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Laguncularia racemosa Phenolics Profiling by Three-Phase Solvent System Step-Gradient Using High-Performance Countercurrent Chromatography with Off-Line Electrospray Mass-Spectrometry Detection

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Abstract: The detailed metabolite profiling of *Laguncularia racemosa* was accomplished by high-performance countercurrent chromatography (HPCCC) using the three-phase system *n*-hexane–*tert*-butyl methyl ether–acetonitrile–water 2:3:3:2 (*v*/*v*/*v*/*v*) in step-gradient elution mode. The gradient elution was adjusted to the chemical complexity of the *L. racemosa* ethyl acetate partition and strongly improved the polarity range of chromatography. The three-phase solvent system was chosen for the gradient to avoid equilibrium problems when changing mobile phase compositions encountered between the gradient steps. The tentative recognition of metabolites including the identification of novel ones was possible due to the off-line injection of fractions to electrospray ionization mass spectrometry (ESI-MS/MS) in the sequence of recovery. The off-line hyphenation profiling experiment of HPCCC and ESI-MS projected the preparative elution by selected single ion traces in the negative ionization mode. Co-elution effects were monitored and MS/MS fragmentation data of more than 100 substances were used for structural characterization and identification. The metabolite profile in the *L. racemosa* extract comprised flavonoids, hydrolysable tannins, condensed tannins and low molecular weight polyphenols.

Keywords: high-performance countercurrent chromatography; off-line MS/MS detection; three-phase solvent system; step-gradient; *Laguncularia racemosa*

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1. Introduction

Countercurrent chromatography (CCC) is an all-liquid method, with no solid support, in which the stationary liquid phase is retained in the apparatus using centrifugal force only [1]. The principle behind this technique underlies the partitioning of a sample in a biphasic liquid solvent system [2]. Among many advantages, the technique is highly versatile; has high loading capacity; is easy to scale up; and eliminates sample loss by chemical degradation and irreversible adsorption [1,3,4]. CCC is a powerful tool in the phytochemical working field as it enables plant extract fractionation with existing major compounds and also isolation or fortification of minor compounds [5,6]. This characteristic is even more noticeable when semi-preparative and preparative scales are employed [7,8]. Standard CCC separation experiments do not provide the full flexibility, solely operating with a two-phase solvent system, and the isocratic elution–extrusion mode [9,10].

To improve the polarity range in the CCC operation field, three-phase solvent systems were developed/applied, due to the great differences in polarities between the upper,

middle and lower phases. Recently, some applications have been reported but only half of them actually were used as three phases in the separation process [11–15]. Tri-phasic systems are built from a two-phase system normally composed of n-hexane, acetonitrile and water in combination with a fourth solvent such as methyl acetate, ethyl acetate, methyl *tert*-butyl-methyl-ether or dichloromethane to create the third phase. Very few tri-phasic systems are described due to limited solvent combinations that form these three stable phases in a convenient volume percentage [11].

In analogy to solid phase chromatography, gradient elution in CCC intends to shorten the duration of a separation process and may also improve resolution. A common way to perform gradient elution is to change the mobile phase polarity over time [16], although gradient elution mode in CCC is less frequently used as the biphasic liquid system is in the equilibrium state and the change of composition in one phase corresponds directly to a change in the respective other liquid phase [17]. However, if a three-phase solvent system is used for gradient elution purposes, all phases involved for the experiment were previously in contact. Disturbance of the equilibrium and collapse of phase layers are omitted during the separation process while maintaining the broad polarity range for the recovery process.

In this work, a three-phase solvent system in step-gradient elution mode high-performance countercurrent chromatography (HPCCC) with off-line ESI-MS/MS detection was used for metabolite profiling of the ethyl acetate partition of the leaves from the mangrove plant *Laguncularia racemosa*. A similar approach was used on *Anogeissus leiocarpus* for compound identification but using centrifugal partition extraction (CPE), and off-line NMR detection [15]. *L. racemosa* (Combretaceae), popularly known as white mangrove, is the only occurring specie in the genus [18], and is considered as a strict mangrove [19], characteristic for growing in brackish coastal environments [20], and with excellent function for stabilizing shorelines against erosion [21]. From aspects of ethno-medicinal use, the plant is applied as astringent and tonic for dysentery and fever [22]. To date, there are only a few studies on the production of secondary metabolites [23–27], probably due to its high complexity of polar natural products.

2. Results and Discussion

The composition of *L. racemosa* ethyl acetate solvent partition (EtOAcPart) was initially investigated by TLC and LC-ESI/TOFMS analysis (Supplementary Figure S1). The liquid mass-spectrometry profile showed a high chemical complexity containing metabolites in a larger polarity range, making this mixture an ideal case study for the application of three-phase gradient elution. Some multiple-solvent biphasic systems have been proposed to great extend the polarity in CCC [28]. However, as the phases are in steady mixing contact during the complete separation process, a change of the mobile phase composition during gradient elution directly influences the stationary phase composition as well, and as consequence, disturbs the equilibrium and could lower or even lead to low chromatographic resolution [29,30]. To circumvent the equilibrium obstacle during the gradient elution procedure, a three-phase solvent system was used instead of two (or more) different biphasic systems. In this approach all phases involved in the separation were previously saturated with each other. The tri-phasic system *n*-hexane–*tert*-butyl-methyl ether–acetonitrile–water 2:3:3:2 (*v*/*v*/*v*/*v*) was used in the semi-preparative purification of *L. racemosa* EtOAcPart.

2.1. HPCCC of L. racemosa Metabolites by Off-Line ESI-MS/MS Profile Detection in the Sequential Order of Recovery

The *L. racemosa* EtOAcPart was separated by semi-preparative HPCCC chromatography, and the off-line injection profiling by injections of recovered fractions to ESI-MS/MS distinguished 17 principal phenolic constituents (Figure 1). However, as a result of the highly concentrated injections of respective HPCCC fractions, the selected single ion-based projection of the HPCCC experiment revealed more than 100 different metabolites (**1**–**109**) (Partially shown in Figure 1 and Supplementary Figure S2). Preliminary LC-ESI/TOF MS

analysis was not capable of detecting all minor compounds due to concentration levels below the detection limits.

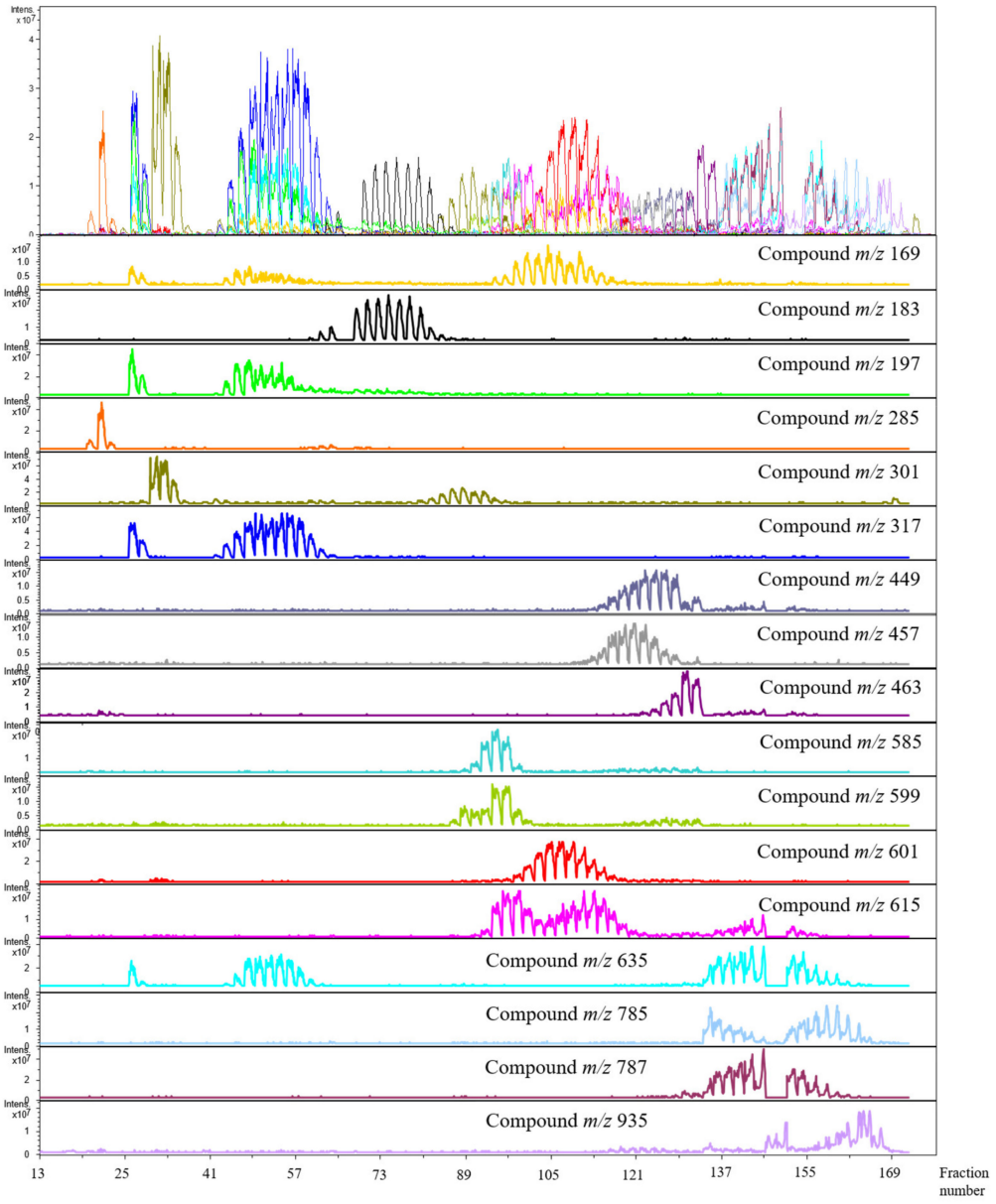

Figure 1. Selected electrospray ionization mass spectrometry electrospray ionization mass spectrometry ions traces (negative mode) of phenolics of *L. racemosa* EtOAcPart detected in the off-line injected high-performance countercurrent chromatography (HPCCC) fractions. HPCCC separation using *n*-hexane- *tert*-butyl-methyl ether–acetonitrile-water 2:3:3:2 (*v*/*v*/*v*/*v*) as triphasic solvent system.

Large advantage of the off-line injection profiling methodology of preparative HPCCC-fractions by an ESI-MS detector in the sequence of recovery is the 'on-the-fly' delivery of the respective molecular weight- and MS/MS-fragmentation data of all ionizeable compounds in one step. This is a very fast process to get the required data for immediate compound identifications in the respective HPCCC-fractions. A full mass-spectrometry guided metabolite profile with more than ten automatically selected precuresur ions for MS/MS on a larger lab-scale preparative HPCCC fractionation can be achieved in a 60 to 100 min experimental mass-spectrometry time frame. This mass-spectromtry approach is roughly by a factor of hundred faster than the single investigation of resepctive HPCCCC fractions by LC-ESI-MS/MS analysis. The results displayed in a single data file are ready

to use and not mutiple analysis sets need to be compared for guiding the decisions in fractionation process. This powerful approach was previously applied by Costa et al. [8] on the complex metabolite mixture extracted from the Brazilian plant *Salicornia gaudichiana*.

In case of the investigated *L. racemosa* ethylacetate solvent partition, the elution ranges of a large selection of higher and lower concentrated target molecules (Table 1) were visualized in the recovered HPCCC-fractions by selected single ion traces for performing the accurate fractionation, recovery and preventing unintentional mixing of already separated compounds. Additionally, the existing compound co-elution effects, and the sequential elution orders of separated isobars/ isomers were clearly detected and visualized (Figure 1).

Table 1. Detected compounds in the HPCCC off-line ESI-MS/MS phenolic profile of *Laguncularia racemosa* EtOAcPart.

Cpd	CCC-Fraction	MS $[M-H]^-$ (*m/z*) MS/MS $[M-H]^-$ (*m/z*)	LC-RT(min)	ESI/TOF MS Formula (Error in ppm)	Identification
Flavonoids and derivatives					
1	11–15	255 237, 226, **209**, 156	n.d.	-	Dihydrocrysin
2	21	269 151	13.7	269.04593 $C_{15}H_9O_5$ (1.4)	Apigenin
3	19–21	271 177, 151	1.9	271.04885 $C_{15}H_{11}O_5$ (45.6) *	Naringenin
4	23	273 167	29.1	273.08108 $C_{15}H_{13}O_5$ (15.5)	Afzelechin
5	19	285 **257**, 151	40.8	285.04413 $C_{15}H_9O_6$ (12.6)	Kaempferol
6	31–41	287 **259**, 151	12.1	287.05883 $C_{15}H_{11}O_6$ (9.5)	Dihydrokaempferol
7	97–115	289 **245**, 205	6.2	289.07438 $C_{15}H_{13}O_6$ (9.0)	(Epi)-catechin
8	29–33	301 179, **151**	35.4	301.03937 $C_{15}H_9O_7$ (13.3)	Quercetin
9	17–19	305 **287**, 249	2.4	305.0706 $C_{15}H_{13}O_7$ (12.8)	(Epi)-gallocatechin
10	21–23	315 300	42.8	315.0466 $C_{16}H_{11}O_7$ (14.1)	Isorhamnetin
11	25–27	317 **179**, 151	28.9	317.03536 $C_{15}H_9O_8$ (16.0)	Myricetin
12	81–93	319 193	9.1	319.04883 $C_{15}H_{11}O_8$ (9.0)	Dihydromyricetin
13	33–41	329 **314**, 299	21.7	329.05816 $C_{17}H_{13}O_7$ (25.9) *	Tricin
14	133–149	393 **317**, 241, 169	-	-	Myricetin derivative
15	29–33	415 301	-	-	Quercetin alkyl derivative
16	127–131	419 305	-	-	(Epi)-gallocatechin alkyl derivative
17	55–57	431 317	-	-	Myricetin alkyl derivative
18	97–105	433 **301**, 179, 151	27.5	433.08215 $C_{20}H_{17}O_{11}$ (10.4)	Quercetin pentoside

Table 1. *Cont.*

Cpd	CCC-Fraction	MS $[M-H]^-$ (*m*/*z*) MS/MS $[M-H]^-$ (*m*/*z*)	LC-RT(min)	ESI/TOF MS Formula (Error in ppm)	Identification
19	89–91	433 319, 193	-	-	Dihydromyricetin alkyl derivative
20	91–95	441 289	19.3	441.08208 $C_{22}H_{18}O_{10}$ (1.5)	(Epi)-catechin gallate
21	105–119	447 301	29.3	447.09851 $C_{21}H_{19}O_{11}$ (11.7)	Quercetin desoxyhexoside
22	115–133	449 **317**, 316	22.4	449.07395 $C_{20}H_{17}O_{12}$ (3.1)	Myricetin pentoside
23	113–127	457 331, 305, **169**	12.1	457.07859 $C_{22}H_{18}O_{11}$ (2.1)	(Epi)-gallocatechin gallate
24	21–23	461 **443**, 381, 301, 193	-	-	Quercetin derivative
25	123–131	463 **317**, 316	24.6	463.09083 $C_{21}H_{19}O_{12}$ (5.7)	Myricetin desoxyhexoside
26	141–145	463 301	25.9	463.09187 $C_{21}H_{19}O_{12}$ (7.9)	Quercetin hexoside
27	153–165	467 458, **391**, 301, 169	-	-	Quercetin derivative
28	67–79	469 317	n.d.	-	Myricetin galatte
29	85–91	471 319, 301, **193**	-	-	Dihydromyricetin alkyl derivative
30	115–131	477 **301**, 179	15.2	477.06812 $C_{21}H_{17}O_{13}$ (1.4)	Quercetin glucuronide
31	133–163	479 **317**, 316	22.1	479.08428 $C_{21}H_{19}O_{14}$ (2.4)	Myricetin hexoside
32	89–97	585 433, **301**	32.7	585.09204 $C_{27}H_{21}O_{15}$ (5.9)	Quercetin pentoside gallate
33	85–95	599 447, **301**	26.8	599.10714 $C_{28}H_{23}O_{15}$ (4.8)	Quercetin desoxyhexoside gallate
34	95–113	601 **449**, 317	28.6	601.08787 $C_{27}H_{21}O_{16}$ (7.3)	Myricetin pentoside gallate
35	29–33	603 301	n.d.	-	Quercetin $[2M-H]^-$
36	125–131	611 305	-	-	(Epi)-gallocatechin $[2M-H]^-$
37	91–115	615 463, **317**, 179	23.4	615.1014 $C_{28}H_{23}O_{16}$ (3.6)	Myricetin desoxyhexoside gallate
38	137–153	615 **463**, 301	31.6	615.10412 $C_{28}H_{23}O_{16}$ (8.1)	Quercetin hexoside gallate
39	113–127	629 **477**, 317, 316, 289	21.5	629.07893 $C_{28}H_{21}O_{17}$ (0.8)	Quercetin glucuronide gallate
40	133–139	631 **479**, 317	28.9	631.09859 $C_{28}H_{23}O_{17}$ (7.2)	Myricetin hexoside gallate
41	45–57	635 317	n.d.	-	Myricetin $[2M-H]^-$

Table 1. *Cont.*

Cpd	CCC-Fraction	MS $[M-H]^-$ (*m*/*z*) MS/MS $[M-H]^-$ (*m*/*z*)	LC-RT(min)	ESI/TOF MS Formula (Error in ppm)	Identification
42	87–91	639 **319**, 301	11.2	639.05562 $C_{29}H_{19}O_{17}$ (11.2)	HHDP Dihydromyricetin
43	25	657 317	-	-	Myricetin derivative
44	89	697 599	-	-	Quercetin desoxyhexoside gallate derivative
45	89–93	737 585, 301	n.d.	-	Quercetin pentoside digalloyl
46	93–103	753 601, 449, 317	32.5	753.09740 $C_{34}H_{25}O_{20}$ (3.9)	Myricetin pentoside digalloyl
47	85–89	773 471, 301	-	-	Quercetin derivative
48	97–103	867 **433**, 301	n.d.	-	Quercetin pentoside $[2M-H]^-$
49	117–127	883 **449**, 317	-	-	Myricetin pentoside derivative
50	115–123	892 **457**, 433	-	-	(Epi)-gallocatechin gallate derivative
51	117–127	899 463, **449**, 317	-	-	Myricetin pentoside derivative
52	87–93	901 **599**, 301	-	-	Quercetin desoxyhexoside gallate derivative
53	111–121	905 469, **457**, 447, 425, 301	-	-	Quercetin desoxyhexoside derivative
54	113–125	907 **449**, 317	-	-	Myricetin pentoside derivative
55	113–123	915 457	-	-	(Epi)-gallocatechin gallate $[2M-H]^-$
56	129–131	927 **463**, 317	n.d.	-	Myricetin desoxyhexoside $[2M-H]^-$
Hydrolisable tannins and deivatives					
57	93–115	169 125	12.1	169.01664 $C_7H_5O_5$ (14.2)	Gallic acid
58	61–81	183 124	5.9	183.01418 $C_8H_7O_5$ (12.2)	Methyl gallate
59	43–57	197 **169**, 125	14.3	197.04741 $C_9H_9O_5$ (9.5)	Ethyl gallate
60	81–93	301 283, 257, **229**, 163	18.5	300.99939 $C_{14}H_5O_8$ (1.3)	Ellagic acid
61	19–21	315 300	6.1	315.01809 $C_{15}H_7O_8$ (10.9)	Ellagic acid methyl ether
62	97–103	321 169	3.9	321.03300 $C_{14}H_9O_9$ (24.3) *	Galloyl gallate
63	133–151	325 169	3.3	325.06016 $C_{14}H_{13}O_9$ (11.3)	Galloyl shikimate
64	33–39	329 314	44.2	329.02154 $C_{16}H_9O_8$ (8.9)	Ellagic acid dimethyl ether

Table 1. *Cont.*

Cpd	CCC-Fraction	MS $[M-H]^-$ (*m*/*z*) MS/MS $[M-H]^-$ (*m*/*z*)	LC-RT(min)	ESI/TOF MS Formula (Error in ppm)	Identification
65	163	331 **271**, 169, 125	11.5	331.06888 $C_{13}H_{15}O_{10}$ (5.5)	Galloyl hexoside
66	81–87	335 183	9.2	335.02817 $C_{15}H_{11}O_9$ (37.9) *	Galloyl methyl gallate
67	21–23	343 328	44.1	343.04787 $C_{17}H_{11}O_8$ (5.6)	Ellagic acid trimethyl ether
68	59–79	349 197	13.9	349.0416 $C_{16}H_{13}O_9$ (42.7) *	Galloyl ethyl gallate
69	105–119	425 301	15.8	425.01469 $C_{20}H_9O_{11}$ (0.8)	Ellagic acid pyrogallol ether
70	103–119	469 425	15.7	469.0039 $C_{21}H_9O_{13}$ (2.1)	Valoneic acid dilactone
71	161–165	481 **439**, 331, 301, 169	1.2	481.06556 $C_{20}H_{17}O_{14}$ (6.6)	HHDP hexoside
72	155–165	483 **439**, 331, 313, 169	11.5	483.07806 $C_{20}H_{19}O_{14}$ (0.1)	Digalloyl hexoside
73	85–89	497 301	23.4	497.03631 $C_{23}H_{13}O_{13}$ (0.3)	Valoneic acid dilactone ethyl ether
74	83–91	625 471, **301**	28.6	625.07458 $C_{26}H_{25}O_{18}$ (28.1) *	Ellagic acid dihexoside
75	147–155	631 **479**, 301	19.4	631.09323 $C_{27}H_{19}O_{18}$ (26.3) *	NHDP hexoside
76	155–165	633 479, **301**	7.8	633.07511 $C_{27}H_{21}O_{18}$ (2.8)	HHDP galloyl hexoside
77	133–151	635 483, **465**, 313	15.6	635.08832 $C_{27}H_{23}O_{18}$ (1.0)	Trigalloyl hexoside
78	135–139	733 635	n.d.	-	Trigalloyl hexoside derivative
79	149	781 631, **301**	3.7	781.06132 $C_{34}H_{21}O_{22}$ (10.7)	Punicalin
80	155–167	783 481, **301**	2.7	783.07063 $C_{34}H_{23}O_{22}$ (2.5)	DiHHDP hexoside
81	133–165	785 633, 481, **301**, 275	9.6	785.08378 $C_{34}H_{25}O_{22}$ (0.7)	HHDP digalloyl hexoside
82	133–155	787 635, 617, 483, 465, **301**	21.1	787.09741 $C_{34}H_{27}O_{22}$ (3.2)	Tetragalloyl hexoside
83	145–167	935 917, **633**, 571, 365, 329, 299, 275	6.0	935.07728 $C_{41}H_{27}O_{26}$ (2.5)	Galloyl diHHDP hexoside
84	131–169	937 **785**, 769, 633, 617, 301	6.0	937.28345 $C_{41}H_{29}O_{26}$ (12.6) *	HHDP trigalloyl hexoside
85	155–167	939 **787**, 769, 617, 465	26.2	939.11228 $C_{41}H_{31}O_{26}$ (1.5)	Pentagalloyl hexoside
86	155–169	951 **907**, 783, 605	-	-	DiHHDP hexoside derivative

Table 1. *Cont.*

Cpd	CCC-Fraction	MS $[M-H]^-$ (*m*/*z*) MS/MS $[M-H]^-$ (*m*/*z*)	LC-RT(min)	ESI/TOF MS Formula (Error in ppm)	Identification
Condensed tannins					
87	121–133	577 **463**, 425, 313, 289	3.9	577.13515 $C_{30}H_{25}O_{12}$ (2.9)	(Epi)-catechin dimer
88	123–125	593 575, 467, **441**, 425, 305	2.6	593.15119 $C_{30}H_{25}O_{13}$ (4.3)	(Epi)-catechin-(epi)-gallocatechin dimer
89	107–119	609 **457**, 439, 321, 169	4.1	609.12858 $C_{30}H_{25}O_{14}$ (5.9)	(Epi)-gallocatechin dimer
90	125–129	897 745, 575, **463**, 449, 423	13.0	897.14880 $C_{44}H_{33}O_{21}$ (3.6)	(Epi)-catechin gallate -(epi)-gallocatechin gallate dimer
91	123–131	913 **463**, 449, 317	8.0	913.14548 $C_{44}H_{33}O_{22}$ (1.5)	(Epi)-gallocatechin gallate dimer
92	137–155	913 **761**, 573, 449, 423	24.6	913.16762 $C_{45}H_{37}O_{21}$ (17.1)	(Epi)-gallocatechin trimer
Others					
93	73–85	109 -	3.0	109.02893 $C_6H_5O_2$ (5.3)	Catechol
94	67–81	124 -	n.d.	-	Amino catechol
95	93–115	125 -	12.1	125.02756 $C_6H_5O_3$ (17.2)	Pyrrogallol
96	77–83	153 109	9.1	153.02038 $C_7H_5O_4$ (6.9)	Protocatechuic acid
97	23–25	167 125	9.1	167.03454 $C_8H_7O_4$ (2.6) *	Vanillic acid
98	67–79	168 124	n.d.	-	Amino protocatechuic acid
99	85–95	193 111	9.1	193.01665 $C_9H_5O_5$ (12.5)	Trihydroxychromone
100	15	209 187, **165**, 125	n.d.	-	Jasmonic acid
101	59–69	217 155	-	-	Unknown
102	13–15	279 277, **243**, 237	73.4	279.23401 $C_{18}H_{31}O_2$ (3.8)	Linoleic acid
103		281 277, **255**	75.8	281.24987 $C_{18}H_{33}O_2$ (4.5)	Oleic acid
104	11–15	295 277, **275**, 265, 251, 249, 185	70.2	295.2304 $C_{18}H_{31}O_3$ (8.6)	Hydroxy linoleic acid
105	125–131	305 221, **219**, 179, 165, 125	12.1	305.06942 $C_{12}H_{17}O_7S$ (2.1)	5′-hydroxysulphonyloxy jasmonic acid
106	11–17	383 **337**	-	-	Unknown

Table 1. *Cont.*

Cpd	CCC-Fraction	MS $[M-H]^-$ (*m*/*z*) MS/MS $[M-H]^-$ (*m*/*z*)	LC-RT(min)	ESI/TOF MS Formula (Error in ppm)	Identification
107	157–159	707 687, 671, 533, **359**	n.d.	-	Integracin D
108	97–99	875 **441**, 433, 289	-	-	Unknown
109	89–93	887 **585**, 301	-	-	Unknown

MS^2 numbers in bold indicate the most intense product ion. * indicate very minor compounds. HHTP = hexahydroxydiphenoyl ester; NHTP = nonahydroxytriphenoyl ester.

One of the special cases of isomer/isobar separation by HPCCC is the selected ion trace $[M-H]^-$ at m/z 615, as the HPCCC experiment separated flavonoid-glycosides with identical molecular weights as displayed in the low resolution ESI-MS injection profile (Figure 1). A set of two partly co-eluting positional isomers of myricetin-desoxy-hexoside-gallate (**37**) (fraction range 91–115) were absolutely separated from the later eluting isobar quercetin-hexoside-gallate (**38**) (fraction range 137–153).

The selected ion trace at m/z 635 displayed two strong HPCCC elution ranges with compeletly separated compound areas with **41** (range 45–57), and **77** (133–151) (Figure 1). However, the metabolite **41** with lower elution volume in the HPCCC run was identified by the ESI-MS/MS profile data as myricetin whereby the ESI-ion-source dimer $[2M-H]^-$ was generated in dominant intensity. This was confirmed by the exact identical position of m/z 317 ($[M-H]^-$) in the HPCCC profile. Nevertheless, the late eluting metabolite **77** was identified as a $[M-H]^-$-signal with a hexosid unit substituted by three galloyl-moieties indicated by MS/MS neutral loss cleavage ($\Delta m/z$ 152) to m/z 483, and 331 of gallic acid releases. The tetra-galloyl-hexoside with $[M-H]^-$ at m/z 787 (**82**) (Table 1) co-eluted in this HPCCC run as seen in Figure 1, as well as the penta-galloyl-hexoside (**85**) (Table 1). A very large elution volume for recovery in the triphasic HPCCC experiment displayed the galloyl diHHDP hexoside (**83**) seen by $[M-H]^-$ at m/z 935 (range 145–167) (Figure 1 and Table 1). Although the constitution of certain compounds had been different, the polarity differences were not sufficient for a successful HPCCC separation as seen for the selected ion traces $[M-H]^-$ m/z 585 (quercetin pentoside gallate, **32**), and m/z 599 (quercetin desoxyhexoside gallate, **33**) (Figure 1).

Using literature to guide the identification process of the minor, and very minor concentrated derivatives, literature was verified and the few previously isolated compounds in *L. racemosa* were listed with molecular weights as a comparative database. Most of the unknown compounds were characterized by ESI-MS/MS fragmentation and indicative neutral loss pattern. High accuracy molecular weights acquired by LC-ESI/TOF MS were used to ratify and/or verify the proposed molecular formulas. Phytochemical investigations describing chemical compounds on other genus of Combretaceae helped to support the results based on chemotaxonomic knowledge. From the aspect of natural product classes, the chemical composition of the EtOAcPart was distinguished in four main groups as flavonoids, hydrolysable tannins, condensed tannins and other low molecular weight polyphenols (Supplementary Figure S2). The chemical structures and substitution patterns of fractionated and identified compounds are shown in Figure 2.

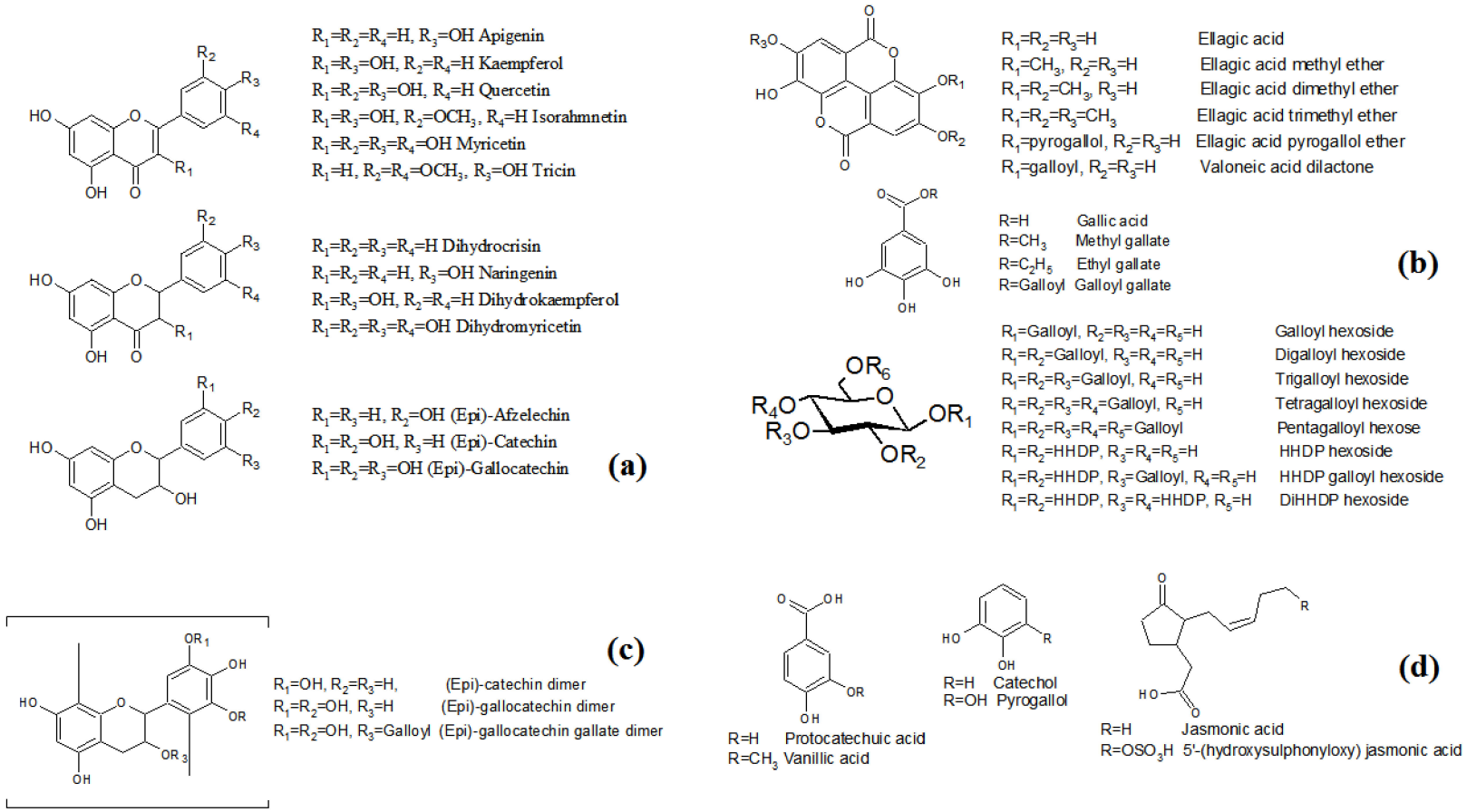

Figure 2. *Laguncularia racemosa* EtOAcPart general structures and tentative substitution patterns of some of the existing compounds. (**a**) Flavonoids, (**b**) hydrolysable tannins, (**c**) condensed tannins and (**d**) other low molecular weight polyphenols.

Fractions had been combined on the basis of TLC analysis and the electrospray mass-spectrometry profiling experiment. Supplementary Figure S3 displays the TLC-analysis on the combined fractions of the HPCCC experiment. Table 1 lists HPCCC chromatographic elution, and ESI-MS/MS informations; LC ESI-TOF-MS data (when present) and tentative identification. Although *L. racemosa* EtOAcPart showed quite complex constituents, most phenolic compounds were well separated.

2.1.1. Flavonoids and Derivatives

Flavonoid derivatives were detected and identified in *L. racemosa* EtOAcPart by ESI-MS/MS as principal compounds in the recovered HPCCC fractions (Table 1, compounds **1-56**). Flavonoids including flavonols, flavones, flavanols and flavanones were found in free form, linked to one sugar unit, as well as in the presence of galloyl substituents. The tentative identification of the flavonoid-aglyca (compounds **1**–**13**) was done by comparison to specific fragmentation patterns, as the spectra of this flavonoid often displayed loss of small neutral fragments contributing to structure information [31–33]. The free flavonoid aglyca eluted during the first step of the gradient before the glycoside linked flavonoids, in accordance to mobile phase/compound polarity in the tail-to-head mode.

The flavonoid-*O*-substituted characteristically exhibited the neutral loss [34] attributed to a pentose unit $[M - H - 132]^-$, hexose unit $[M - H - 162]^-$, desoxy-hexose unit $[M - H - 146]^-$, glucuronyl unit $[M - H - 176]^-$, galloyl moiety $[M - H - 152]^-$ and combination of these substituents. A set of quercetin-*O*-pentoside, -*O*-desoxy-hexoside, -*O*-hexoside, -*O*-glucuronide and myricetin-*O*-pentoside, -*O*-desoxy-hexoside, -*O*-hexoside were detected in compounds **18**, **21**, **22**, **25**, **26**, **30** and **31** [35–41]. The substituent gallate was found connected to (epi)-catechin (20), (epi)-gallocatechin (23) and myricetin (28) as well as in glycosylated forms of quercetin and myricetin (**32**–**34**, **37**–**40**) [41]. The digallate derivative of quercetin and myricetin-*O*-pentoside were also recognized in compounds **45** and **46**.

Aglycones apigenin, kaempferol, quercetin and tricin were previously reported in *L. racemosa* [8,26] in addition to the glycosylated derivatives quercetin-3-*O*-arabinoside and quercetin-3-*O*-rhamnoside [24]. Not fully identified derivatives could be distinguished by observed aglycone fragment ions in MS/MS.

2.1.2. Hydrolysable Tannins

Hydrolysable tannins, well-known in Combretaceae, were the second main class of natural compounds detected by the HPCCC and off-line injection ESI-MS/MS experiment (Table 1, compounds **57**–**86**) [36–40,42,43]. It included derivatives of gallic acid, ellagic acid, gallotannins and ellagitannins. Some of the ellagic acid and its methyl-, dimethyl- and trimethyl ether derivatives were previously reported in *L. racemosa* [26]. Several studies describing the detection of hydrolysable tannins in species of Combretaceae can be found [44–46].

Common neutral loss cleavages observed in the MS/MS for simple gallic acid and its derivatives were related to the cleavage of carboxyl $[M - H - 44]^-$, methyl $[M - H - 15]^-$, ethyl $[M - H - 29]^-$ and galloyl $[M - H - 152]^-$. They were found as ester or ether arrangements. Compounds **57**–**59**, **62**, **63**, **66** and **68** were identified as gallic acid, methyl gallate, ethyl gallate, galloyl gallate, galloyl shikimate, galloyl methyl gallate and galloyl ethyl gallate, respectively [37,38,42].

Ellagic acid derivatives were characterized by the fragment ion m/z 301. At this point, LC ESI-TOF-MS was essential to distinguish derivatives from quercetin and ellagic acid. The sequence of compounds comprised ellagic acid itself and the -methyl, -dimethyl, -trimethyl, -pyrogalloyl and dihexoside ether forms (**60**, **61**, **64**, **67**, **69**, **74**) [36,39,40,43]. Additionally, valoneic acid dilactone (**70**) and its ethyl ether derivative (**73**) were detected [36,40].

By comparison to literature [47], the molecular masses of compounds **65**, **72**, **77**, **82** and **85** showed that they consist of a gallotannin series of molecules (mono-, di-, tri-,

tetra- and penta-galloyl hexosides) [37,38,42]. A similar series of monomeric ellagitannins (HHDP-, NHDP-, HHDP galloyl-, diHHDP-, HHDP digalloyl-, diHHDP galloyl- and HHDP trigalloyl-) were found in compounds **71**, **75**, **76**, **80**, **81**, **83** and **84** [36,38,40]. The ellagic acid punicalin (**79**) was further detected at m/z 781.

2.1.3. Condensed Tannins

Condensed tannins (proanthocyanidins), formerly observed in *L. racemosa* wood and leaves [48–50], were recognized and characterized based on the detected flavanol-aglyca (**4**, **7**, **9**) and its gallate derivatives (**20**, **23**). They were found as homo-dimers consisting of (epi)-catechin (**87**), (epi)-gallocatechin (**89**) and (epi)-gallocatechin gallate (**91**) [40]. Additionally, as hetero-dimers, existing as (epi)-catechin-(epi)-gallocatechin (**88**) and (epi)-catechin gallate-(epi)-gallocatechin gallate (**90**). The trimeric (epi)-gallocatechin (**92**) was also encountered. Compounds had fragmentation patterns related to the cleavage of flavanol units according to literature [51]: $[M - H - 289]^-$ for (epi)-catechin loss, $[M - H - 305]^-$ (epi)-gallocatechin loss, $[M - H - 441]^-$ (epi)-gallocatechin gallate loss and $[M - H - 162]^-$ for gallate loss.

Considering the elution order of compounds in respect to gradient polarity range, the flavonol-aglyca eluted before the gallate derivatives, both in the first step, while dimers and trimers stayed retained in the column until extrusion started.

2.1.4. Low Molecular Weight Polyphenols

Other compounds were recognized and characterized based on precursors/derivatives of existing identified compounds in the off-line ESI-MS/MS profile or on the *L. racemosa* chemical database. Simple phenolic compounds included catechol (**93**) and pyrogallol (**95**), common occurring products in the hydrolysable tannins pathway [35]. Benzoic acid derivatives with frequent $[M - H - 44]^-$ corresponding to the neutral loss of CO_2, comprised protocatechuic (**96**) and vanillic (**97**) acids [37]. The amino derivatives aminocatechol (**94**) and amino protocatechuic acid (**98**) were also detected [52]. The chromone detected at m/z 193, was identified as trihydroxy-chromone (**99**) and had its molecular formula confirmed by HRMS.

The jasmonic acid (**100**) and its sulphated derivative 5′-hydroxy-sulphonyloxy jasmonic acid (**105**), earlier isolated from the *L. racemosa* twigs and leaves [23], could be found at m/z 209 and 305, respectively. ESI/TOF MS data confirmed the proposed compounds. Ordinary oleic and linoleic fatty acids (**102-104**), jasmonic acid biosynthetic precursor, were further encountered. Another sulphated derivative isolated from *L. racemosa* leaves [26] was found at $[M - H]^-$ at m/z 707 and was identified as integracin D (**107**) [26]. Due to concentration limits, the compound could not be detected in the ESI/TOF MS analysis and structure was not fully confirmed.

3. Materials and Methods

3.1. Chemical Reagents and Solvents

Preparation of extracts was carried out with analytical grade solvents from Tedia Brazil (Rio de Janeiro, Brazil). LC-ESI/TOF-MS/MS analyses used HPLC grade solvents from Tedia Brazil (Rio de Janeiro, Brazil). HPCCC separations were performed with analytical grade solvents from Fisher Chemicals (Loughborough, UK). ESI-MS/MS analyses were done with HPLC grade solvents from VWR Chemicals (Radnor, PA, USA). NMR analyses used deuterated solvents from Cambridge Isotope Laboratories (Tweksbury, MA, USA) and TMS as internal standard. All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Thame, UK).

3.2. Preparation of the Extract

Laguncularia racemosa (3 kg) was collected at Guaratiba Biological and Anthropological Reserve (Rio de Janeiro, Brazil) in November 2010. Specialist researchers from the Nucleus of Mangrove Studies (University of the State of Rio de Janeiro) helped in the

localization, identification and collection of the plant. The leaves were dried and grounded in a laboratory mill (Laboratory Retsch mill, Haan, Germany) and 1800 g were submitted to maceration with ethanol–water 8:2 (*v*/*v*) in 10 cycles of 24 h. The solvent was evaporated under reduced pressure at 50 °C and the crude extract (255 g) was partitioned between water and organic solvents, affording different extracts: n-hexane (4 g), dichloromethane (8 g), ethyl acetate (15 g) and aqueous (215 g).

3.3. Thin Layer Chromatography

Preliminary analyses of EtOAcPart, solvent system evaluation tests and CCC fraction analyses were done by thin layer chromatography (TLC) on normal phase silica gel TLC plates (SiO_2-60, F254, Merck, Darmstadt, Germany, gel 60 RP-18, F254S) developed with EtOAc–acetone–H_2O 25:15:10 (*v*/*v*/*v*), and acetonitrile-H_2O 1:1 (*v*/*v*) for reversed phase C18-plates (RP18W, Macherey and Nagel, Düren, Germany). Results were visualized by using spray-reagent H_2SO_4 (10% *m*/*v*) in methanol with vanillin 5% in ethanol and flash heating on a hot plate 105 °C.

3.4. LC-ESI/TOF MS Preliminary Analysis

The EtOAcPart was also analysed by LC–ESI/TOF-MS with a 1200 Series LC-chromatograph (Agilent, Palo Alto, CA, USA) coupled with a MicrOTOF II time-of-flight mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA). 5 µL injection was performed with an autosampler on a Poroshell EC-C18 column (100 × 2.1 mm; 2.7 µm, Agilent, Palo Alto, CA, USA). The source temperature was set at 200 °C, the drying gas (nitrogen) flow rate was 10.0 L/min and the nebulizer gas (nitrogen) pressure was 4 bar. Data were acquired in negative mode in the range of *m*/*z* 100–1500. The capillary voltage was 3.8 kV, the capillary exit voltage was −150 V, the skimmer 1 and 2 voltages were 50 V and 23 V, respectively, the hexapole 1 voltage was set to −23 V, the hexapole RF voltage was 120 Vpp, lens 1 transfer was 68 µs and lens 1 pre plus stage was 7 µs. Mass calibration was achieved by infusing ammonium formate in an isopropanol–water mixture (1:1, *v*/*v*) as an external standard. All data were analysed using Bruker Daltonics ESI Compass Data Analysis Version 4.0 SP 1 (Bruker Daltonics Inc., Billerica, MA, USA). The mobile phase consisted of spectroscopic grade methanol (B) and ultrapure water (A) containing 0.05% (*v*/*v*) formic acid. The linear gradient elution was set from 10% to 100% of B in 90 min at a flow rate of 0.3 mL/min.

3.5. High Performance Countercurrent Chromatography

3.5.1. Equipment

CCC separations were performed on a semi-preparative HPCCC system (model Spectrum, Dynamic Extractions Ltd., Gwent, UK) equipped with two counter-balanced bobbins with perfluoroalkoxypolymer (PFA) tubing (1.6 mm i.d.) wound in multi-layer coiled-columns, resulting in 143.5 mL total volume (V_C). The rotation speed was adjusted to the maximum velocity of 1600 rpm (240 g). Solvent phase systems were delivered by a constant flow pump (Agilent HP1200, Palo Alto, CA, USA) to the HPCCC system. A semi-preparative sample loop (7.15 mL) was used to inject the dissolved sample over a low-pressure valve (Upchurch Model V-450, with 1.6 mm i.d. fittings) to the chromatographic system. Fractions were collected by a fraction collector (Agilent HP1200, Palo Alto, CA, USA).

3.5.2. Three-Phase Solvent System Test Evaluation

The three-phase solvent systems were composed of *n*-hexane–methyl acetate –acetonitrile–water and *n*-hexane–*tert*-butyl methyl ether–acetonitrile–water [11,53,54]. For the experiments for solvent system evaluation, 2 mg of the EtOAcPart were dissolved in a test tube containing 2 mL of each phase of the thoroughly equilibrated solvent systems. The test tubes were shaken vigorously for compound partition. After the phase layers had completely separated and distribution equilibrium was established, the resulting phase layers were analyzed by TLC (Supplementary Figure S4).

3.5.3. Solvent System and Sample Preparation

The selected solvent system *n*-hexane–*tert*-butyl methyl ether–acetonitrile–water (2:3:3:2, *v*/*v*/*v*/*v*) was thoroughly equilibrated in a separatory funnel at room temperature. The three phases were separated shortly before use and degassed by ultra-sonication for 5 min. The sample solution was prepared by dissolving the sample at fixed concentration (100 mg/mL) and coil-volume (5% V_C) in the lower aqueous phase only.

3.5.4. HPCCC Separation Procedure

Separation was performed in a normal step-gradient elution mode. The more aqueous lower phase was used as the stationary phase while organic upper and middle phases were used as mobile phases as shown in Figure 3. The system was completely filled with the lower aqueous stationary phase. Rotation was set to 1600 rpm. For the separation, the upper organic mobile phase was pumped at 4.0 mL/min. After reaching hydrodynamic equilibrium, the sample was injected to the HPCCC column. For the first elution step, 214.5 mL mobile phase (1.5 V_C) of upper phase was pumped through. For the second elution step, 2 V_C (286 mL) of the middle phase was pumped through the HPCCC system. Fractions were collected at 1 min intervals. For the extrusion step, rotation was reduced to 200 rpm and the column contents were pushed out of the system by lower phase at 8.0 mL/min and fractions were collected at 30 s intervals. The temperature control was maintained at 30 °C.

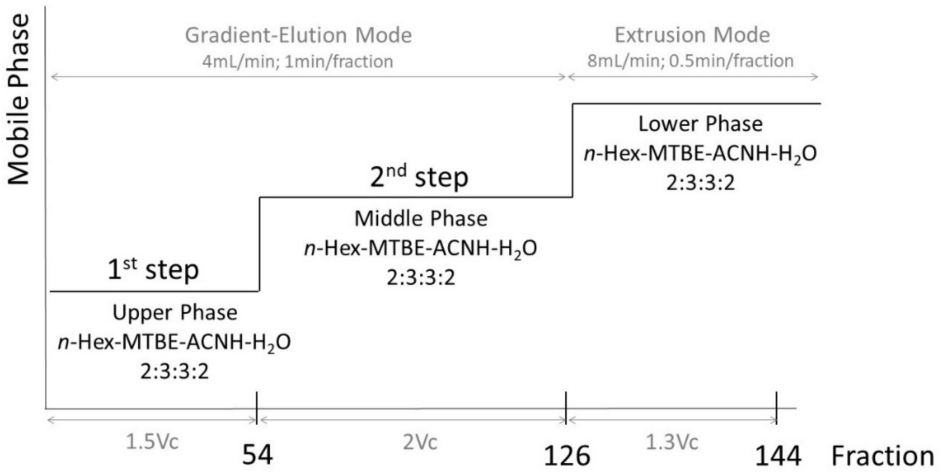

Figure 3. HPCCC three phase solvent system step-gradient procedure.

3.6. Metabolite Profiling by Offline Injections to ESI-MS/MS

The molecular weight profiles of the recovered HPCCC fractions were monitored by off-line ESI-MS/MS and were recorded in a single data file using the ion-trap mass-spectrometer HCT-Ultra ETD II (Bruker Daltonics, Bremen, Germany). Aliquots of 0.75 µL of odd numbered CCC fractions were directly filled to vials, dried and redissolved in 1.0 mL of methanol for conducting the ESI-MS analysis. Fractions were delivered to the ESI-MS/MS by a HPLC-pump (binary pump, G1312 A, 1100 Series, Agilent, Waldbronn, Germany) using the make-up solvent system with a flow rate of 0.5 mL/min composed of acetonitrile and water (1:1, *v*/*v*). ESI-MS/MS parameter settings were in the negative ionization mode, with scan-range between *m*/*z* 100–2000, where mostly deprotonated $[M - H]^-$ ion signals were generated. An auto-MS/MS method fragmented the nine most intense peaks and to monitor and characterize co-eluting compounds. Drying gas was nitrogen (flow rate 10.0 L/min, 310 °C), and nebulizer pressure was set to 60 psi. Ionization voltage at HV capillary was 3500 V, HV end plate off set −500 V, trap drive 61.8, octupole RF amplitude 187.1 Vpp, lens 2 60.0 V, Cap Ex −115.0 V, max. accumulation time 200 ms, averages 5 spectra, trap drive level 120%, target mass range: *m*/*z* 500, compound stability 80%, Smart ICC target 70.000, ICC charge control 'on' and smart parameter setting 'active'.

4. Conclusions

The combination of analysis of preparative HPCCC fractions with off-line injections to an ESI-MS/MS device was proven to be highly effective for a full metabolite chemical profile for polyphenols using the negative ionization mode. The use of a three-phase solvent system for HPCCC in a step-gradient elution mode was adequate to maintain equilibrium and chromatographic resolution while improving mobile phase strength. The ESI-MS/MS projection of the semi-preparative HPCCC experiment visualized over 100 compounds by selected single ion traces and was an adequate confirmation of the LC–ESI/TOF MS analysis. This study detected a variety of metabolites from different classes occurring in *L. racemosa* EtOAcPart and used chemotaxonomic data to guide the MS/MS putative structure elucidation.

Supplementary Materials: The following are available online, Figure S1: EtOAcPart preliminary analyses by LC-ESI-TOF-MS. The mobile phase was methanol (B) and water (A) containing 0.05% (*v*/*v*) formic acid. The linear gradient elution was set from 10% to 100% of B in 90 min. Figure S2: HPCCC off-line injection ESI-MS/MS profile of the the EtOAc Part by use of selected single ion traces for target compounds or classes. (a) Flavonoids, (b) hydrolysable tannins, (c) condensed tannins and (d) other compounds. Figure S3: TLC analysis of *L. racemosa* CCC combined fractions. On the left: reversed phase silica gel TLC plates developed with acetonitrile-H_2O 1:1 (*v*/*v*). On the right: Normal phase silica gel TLC plates developed with EtOAc-acetone-H_2O 25:15:10 (*v*/*v*/*v*). Visualization was done using 254 nm UV light and spray-reagent H_2SO_4 10% and vanillin 5%. F means a group of jointed fractions according to TLC similarity. Figure S4: Three-phase solvent system test by TLC. CCC solvent system: *n*-hexane–MTBE–ACN-H_2O (1) 1-1-2-1, (2) 2-1-3-2, (3) 2-2-3-2, (4) 2-3-3-2 and (5) 3-5-5-2 (*v*/*v*); (U) upper, (M) middle and (L) lower phases. Normal phase silica gel TLC plates developed with EtOAc–acetone-H_2O 25:15:10 (*v*/*v*/*v*), visualized using λ 254 nm UV light and spray-reagent H_2SO_4 10% and vanillin 5%.

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