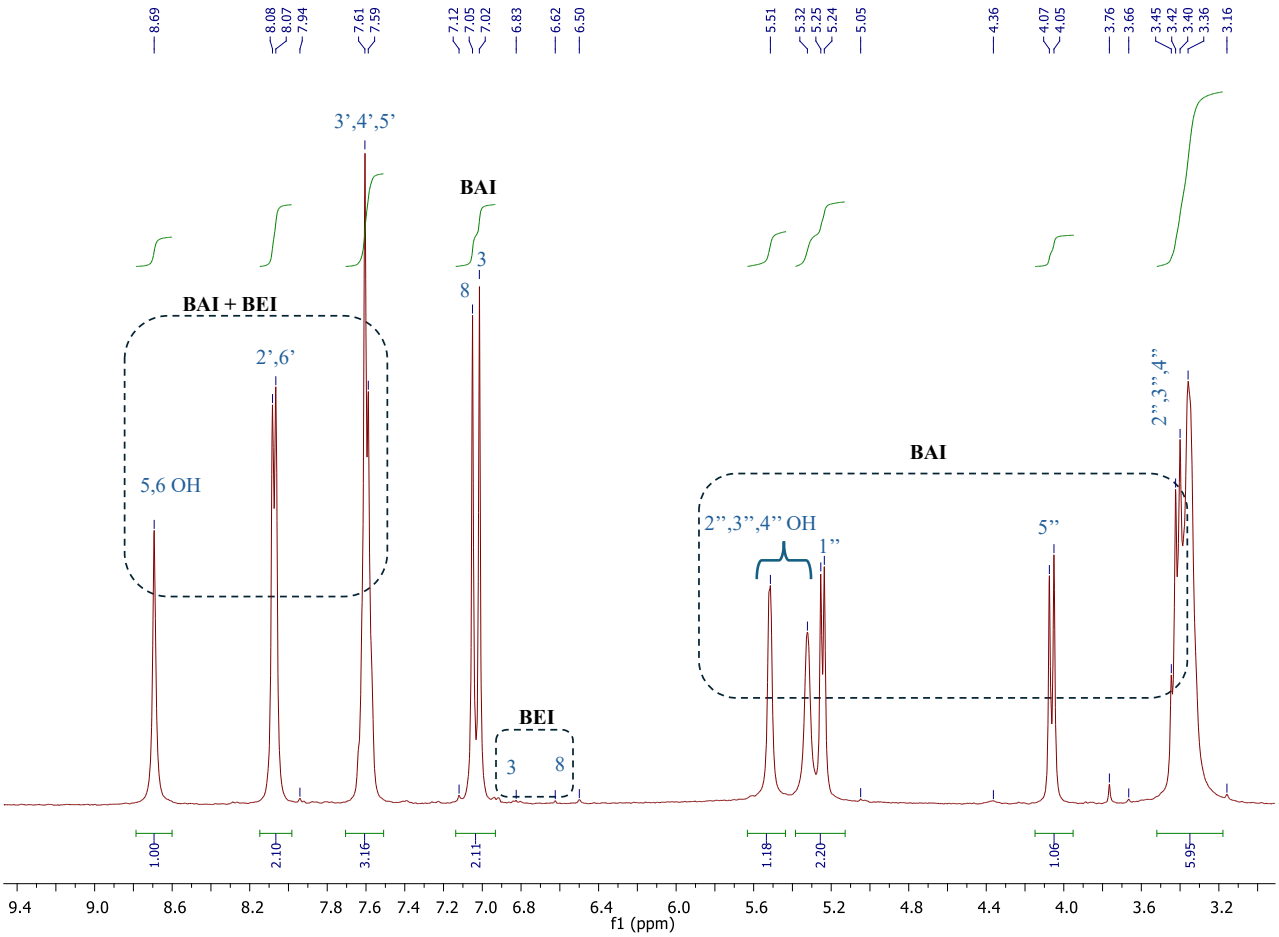


Figure SI5 - ^{1}H-NMR spectrum of the commercial Baicalin-95%, recorded using DMSO-d_6 as solvent.

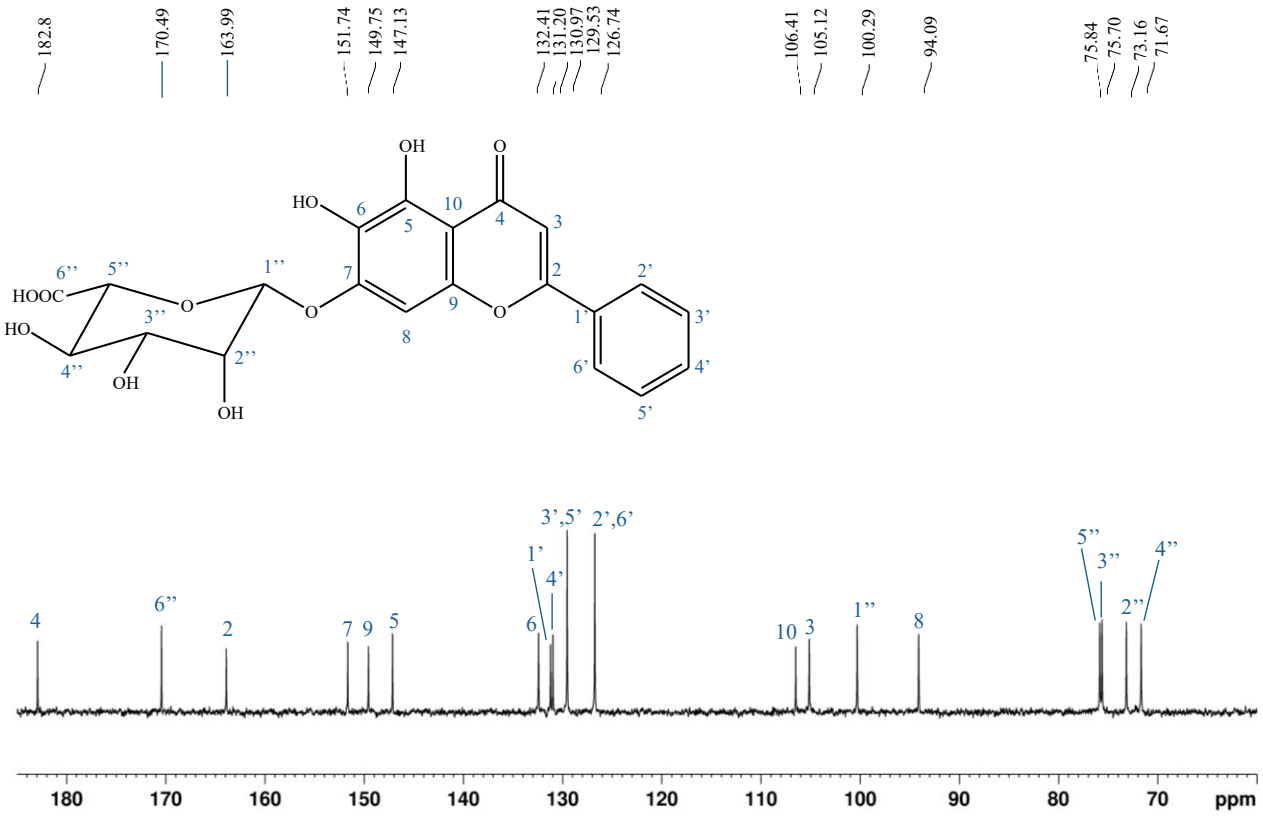
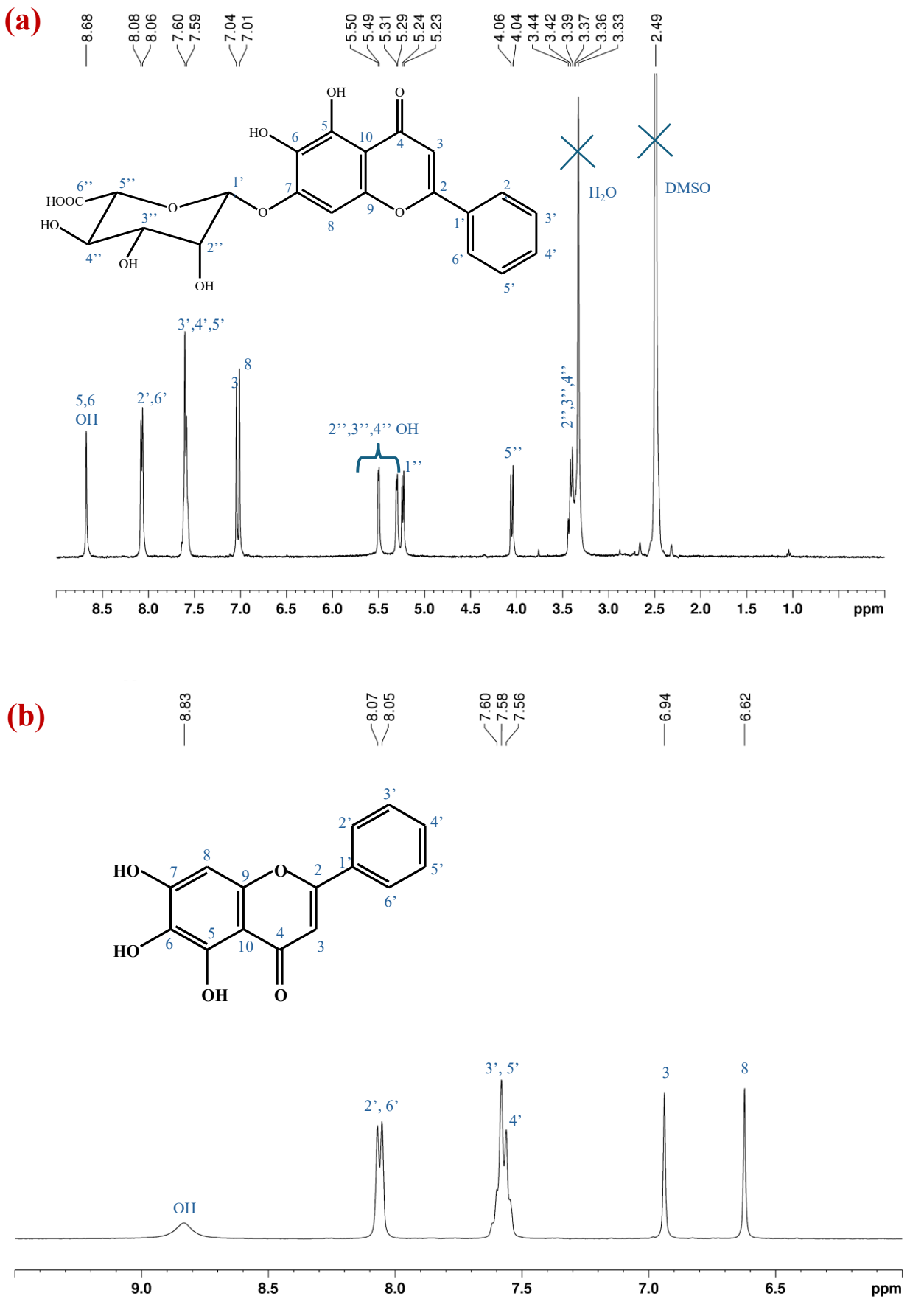


Figure SI6 - ^{13}C-NMR spectrum of the commercial Baicalin-95%, recorded using DMSO-d_6 as solvent.



Figures SI7 - ^{1}H-NMR spectra of a) Baicalin and b) Baicalein standard compounds, recorded using DMSO-d_6 as solvent.

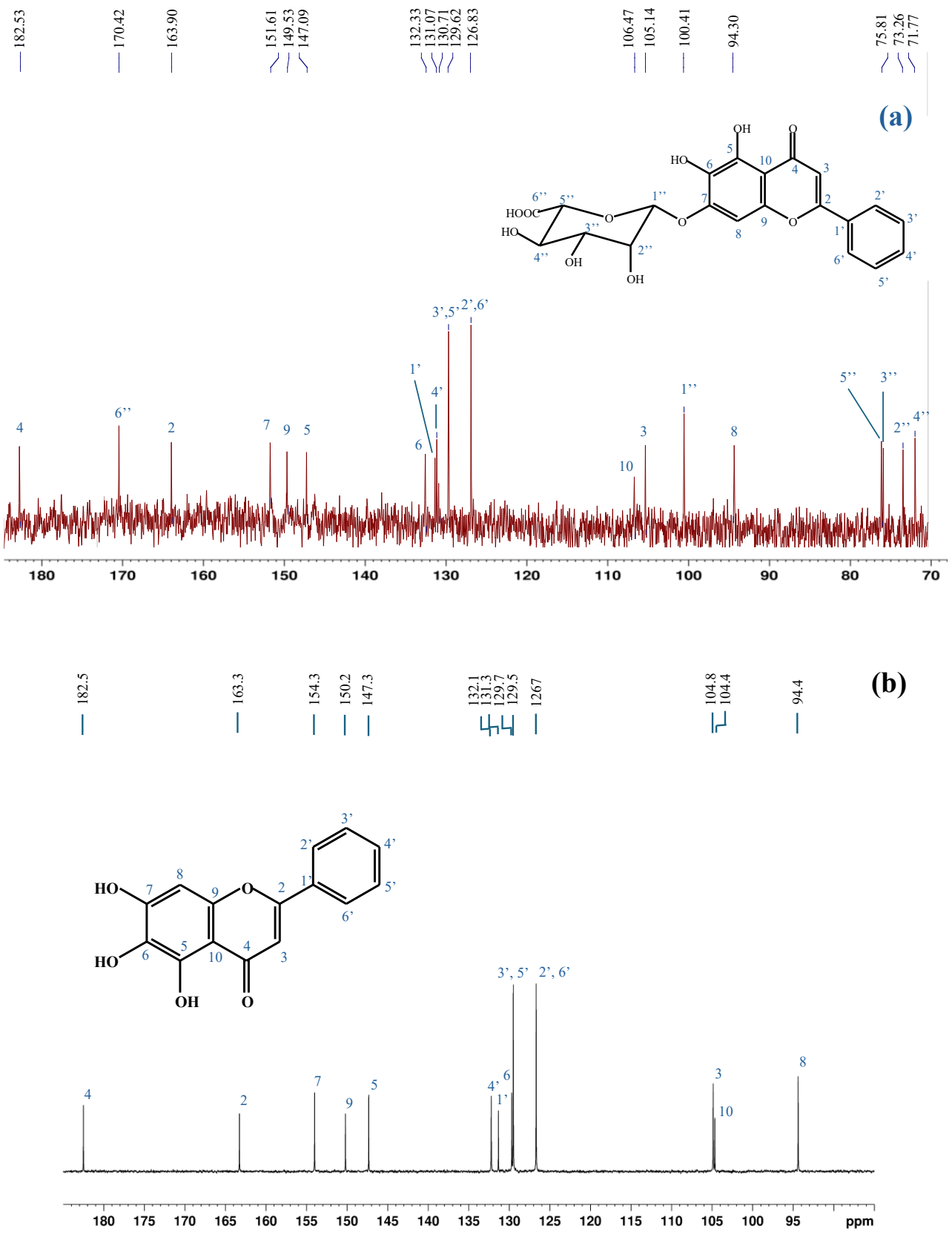


Figure SI8 - ^{13}C-NMR spectra of a) Baicalin and b) Baicalein standard compounds, recorded using DMSO-d_6 as solvent.

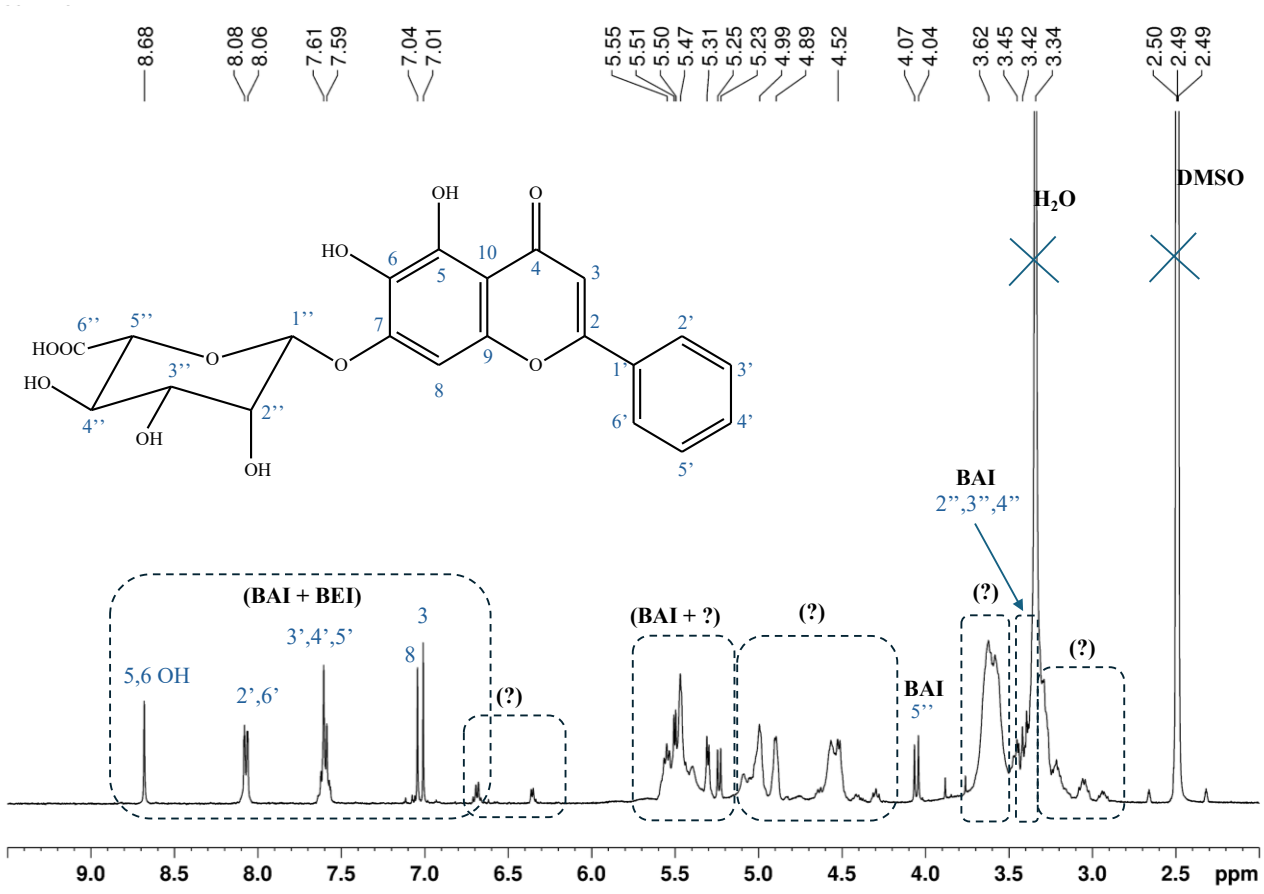


Figure SI9 - ^{1}H-NMR spectrum of neat Baicalin-30% sample in DMSO-d_6 as solvent.

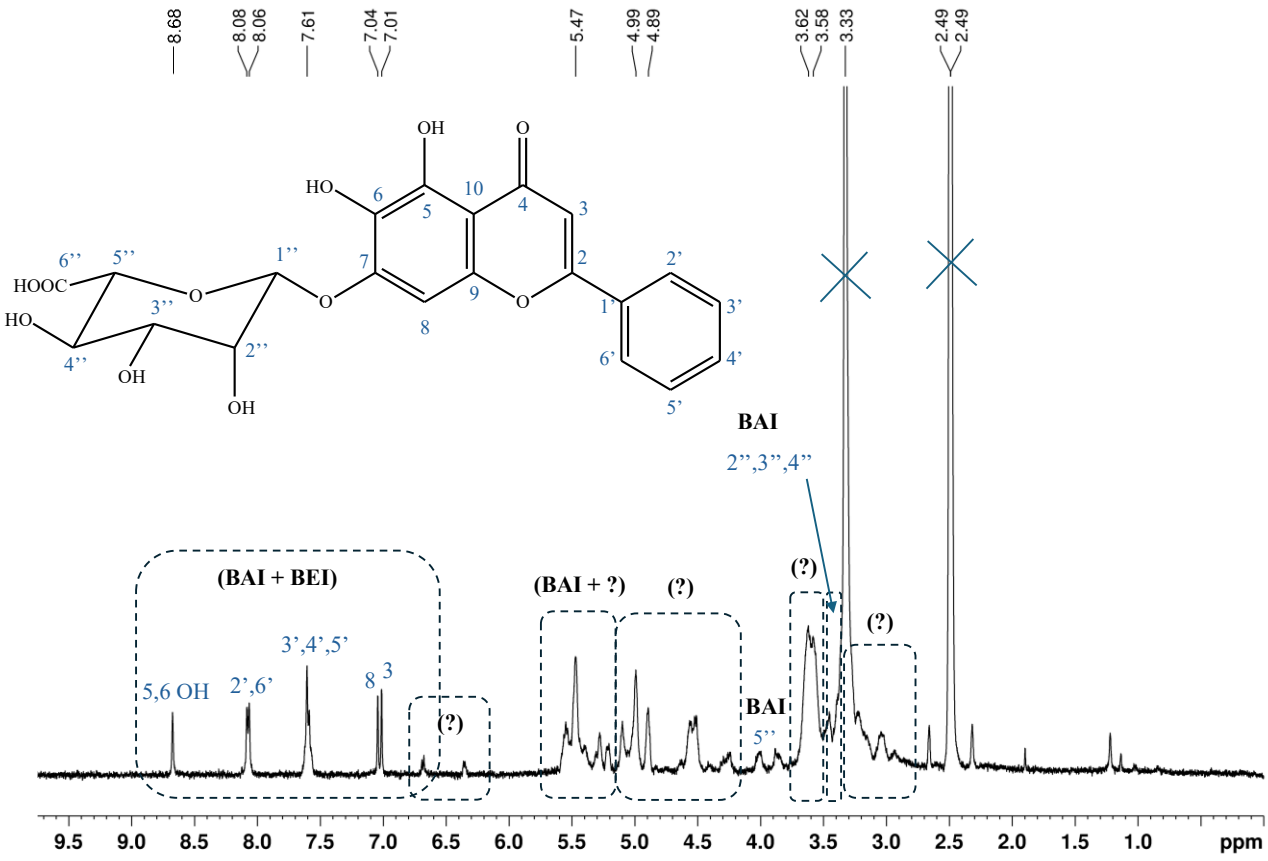


Figure SI10 -^{1}H-NMR spectrum of the soluble fraction in the 2-propanol/water solvent mixture (70:30 v/v), extracted from the commercial Baicalin-30% sample.

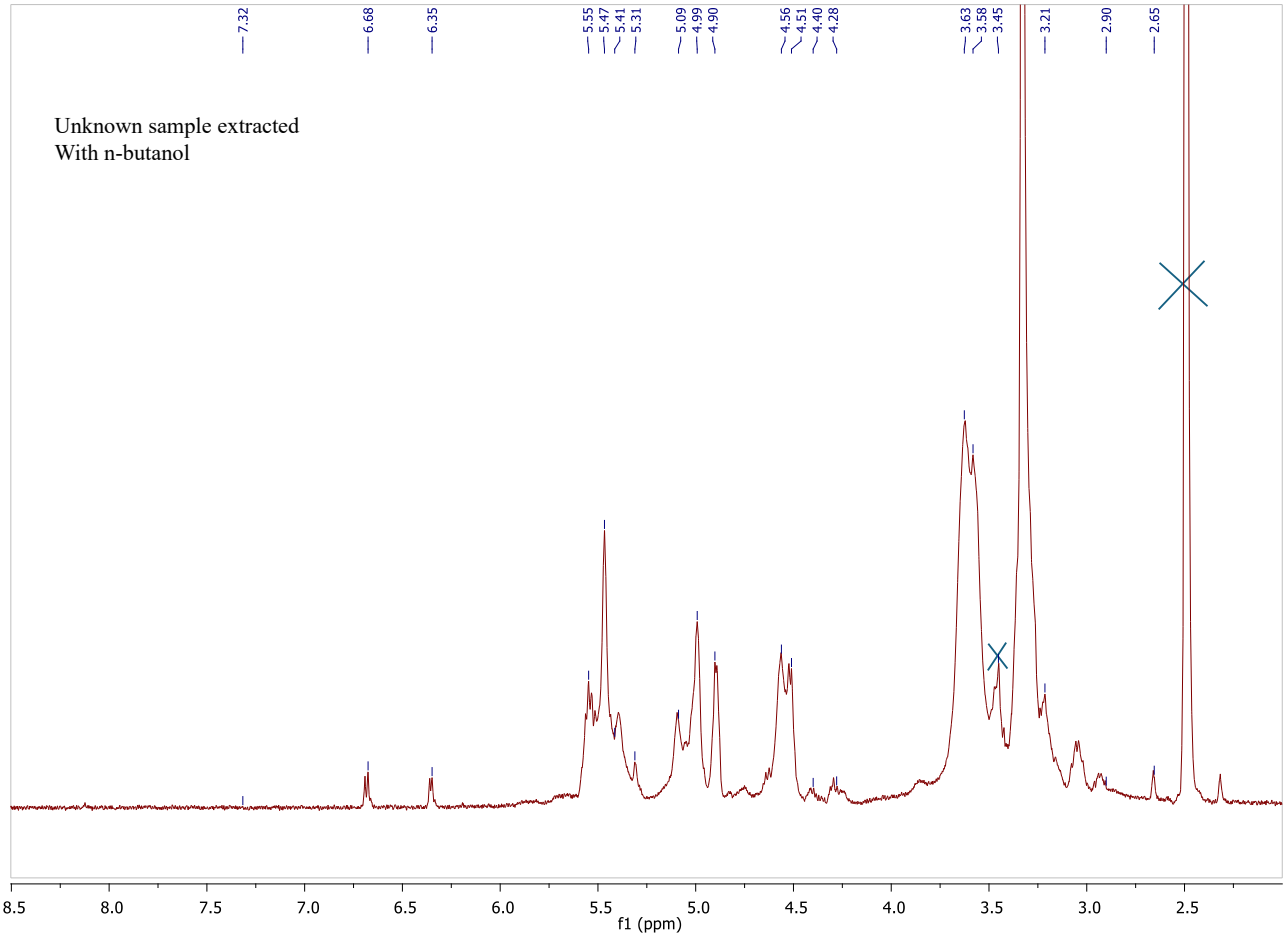


Figure SI11 -^{1}H-NMR spectrum of unknown butanol soluble component extracted from the soluble fraction in 2-propanol/water solvent mixture (70:30 v/v) extracted from the commercial Baicalin-30% sample.

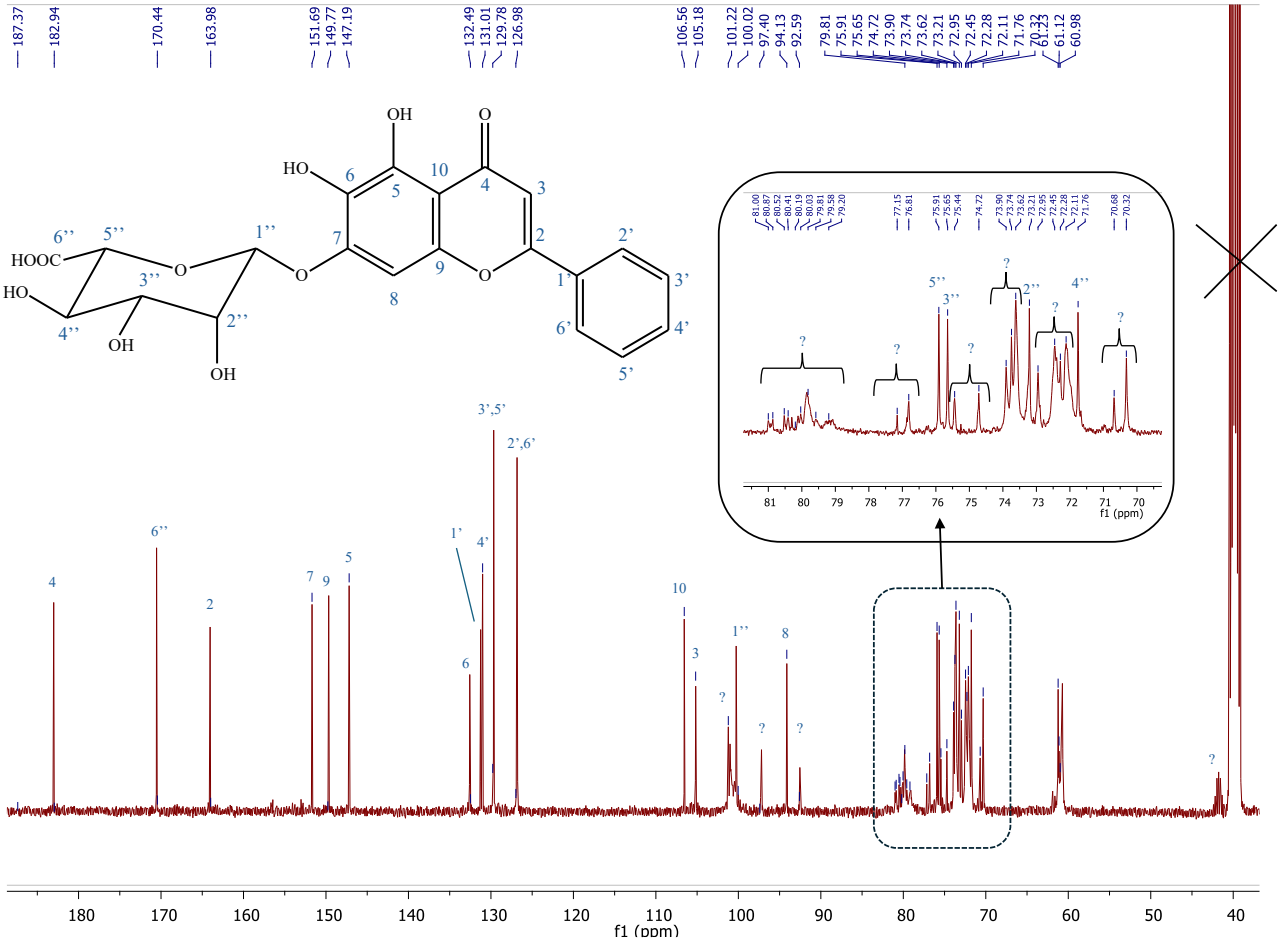
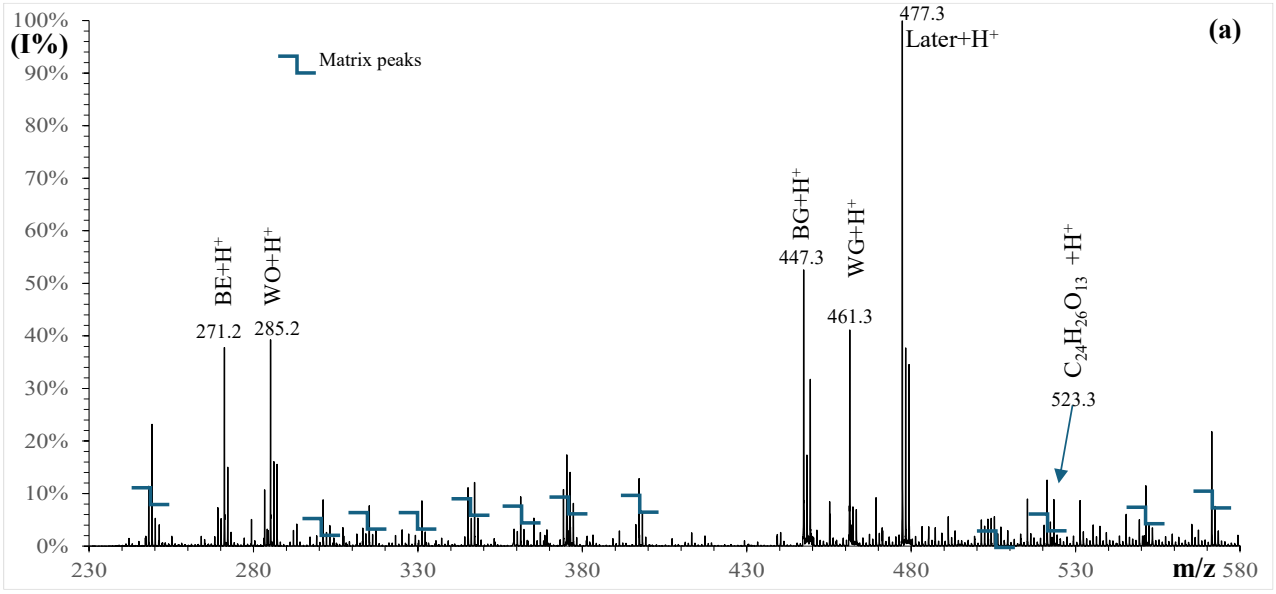
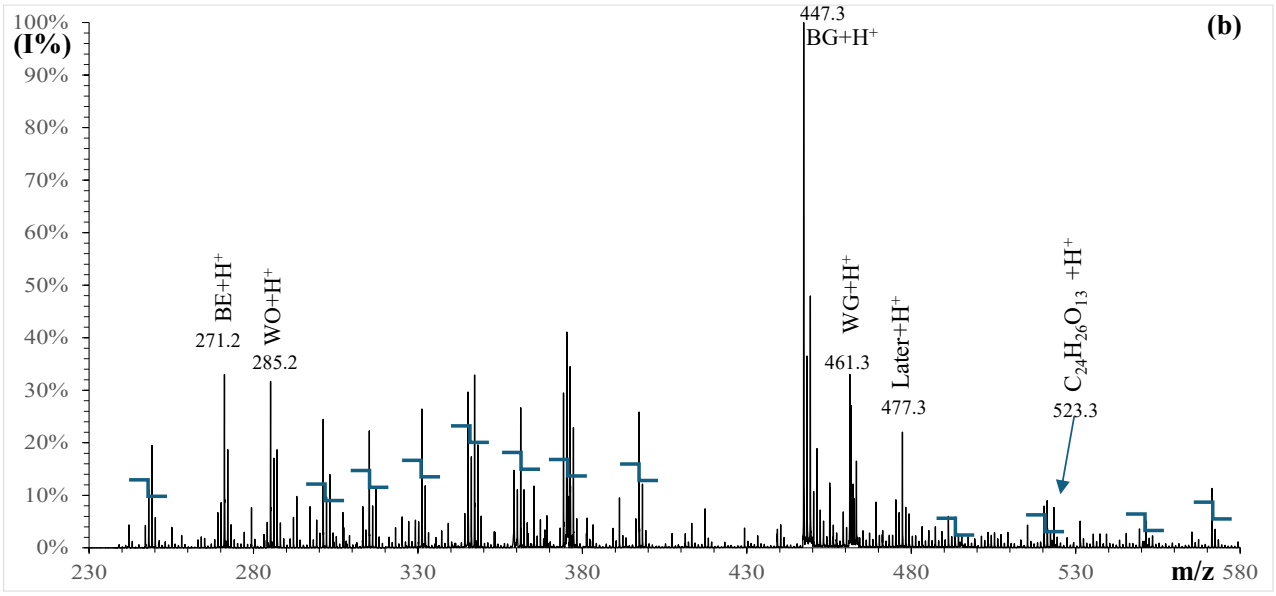


Figure SI12- ^{13}C-NMR spectrum of the neat baicalin-30% sample


(a)
100%
(I%)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Matrix peaks
477.3
Later+H+
BG+H+
447.3
WG+H+
461.3
BE+H+
271.2
WO+H+
285.2
C24H26O13 +H+
523.3
230
280
330
380
430
480
530
m/z 580


(b)
100%
(I%)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
447.3
BG+H+
WG+H+
461.3
Later+H+
477.3
BE+H+
271.2
WO+H+
285.2
C24H26O13 +H+
523.3
230
280
330
380
430
480
530
m/z 580

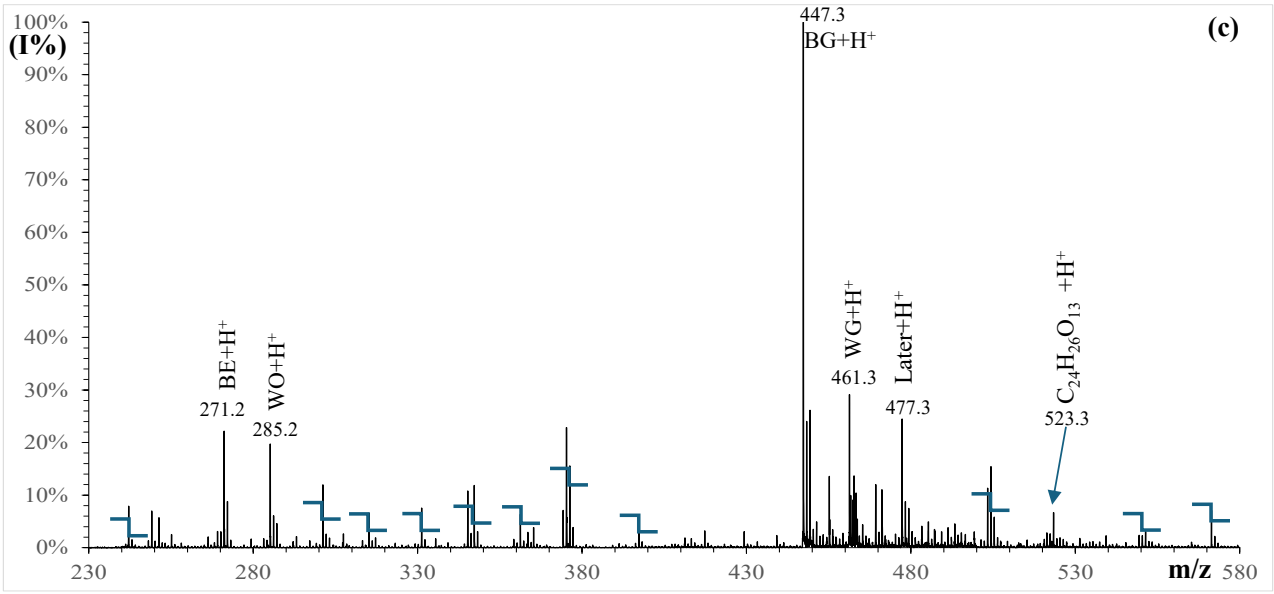

(c)
100%
(I%)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
447.3
BG+H+
WG+H+
461.3
Later+H+
477.3
BE+H+
271.2
WO+H+
285.2
C24H26O13 +H+
523.3
230
280
330
380
430
480
530
m/z 580

Figure SI13– MALDI-TOF mass spectra of baicalin fraction extracted from root of *S. baicalensis* at 60°C for 6 h in (a) isopropanol, (b) purified in butanol and (c) crystallized, recorded using CHCA as matrix.