Thieme

Application of UHPLC-ESI-QTOF-MS in Phytochemical Profiling of Sage (*Salvia officinalis*) and Rosemary (*Rosmarinus officinalis*)

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ABSTRACT

UHPLC with QTOF-MS is widely used as a powerful tool for metabolomic analysis. This technology has recently been applied to the analysis of polyphenols in food and herb extracts. Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*), belonging to the family Lamiaceae, are known for their potent antioxidant properties due to the presence of polyphenols. We have developed a sensitive and reproducible UHPLC-QTOF-MS/MS-based method for comprehensive phytochemical profiling and the identification and quantitation of specific polyphenolic compounds present in sage and rosemary leaves. The herbs were extracted ultrasonically using methanol as the solvent. In sage, rosmarinic acid ($17\,678.7 \pm 673.4\,\mu g/g$) and 12-methoxy carnosic acid ($21\,918.3 \pm 715.4\,\mu g/g$) were found in the highest concentrations among all polyphenols. In contrast, rosmarinic acid ($14\,311.0 \pm 636.4\,\mu g/g$), luteolin-3'-acetyl-*O*-glucuronide ($1488.50 \pm 47.58\,\mu g/g$), and luteolin-7-*O*-glucuronide ($1053.68 \pm 68.83\,\mu g/g$) were observed in the highest concentrations in rosemary. Sagerinic acid, rosmanol, rosmadial, carnosol, and carnosic acid were found in abundance in both sage and rosemary. The pentacyclic triterpenoid, corosolic acid ($[M - H]^- \ m/z\ 471.35$), was detected for the first time in both plants. Of the 47 polyphenolic compounds identified in each plant, 38 compounds were found in common in rosemary and sage. A flavonoid compound, baicalin ($[M - H]^- \ m/z\ 445.08$), was identified for the first time in *S. officinalis*. Also, pectolinarigenin ($[M - H]^- \ m/z\ 313.07$), a dimethoxyflavone, was detected for the first time in both sage and rosemary leaves.

Introduction

UHPLC is an advanced technology providing improved speed of analysis while maintaining chromatographic resolution. Quadrupole time-of-flight mass spectrometry (QTOF-MS/MS) delivers rapid acquisition speed, high resolution, superior sensitivity, and excellent mass accuracy for investigating samples containing complex mixtures of compounds [1, 3]. UHPLC hyphenated to atmospheric MS with its hybrid form, QTOF offers refined chromatographic peak separation and hence the most widely used tool in the profiling of polyphenolic compounds in crude samples and can identify elemental composition for both parent and fragment ions [4, 6]. The integration of quantitative analysis and qualitative analysis is one of the important applications of this technique, which in combination with the sequential window acquisition of all theoretical fragment-ion spectra (SWATH) window of selected mass range, can be used for structural elucidation of polyphenols [7, 8]. A run time of less than 20 min is sufficient to carry out UHPLC analysis using smaller particle size (<3 µM) analytical columns operating up to 15 000 psi., which is much shorter than that under the conventional HPLC-MS method. The ESI Q-TOF detector offers excellent full-mass range detection sensitivity and a fast data acquisition rate [9].

Sage (*Salvia officinalis* L.) and rosemary (*Rosmarinus officinalis* L.), belonging to the family Lamiaceae, are the two potent aromatic and medicinal plant species used in traditional medicine, phytopharmaceutical preparations, food preservation, and aromatherapy [10, 11]. Both herbs contain essential oils and share similar chemical and pharmacological properties. Sage is mainly used to improve cognition and is used in the treatment of cardiovascular diseases, excessive sweating, nervous disorders, depression, and cerebral ischemia [11, 14]. It has been found to have a wide range of medicinal uses including antioxidant, antimicrobial, anti-inflammatory, anticancer, anti-dementia, hypoglycemic, and hypolipidemic effects [15, 18]. Similarly, rosemary is traditionally used to strengthen memory functions as well as reduce headaches, tension, insomnia, fever, and respiratory system diseases [19, 20]. It is also used as a cardiac stimulant, a strong antiseptic, antispasmodic, aromatic, carminative, emmenagogue, and nervine stimulant, and is used to cure rheumatism and dandruff [21, 22]. The essential oil of both herbs is used in perfumes, as an antimicrobial, deodorant, insecticide, and fragrant repellant [12, 19]. Sage and rosemary are rich sources of polyphenolic compounds and are the basis of widely commercialized plant extracts known for their potent antioxidant activity [14, 23].
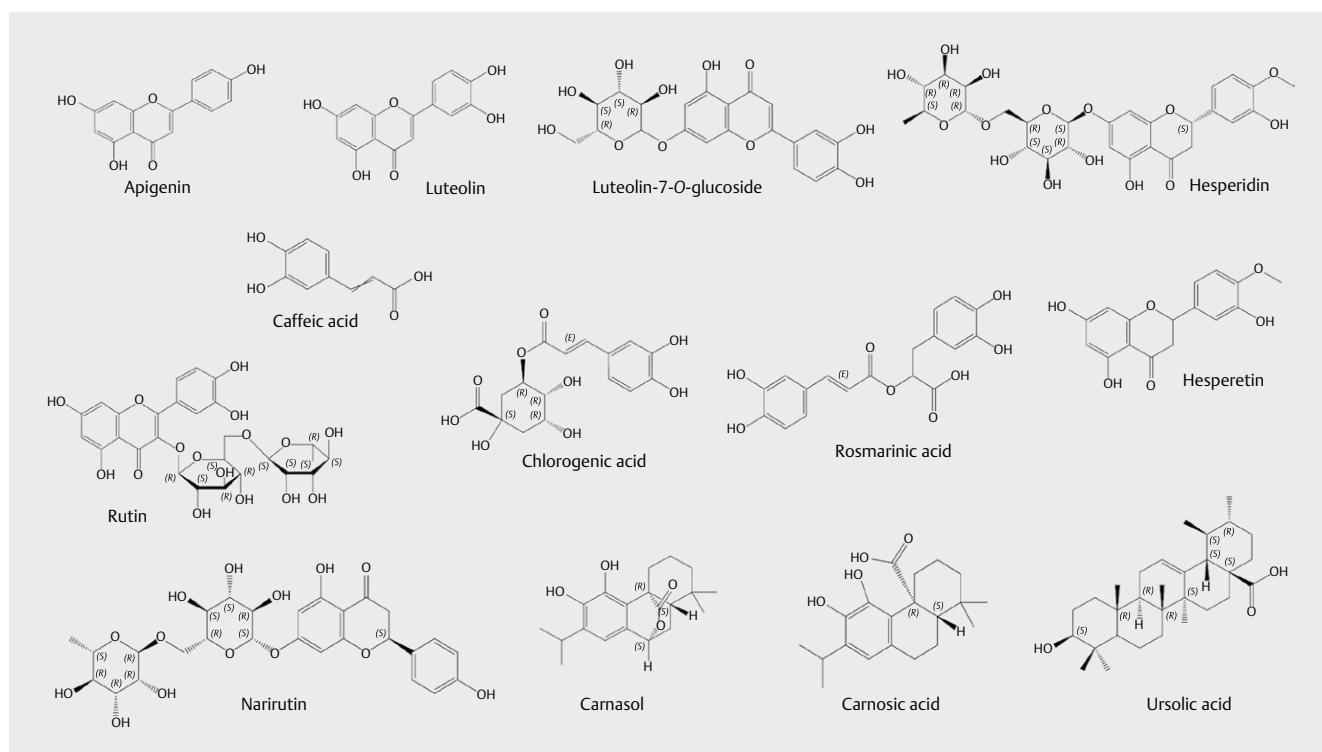
Caffeic acid, rosmarinic acid, salvianolic acids, sagecoumarin, sagerinic acid, ferulic acid, luteolin, apigenin, hispidulin, kaempferol, and quercetin were the most abundant polyphenols present in sage [24, 25], whereas in rosemary, surplus quinic acid, rosmarinic acid, gallocatechin, carnosic acid, and carnosol were reported in previous literatures [26, 27]. Earlier research has employed LC-MS methodologies to investigate the phytochemical composition of both rosemary and sage [27, 28]. We have applied the higher resolution and discriminating power of UHPLC-QTOF-MS/MS, developing a qualitative and quantitative methodology for simultaneously identifying and quantitating the diversity of polyphenolic compounds present in sage and rosemary leaves. The method, which integrates data-independent acquisition (DAI) with the SWATH-based fragmentation protocol and optimized UHPLC conditions, is capable of virtually exhaustive profiling of the highly complex range of phytochemicals present in both sage and rosemary. Besides this, the study also aimed at a tandem MS driven non-targeted screening of bioactive constituents by generating an accurate empirical formula aiding in orthogonal data generation. Further, the application of data evaluation platforms and compound databases can help identify compounds that lack reference standards by searching for the fragmentation spectra aiding in an untargeted screening approach.

Results and Discussion

The standard mixtures of 13 polyphenol and terpenoid compounds, apigenin, luteolin, luteolin-7-*O*-glucoside, hesperidin, hesperetin, rutin, narirutin, caffeic acid, chlorogenic acid, rosmarinic acid, carnosol, carnosic acid, and ursolic acid, were analyzed using the optimized UHPLC-QTOF-MS/MS method in SWATH MS^2 mode. The extraction window for the TOFMS mode was set at 0.02 Da for the deprotonated molecular ions. Limits of detection (LOD) for each analyte in the standard mixture was estimated using the standard deviation (STDEV) from actual values for replicate samples fortified at the limits of quantification (LOQ), applying the equation LOD = STDEV × t$_{0.99}$ (t$_{0.99}$ is the t value at the 99% confidence level at n-1 degrees of freedom, where n = 6, the number of replicates) [29]. Using the proposed UHPLC-QTOF-MS/MS method, 13 precursor polyphenols and their related characteristic compounds were successfully identified and quantified in an ultrasonic methanolic extraction of sage and rosemary. The structures of these reference standards are depicted in ▶ **Fig. 1**. The rosmarinic acid standard curve was used to quantify rosmarinic acid-3-*O*-glucoside, sagerinic acid, rosmanol, rosmadial, rosmanol methyl ether, and rosmaridiphenol. 12-Methoxy carnosic acid was quantified from the carnosic acid calibration curve, whereas micromeric acid was quantified from the ursolic acid standard curve. 6-Hydroxyluteolin-7-*O*-glucoside, isorhamnetin-3-*O*-glucoside, luteolin-7-*O*-glucuronide, luteolin-7-*O*-rutinoside, scutellarin, and luteolin-3'-acetyl-*O*-glucuronide were all quantified by using the luteolin-7-*O*-glucoside standard curve, whereas apigenin-7-*O*-glucoside was quantified as apigenin. Concentrations of phenolic and terpenoid compounds (µg/g) in sage and rosemary leaf extracts analyzed through UHPLC-ESI-QTOF-MS are presented in ▶ **Table 1**.

The quantitative analysis of the sage extract showed that 12-methoxy carnosic acid (21 918.33 ± 715.36 µg/g) was at the highest concentration, followed by rosmarinic acid (17 678.67 ± 673.37 µg/g). Another group of abundant polyphenolic compounds was flavonoids, especially luteolin and its derivatives, phenolic diterpenoids such as rosmadial, rosmanol, and their isomers, carnosol and carnosic acid. The high content of luteolin derivatives and rosmarinic acid was also reported in previous studies on sage polyphenol analysis [30, 31]. The concentration of phenolic acids, sagerinic acid, caffeic acid and its derivatives and rosmarinic acid derivatives were also high in the sage extract. Rosmarinic acid (14 311.00 ± 636.41 µg/g) was found in the highest concentration in rosemary, followed by luteolin 3'-acetyl-*O*-glucuronide (1488.50 ± 47.58 µg/g) and luteolin-7-*O*-glucuronide (1053.68 ± 68.83 µg/g). Hesperidin, isorhamnetin-3-*O*-glucoside, scutellarin, and rosmarin-

▶ **Fig. 1** Structure of phenolic compounds quantified in sage and rosemary leaves by UHPLC-QTOF-MS.

ic acid-3-*O*-glucoside were among the most abundant flavonoids, and the most abundant phenolic acids after rosmarinic acid were sagerinic acid and chlorogenic acid. High concentrations of phenolic diterpenoids such as rosmanol, rosmadial, and their isomers, carnosol, carnosic acid, and 12-methoxy carnosic acids were also found in both herbs. Similar results were obtained in rosemary leaves analyzed through liquid chromatography [26, 27].

Among the pentacyclic triterpenoids, micromeric acid was observed to be higher in rosemary leaves than sage, whereas the quantity of ursolic acid present in both sage and rosemary was almost equal. As compared to sage extract, rosemary extract contained higher amounts of hesperidin and rosmarinic acid-3-*O*-glucoside. It also contained a significant quantity of scutellarin, narirutin, and chlorogenic acid, which was absent in sage extract. Rutin and caffeic acid-3-*O*-glucoside were detected only in sage. Even though the chemical constituents present in sage and rosemary were similar, the concentrations of specific flavonoids, phenolic acids, and terpenoids varied significantly, showing a wide variation in their polyphenol profiles. The developed analytical method proved to be efficient, sensitive, and reproducible for the quantitative analysis of 13 compounds and their phenolic acids in the leaf extracts. All analyte peaks were well resolved within 20 min, and the analysis simultaneously collected all of the data required for the untargeted analysis of the samples.

The high-resolution, accurate mass, UHPLC-ESI-QTOF-MS analysis used in this study not only enabled the quantitative characterization of 13 compounds for which reference materials were available, but simultaneously enabled comprehensive profiling (identification and semiquantitation) of previously unknown compounds based on their molecular formulae, exact mass measurements, and MS/MS fragmentation patterns. Negative ionization [M - H]⁻ was reported to be more sensitive for the analysis of phenolic acids and flavonoids compared to the positive ionization mode. Hence, the analysis was carried out under the negative ionization mode [5, 32, 33]. The phenolic compounds identified in the study along with retention time, mass [M - H]⁻, and MS^2 (*m/z*) ion fragments of sage and rosemary extracts are presented in ▶ **Tables 2** and ▶ **3**, respectively. The compounds without reference standards were identified tentatively by comparing the mass spectra data, ion fragmentation, and molecular weight (*m/z*) with data available in the literature and a mass spectral library obtained from the National Institutes of Standards and Technology (NIST) [26, 28, 31]. In metabolomics and especially in non-targeted metabolomic analysis, compounds are routinely identified by data processing tools that match MS/MS spectra against mass spectral reference libraries and use cheminformatics to provide spectral interpretation [34, 36]. SWATH is a DIA in LCMS/MS and provides a more comprehensive untargeted acquisition of molecular data. We used DIA methods to obtain all fragment ions for all precursors simultaneously, thereby increasing the coverage of observable molecules and reducing false negative identifications. The SWATH acquisition covered a larger number of polyphenolic compounds in the negative ionization mode.

The chromatograms of the UHPLC-ESI-QTOF-MS analysis of sage and rosemary extracts are presented in ▶ **Figs. 2** and ▶ **3**, respectively. A total number of 47 phenolic and terpenoid compounds were tentatively identified in sage and rosemary leaf extracts (▶ **Tables 2** and ▶ **3**). About 22 flavonoid compounds were detected in both samples, mainly representing luteolin, isorhamnetin, hispidulin, hesperidin, apigenin, and their derivatives, scutellarin,

▶ **Table 1** Polyphenol and terpenoid compounds concentration (µg/g) in sage and rosemary analyzed by UHPLC-QTOF-MS.

	Phenolic and terpenoid compounds	Concentration (µg/g)		Quantified as
		Sage	Rosemary	
1	Apigenin	24.80 ± 1.31	6.90 ± 0.59	
2	Apigenin-7-O-glucoside	125.94 ± 9.80	14.48 ± 0.94	Apigenin
3	Luteolin	10.93 ± 1.24	11.13 ± 1.25	
4	Luteolin-7-O-glucoside	294.33 ± 19.44	70.87 ± 7.20	
5	6-Hydroxyluteolin-7-O-glucoside	29.01 ± 1.27	49.03 ± 5.07	Luteolin-7-glucoside
6	Isorhamnetin-3-O-glucoside	75.26 ± 3.51	416.37 ± 17.99	Luteolin-7-glucoside
7	Luteolin 7-O-glucuronide	747.64 ± 30.31	1053.68 ± 68.83	Luteolin-7-glucoside
8	Luteolin-7-O-rutinoside	153.04 ± 6.29	-	Luteolin-7-glucoside
9	Scutellarin	-	152.60 ± 11.87	Luteolin-7-glucoside
10	Luteolin 3'-acetyl-O-glucuronide	-	1488.50 ± 47.58	Luteolin-7-glucoside
11	Rutin	10.54 ± 1.26	-	
12	Narirutin	-	1.98 ± 0.14	
13	Hesperidin	7.67 ± 1.03	729.22 ± 39.36	
14	Hesperetin	1.99 ± 0.26	0.40 ± 0.08	
15	Rosmarinic acid-3-O-glucoside	29.65 ± 1.64	331.36 ± 14.42	Rosmarinic acid
16	Rosmarinic acid-3-O-glucoside isomer	20.43 ± 2.02	-	Rosmarinic acid
17	Caffeic acid-3-O-glucoside	558.83 ± 42.26	-	Caffeic acid
18	Caffeic acid	164.85 ± 18.59	39.08 ± 2.73	
19	Chlorogenic acid	-	241.07 ± 10.82	
20	Rosmarinic acid	17678.67 ± 673.37	14311.00 ± 636.41	
21	Sagerinic acid	867.40 ± 43.79	819.93 ± 46.07	Rosmarinic acid
22	Epirosmanol	200.16 ± 6.31	225.94 ± 4.49	Rosmarinic acid
23	Rosmanol	87.42 ± 2.97	218.48 ± 11.70	Rosmarinic acid
24	Epiisorosmanol	69.55 ± 4.39	182.98 ± 7.36	Rosmarinic acid
25	Rosmanol isomer	29.20 ± 1.01	-	Rosmarinic acid
26	Rosmadial isomer-I	268.42 ± 20.91	422.03 ± 19.93	Rosmarinic acid
27	Rosmadial	342.85 ± 6.64	588.64 ± 24.14	Rosmarinic acid
28	Rosmadial isomer-II	371.97 ± 17.21	358.32 ± 26.60	Rosmarinic acid
29	Rosmadial isomer-III	69.33 ± 3.23	-	Rosmarinic acid
30	Rosmanol methyl ether	47.12 ± 3.08	114.15 ± 13.27	Rosmarinic acid
31	Rosmanol methyl ether isomer	122.13 ± 7.25	42.37 ± 1.46	Rosmarinic acid
32	Rosmaridiphenol	25.57 ± 0.94	-	Rosmarinic acid
33	Carnosol	544.97 ± 33.23	698.78 ± 21.07	
34	Carnosic acid	555.23 ± 23.68	797.75 ± 32.70	
35	12-Methoxy carnosic acid	21918.33 ± 715.36	982.78 ± 32.77	Carnosic acid
36	Ursolic acid	61.46 ± 2.48	59.03 ± 3.95	
37	Micromeric acid	4.80 ± 0.14	83.22 ± 2.38	Ursolic acid

rutin, baicalin, pectolinarigenin, and genkwanin. In sage, a dihydroxyflavone, baicalin ([M - H]⁻ m/z 445.08), was detected for the first time, and the flavonoid was not identified in *S. officinalis* before. Baicalin is a flavone glycoside commonly found in roots of *Scutellaria baicalensis*, and has been reported as having anti-inflammatory, antiviral, anti-allergic, cognitive, and neuroprotective properties [37, 38]. A trihydroxy-methoxyflavone, diosmetin ([M - H]⁻ m/z 299.05), detected in the rosemary sample was previously described by Borras-Linares et al. with a similar fragmentation pattern [39]. Pectolinarigenin ([M - H]⁻ m/z 313.07) was yet another flavone reported for the first time in both sage and rosemary.

Pectolinarigenin was found in *Salvia hypoleuca* and *Salvia pedicellata* species, and it was also reported before in rosemary as a dimethoxyflavone with the same fragment ions [24, 27]. Both diosmetin and pectolinarigenin flavonoids are said to have potent anti-inflammatory and anticancer properties [40, 41]. Among the nine different identified phenolic acids, ferulic acid ([M-H]⁻ at m/z 193.95), rosmarinic acid ([M - H]⁻ at m/z 359.08), salvianolic acid K ([M - H]⁻ at m/z 555.12), and methyl rosmarinate ([M - H]⁻ at m/z 373.09) shared many of the same MS/MS (m/z) ion fragments, which are related to caffeic acid. Many phenolic acids of the Salvia species were previously reported to be caffeic acid derivatives, mostly

▶ Table 2 Polyphenolic and terpenoid compounds identified by UHPLC-ESI- QTOF-MS analysis in sage (*S. officinalis*).

Sl No	Compound	RT (min)	Mass [M - H]⁻ (*m/z*)	Formula	MS² (*m/z*) Fragments
1	Caffeic acid 3-*O*-glucoside	0.23	341.09	$C_{15}H_{18}O_9$	179.0551 (67)
2	Caffeic acid	0.61	179.04	$C_9H_8O_4$	135.0432 (100), 134.0354 (28), 105.0335 (5)
3	Gallocatechin	0.74	305.07	$C_{15}H_{14}O_7$	225.1112 (73), 96.9579 (38)
4	Luteolin-7-*O*-rutinoside	0.95	593.12	$C_{27}H_{30}O_{15}$	287.0548 (62), 285.0386 (45)
5	Vanillic acid	1.01	167.03	$C_8H_8O_4$	108.0208 (19), 65.0388 (18)
6	Ferulic acid	1.35	193.05	$C_{10}H_{10}O_4$	134.0366 (100), 179.0269 (41), 133.0292 (18)
7	6-Hydroxyluteolin -7-*O*-glucuronide	1.44	477.07	$C_{21}H_{18}O_{13}$	301.0345 (100)
8	6-Hydroxyluteolin-7-*O*-glucoside	1.57	463.09	$C_{21}H_{20}O_{12}$	287.0576 (25), 301.0338 (10)
9	Scutellarin	1.76	461.07	$C_{21}H_{18}O_{12}$	285.0385 (100)
10	Rutin	1.84	609.15	$C_{27}H_{30}O_{16}$	300.0350 (10)
11	Luteolin-7-*O*-glucoside	1.92	447.09	$C_{21}H_{20}O_{11}$	285.0403 (100)
12	Isorhamnetin-3-*O*-glucoside	1.94	477.10	$C_{22}H_{22}O_{12}$	315.0694 (24)
13	Baicalin	2.03	445.08	$C_{21}H_{18}O_{11}$	269.0445 (100)
14	Sagerinic acid	2.10	719.16	$C_{36}H_{32}O_{16}$	359.0724 (100)
15	Rosmarinic acid	2.13	359.08	$C_{18}H_{16}O_8$	161.0216 (100), 179.0325 (59), 197.0432 (31)
16	Salvianolic acid K	2.30	555.12	$C_{27}H_{24}O_{13}$	359.0734 (100), 493.1082 (44), 179.0323 (16), 135.0426 (12)
17	Apigenin-7-*O*-glucoside	2.32	431.10	$C_{21}H_{20}O_{10}$	269.0825 (17)
18	Hispidulin glucuronide	2.34	475.09	$C_{22}H_{20}O_{12}$	299.0552 (100), 284.0320 (10)
19	Hesperidin	2.65	609.18	$C_{28}H_{34}O_{15}$	301.0697 (100)
20	4,5,7-Trihydroxy flavone	2.70	269.05	$C_{15}H_{10}O_5$	225.1684 (3), 201.0567 (2), 183.0452 (2)
21	Methyl rosmarinate	2.70	373.09	$C_{19}H_{18}O_8$	179.0343 (100), 197.0453 (15), 135.0443 (9)
22	Luteolin	2.88	285.04	$C_{15}H_{10}O_6$	133.0300 (19), 151.0042 (12), 175.0409 (9), 199.0406 (9)
23	Isorhamnetin	2.99	315.05	$C_{16}H_{12}O_7$	300.0268 (100)
24	Salvianolic acid B	3.23	717.14	$C_{36}H_{30}O_{16}$	519.0935 (100), 339.0511 (17)
25	4,5,7-Trihydroxy flavone isomer	3.26	269.05	$C_{15}H_{10}O_5$	225.0570 (8), 159.0454 (4), 201.0568 (3)
26	Hispidulin	3.36	299.06	$C_{16}H_{12}O_6$	284.0315 (100), 136.9866 (18), 200.0475 (9), 65.0019 (4)
27	Hesperetin	3.48	301.07	$C_{16}H_{14}O_6$	284.0319 (100), 136.9878 (20), 164.0113 (5)
28	Apigenin	3.50	269.05	$C_{15}H_{10}O_5$	117.0346 (19), 149.0244 (14), 107.0143 (4)
29	Epirosmanol	3.82	345.17	$C_{20}H_{26}O_5$	284.1679 (12), 283.8697 (4)
30	Pectolinarigenin	3.94	313.07	$C_{17}H_{14}O_6$	284.0266 (100), 298.0467 (100), 227.0346 (13), 117.0337 (7), 163.0035 (10)
31	Rosmanol	4.04	345.17	$C_{20}H_{26}O_5$	301.1786 (100), 284.1701 (22)
32	Rosmadial isomer	4.31	343.15	$C_{20}H_{24}O_5$	284.1410 (39)
33	Epiisorosmanol	4.36	345.17	$C_{20}H_{26}O_5$	284.1680 (15), 283.8695 (5)
34	Genkwanin	4.38	283.06	$C_{16}H_{12}O_5$	268.0382 (85), 117.0343 (10), 240.0438 (12)
35	Rosmanol isomer	4.87	345.17	$C_{20}H_{26}O_5$	301.1786 (100)
36	Asiatic acid	5.17	487.34	$C_{30}H_{48}O_5$	-
37	Rosmadial	5.32	343.15	$C_{20}H_{24}O_5$	300.1660 (100), 284.1410 (39)
38	Rosmanol methyl ether	5.47	359.99	$C_{21}H_{28}O_5$	283.1657 (100), 284.1679 (79), 268.1440 (20), 329.1699 (12)
39	Carnosol	5.58	329.18	$C_{20}H_{26}O_4$	286.1847 (100), 285.05858891 (100), 270.1605 (8), 201.0885 (5)
40	Rosmadial isomer	5.85	343.19	$C_{20}H_{24}O_5$	299.2016 (100), 284.1791 (45), 269.1556 (9)
41	Rosmaridiphenol	5.97	315.20	$C_{20}H_{28}O_3$	284.1768 (34)
42	Carnosic acid	6.38	331.19	$C_{20}H_{28}O_4$	288.2043 (100), 287.2015 (39), 244.1476 (19)
43	Corosolic acid	6.44	471.35	$C_{30}H_{48}O_4$	-
44	12-Methoxy-carnosic acid	6.82	345.21	$C_{21}H_{30}O_4$	301.2138 (100), 287.1911 (89)
45	Micromeric acid	7.51	453.34	$C_{30}H_{46}O_3$	-
46	Betulinic acid	7.71	455.35	$C_{30}H_{48}O_3$	-
47	Ursolic acid	7.91	455.35	$C_{30}H_{48}O_3$	-

▶ **Table 3** Polyphenolic and terpenoid compounds identified by UHPLC-ESI-QTOF-MS analysis in rosemary (*R. officinalis*).

| Sl No	Compound	RT (min)	Mass [M-H]⁻ (m/z)	Formula	MS² (m/z) Fragments
1	Caffeoyl-fructosyl-glucose	0.24	503.13	$C_{21}H_{28}O_{14}$	191.0543 (81), 145.0618 (72),179.0549 (63)
2	Quinic acid	0.43	191.06	$C_7H_{12}O_6$	85.0293 (45), 127.0396 (31), 93.0345 (30)
3	Chlorogenic acid	0.48	353.09	$C_{16}H_{18}O_9$	191.0534 (100), 161.0239 (6)
4	Gallocatechin	0.58	305.07	$C_{15}H_{14}O_7$	225.1078 (43), 96.9559 (33), 135.0793 (31)
5	Vanillic acid	0.67	167.03	$C_8H_8O_4$	152.0110 (60), 108.0210 (35), 123.0440 (10), 91.0195 (5)
6	Caffeic acid	0.85	179.03	$C_9H_8O_4$	135.0457 (100), 134.0380 9 (42), 79.0562 (6)
7	Ferulic acid	1.60	193.05	$C_{10}H_{10}O_4$	134.0375 (100), 179.0275 (50), 133.0295 (18), 149.0611 (14)
8	6-Hydroxyluteolin -7-O-glucuronide	1.66	477.07	$C_{21}H_{18}O_{13}$	301.0346 (100)
9	6-Hydroxyluteolin-7-O-glucoside	1.93	463.09	$C_{21}H_{20}O_{12}$	301.0331 (15)
10	Scutellarin	2.16	461.07	$C_{21}H_{18}O_{12}$	286.0417 (100), 285.0388 (75), 113.0228 (10), 175.0240 (5)
11	Narirutin	2.20	579.18	$C_{27}H_{32}O_{14}$	271.0603 (79), 151.0038 (9)
12	Rosmarinic acid-3-O-glucoside	2.22	521.13	$C_{24}H_{26}O_{13}$	359.0759 (100), 341.0851 (54)
13	Luteolin-7-O-glucoside	2.23	447.09	$C_{21}H_{20}O_{11}$	285.0382 (10)
14	Isorhamnetin-3-O-rutinoside	2.24	623.16	$C_{28}H_{32}O_{16}$	315.0740 (48)
15	Salvianolic acid B	2.25	717.14	$C_{36}H_{30}O_{16}$	519.0863 (100)
16	Isorhamnetin-3-O-glucoside	2.28	477.10	$C_{22}H_{22}O_{12}$	315.0740 (22)
17	Rosmarinic acid	2.46	359.07	$C_{18}H_{16}O_8$	161.0225 (100), 179.0322 (77), 197.0425 (63), 133.0281 (57)
18	Sagerinic acid	2.46	719.16	$C_{36}H_{32}O_{16}$	359.0735 (100), 179.0337 (7)
19	Luteolin-7-O-glucuronide	2.57	461.07	$C_{21}H_{18}O_{12}$	285.0385 (100)
20	Apigenin-7-O-glucoside	2.58	431.10	$C_{21}H_{20}O_{10}$	269.0445 (35)
21	Hispidulin glucuronide	2.65	475.09	$C_{22}H_{20}O_{12}$	299.0546 (34)
22	Hesperidin	2.65	609.18	$C_{28}H_{34}O_{15}$	301.0697 (100)
23	Luteolin	2.88	285.04	$C_{15}H_{10}O_6$	133.0300 (24), 151.0042 (12), 175.0409 (9), 199.0406 (9)
24	Methyl rosmarinate	2.94	373.09	$C_{19}H_{18}O_8$	175.0407 (100), 179.0356 (58), 197.0463 (45), 135.0458 (35)
25	Luteolin 3'-acetyl-O-glucuronide	3.07	503.08	$C_{23}H_{20}O_{13}$	443.0579 (100), 285.7495 (9)
26	Apigenin	3.47	269.04	$C_{15}H_{10}O_5$	117.0363 (18), 151.0053 (13), 225.0583 (8)
27	Hesperetin	3.48	301.07	$C_{16}H_{14}O_6$	284.0319 (100), 136.9878 (20), 164.0113 (5)
28	Diosmetin	3.56	299.05	$C_{16}H_{12}O_6$	284.0331 (100), 136.9876 (20), 227.0346 (11)
29	Epirosmanol	3.97	345.17	$C_{20}H_{26}O_5$	284.1679 (12), 283.8697 (4)
30	Pectolinarigenin	4.07	313.07	$C_{17}H_{14}O_6$	298.0471 (100), 283.0241(73), 255.0289 (28), 117.0350 (6)
31	Rosmanol	4.18	345.17	$C_{20}H_{26}O_5$	301.1803 (100), 284.0282 (6)
32	Rosmadial isomer	4.42	343.15	$C_{20}H_{24}O_5$	284.1418 (12), 299.8915 (7)
33	Epiisorosmanol	4.46	345.17	$C_{20}H_{26}O_5$	284.1701 (22), 283.8717 (8)
34	Genkwanin	4.46	283.06	$C_{16}H_{12}O_5$	268.0378 (94), 240.0433 (13), 117.0354 (6), 211.0405 (5)
35	Rosmadial	4.60	343.15	$C_{20}H_{24}O_5$	299.8914 (8), 285.1857 (6)
36	Rosmanol isomer	4.97	345.17	$C_{20}H_{26}O_5$	301.1803 (100), 263.2028 (6)
37	Asiatic acid	5.24	487.35	$C_{30}H_{48}O_5$	-
38	Rosmanol methyl ether	5.38	359.18	$C_{21}H_{28}O_5$	283.8754 (100), 329.1709 (10), 300.1700(15)
39	Carnosol	5.63	329.17	$C_{20}H_{26}O_4$	285.1831 (100), 201.0909 (4), 270.1615 (3), 214.1001 (3)
40	Carnosic acid	5.63	331.19	$C_{20}H_{28}O_4$	286.1848 (100), 287.0588 (72)
41	Rosmadial isomer	5.90	343.15	$C_{20}H_{24}O_5$	299.2005 (100), 284.1779 (67), 269.1556 (9)
42	Unknown compound	6.17	469.34	$C_{30}H_{46}O_4$	425.3430 (10)
43	Corosolic acid	6.48	471.35	$C_{30}H_{48}O_4$	-
44	12-Methoxy-carnosic acid	6.85	345.20	$C_{21}H_{30}O_4$	301.2161 (100), 287.1956 (39)
45	Micromeric acid	7.53	453.33	$C_{30}H_{46}O_3$	-
46	Betulinic acid	7.80	455.35	$C_{30}H_{48}O_3$	-
47	Ursolic acid	7.94	455.35	$C_{30}H_{48}O_3$	-
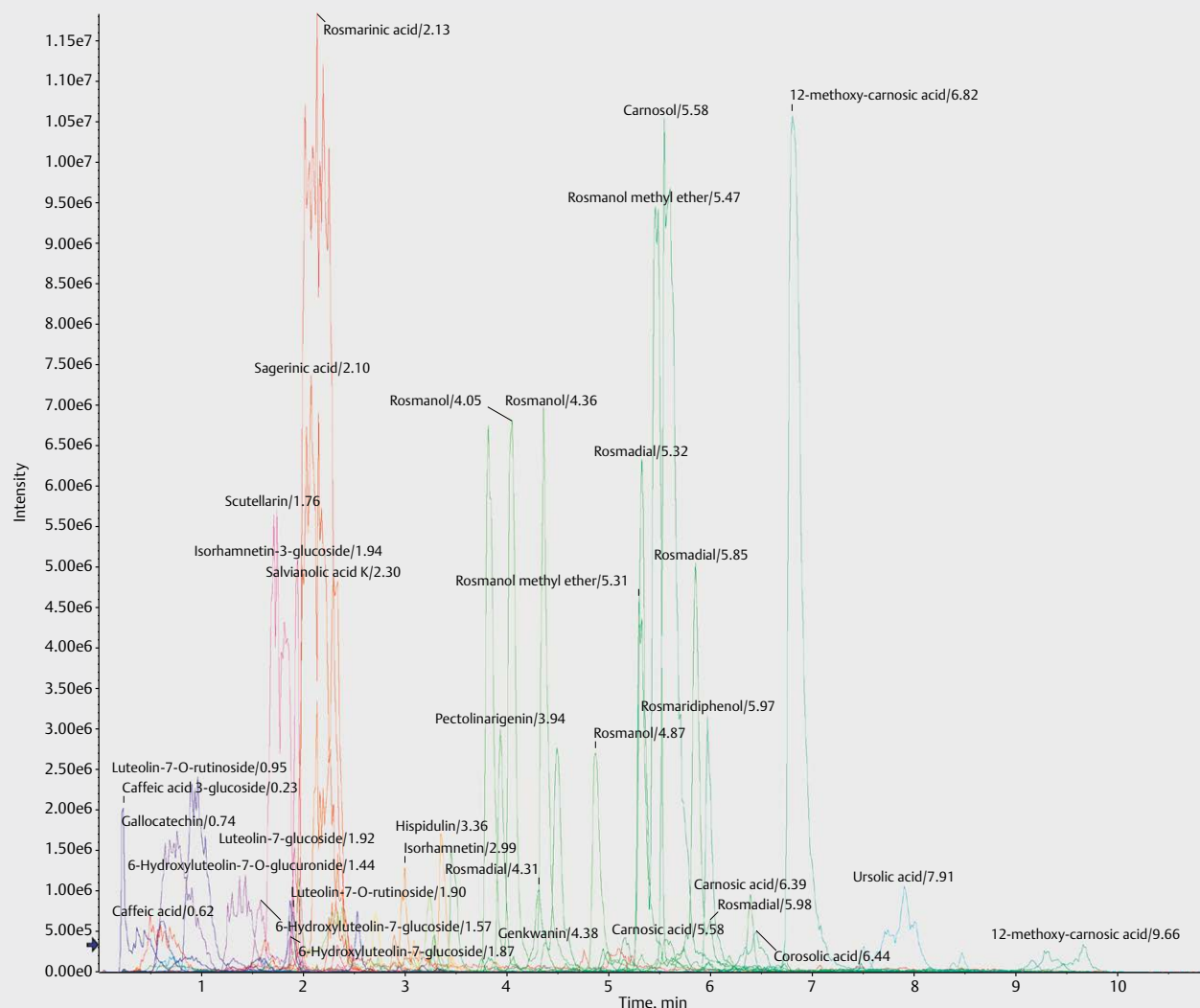
▶ Fig. 2 Chromatogram representing relative abundance of polyphenols in sage leaves analyzed through UHPLC-ESI-QTOF-MS (intensity vs. elution time).

formed by esterification of caffeic acid [42]. Similarly, sagerinic acid ([M - H]$^-$ m/z 719.16) shares an MS/MS ion fragment (359.07) of rosmarinic acid ([M - H]$^-$ m/z 359.08), as has been reported previously by Lu and Foo [43].

A large group of phenolic terpenoid compounds was detected in both sage and rosemary leaves. Rosmanol, rosmadial, and their isomers, carnosol, carnosic acid, rosmanol methyl ether, and 12-methoxy carnosic acids were the major diterpenoids found in the extracts. The presence of more than one peak corresponding to the same molecular mass but with different elution times was due to the presence of isomers. Rosmanol ([M - H]$^-$ m/z 345.17) generated four peaks at different intervals with the same fragmentation (MS^2 m/z fragment 284.1 and 301.18) (▶ **Figs. 2** and ▶ **3**). Similarly, three peaks were observed for the rosmadial molecule ([M - H]$^-$ m/z 343.15), sharing the same fragmentation patterns. Elution of multiple peaks with the same molecular mass and fragment ions at different retention times indicates the presence of isomers of the same molecules, and similar results were reported in

sage and rosemary in previous literature [28, 44, 45]. A total of five pentacyclic triterpenoid compounds, asiatic acid ([M - H]$^-$ m/z 487.34), betulinic acid and ursolic acid ([M - H]$^-$ m/z 455.35), corosolic acid ([M - H]$^-$ m/z 471.34, and micromeric acid ([M-H]$^-$ m/z 453.33), have been identified both in sage and rosemary. Among them, betulinic acid and ursolic acid were detected before in sage and rosemary leaves [12, 26, 27, 46, 47]. Despite the similar molecular weight of ursolic acid and betulinic acid ([M - H]$^-$ at m/z 455.35), the former was identified through the reference standard, and the later molecule was confirmed by comparison to the NIST mass spectral library. Betulinic acid in the herbs were found to have potent antiviral activity against severe acute respiratory syndrome coronavirus [48]. Although asiatic acid was previously reported in rosemary, it was not reported in the previous literature of *S. officinalis* [26]. A triterpenoid compound, corosolic acid ([M - H]$^-$ m/z 471.34), was detected in both herbs for the first time, yet corosolic acid was identified before in roots of some Salvia species [49, 50]. Even though micromeric acid ([M - H]$^-$ m/z 453.33) has been re-
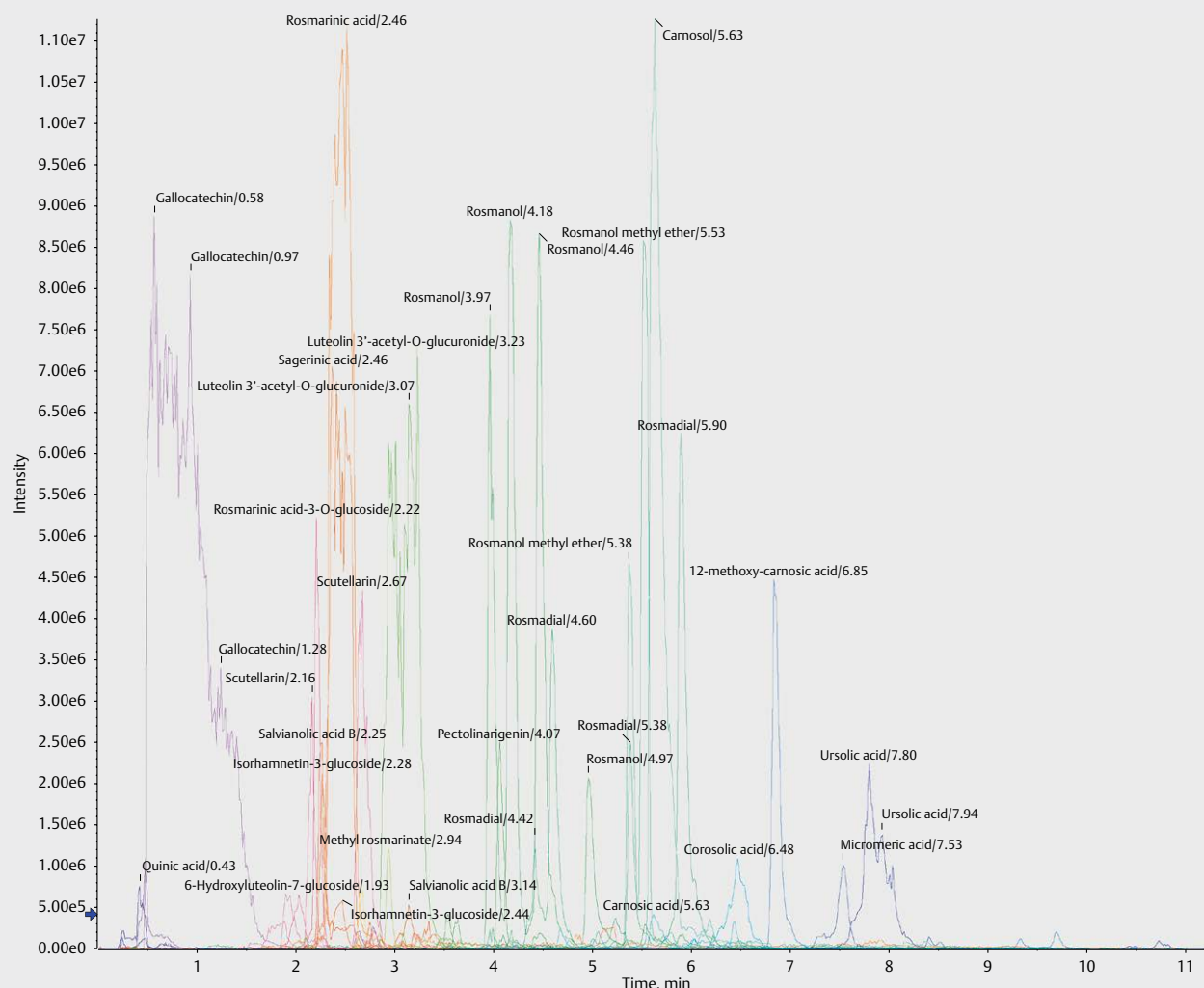
▶ **Fig. 3** Chromatogram representing relative abundance of polyphenols in rosemary leaves analyzed through UHPLC-ESI-QTOF-MS (intensity vs. elution time).

ported in rosemary, it was found for the first time in sage leaves [26]. Triterpene content in rosemary samples was remarkably high compared to sage leaves. Pentacyclic triterpenoids have been reported as having several medicinal properties, especially anti-inflammatory, anticancer, and antidiabetic potential [51, 52].

This study developed a high-throughput, sensitive, and reproducible UHPLC-QTOF-MS/MS method that can serve two purposes. First, it was validated for the identification and quantification of 13 polyphenols in sage and rosemary and could be used for quantitative analysis of any other polyphenol for which reference material is available. Results obtained from validation studies indicated that the developed method was highly selective, sensitive, accurate, and reproducible for the detection and quantitation of target phenolic compounds in complex biological matrices. It has demonstrated great value for qualitative analysis to support the structural elucidation of expected and unexpected metabolites and has been evaluated for its potential to handle quantitative measurements [53]. To the best of our knowledge, this is the most sensitive and comprehensive UHPLC-QTOF-MS/MS-based method for tar-

geted analysis of leaf extracts of sage and rosemary. Minor modifications of this method could be applicable as a quantitative tool for accurately and efficiently determining the content of specific phytochemicals present in a wide range of medicinal herbs and extracts thereof given its fast acquisition speed with full-scan sensitivity, enhanced mass resolution, and accurate mass measurement capabilities. Here, we described an optimized and efficient procedure for the UHPLC MS-based analysis of tissue samples from sample preparation to spectral processing and data analysis.

Second, this same UHPLC-QTOF-MS/MS-based method was found to be directly applicable for semiquantitative profiling of the full range of polyphenols in sage and rosemary. The untargeted dimension of this analytical method, enabled by the SWATH protocol, generated retention time, molecular ion *m/z*, and mass fragmentation pattern data for virtually every phytochemical present in the sage and rosemary extracts. Many of these phytochemicals were tentatively identified based on the comparison of their molecular *m/z* and mass fragmentation patterns to data in several mass spectral databases. An equal or larger number of phytochemicals

▶ **Table 4** Results of analysis of calibration curve and limits of quantification and limits of detection of reference standards.

	Standard	Purity (%)	Formula	Molecular weight	Calibration range (ng/mL)	LOQ	LOD	Calibration equations	Slope (R²)
1	Apigenin	100.0	$C_{15}H_{10}O_5$	270.24	3.91–250	3.81	1.65	$y = 0.0311x + 0.01777$	0.9926
2	Luteolin	99.6	$C_{15}H_{10}O_6$	286.24	3.91–250	3.91	1.22	$y = 0.04458x + 0.00937$	0.9939
3	Luteolin-7-*O*-glucoside	98.0	$C_{21}H_{20}O_{11}$	448.38	3.91–250	3.91	1.62	$y = 0.02301x - 0.00426$	0.9966
4	Hesperidin	91.1	$C_{28}H_{34}O_{15}$	610.57	3.91–250	4.14	0.97	$y = 0.01901x - 0.0134$	0.9900
5	Hesperitin	99.6	$C_{16}H_{14}O_6$	302.28	3.91–250	3.70	0.88	$y = 0.05678x + 0.04536$	0.9907
6	Rutin	96.9	$C_{27}H_{30}O_{16}$	610.56	3.91–250	4.14	0.96	$y = 0.01645x - 0.02858$	0.9892
7	Narirutin	98.6	$C_{27}H_{32}O_{14}$	580.50	3.91–250	4.12	1.45	$y = 0.01884x - 0.025$	0.9906
8	Caffeic acid	98.0	$C_9H_8O_4$	180.00	3.91–250	3.91	1.87	$y = 0.01048x + 0.01989$	0.9964
9	Chlorogenic acid	99.0	$C_{16}H_{18}O_9$	354.31	3.91–250	3.81	1.41	$y = 0.01472x + 0.00617$	0.9960
10	Rosmarinic acid	98.0	$C_{18}H_{16}O_8$	360.31	3.91–250	3.72	1.03	$y = 0.02319x + 0.59972$	0.9943
11	Carnosol	100.0	$C_{20}H_{26}O_4$	330.40	3.91–250	3.85	1.25	$y = 0.04408x + 0.44018$	0.9960
12	Carnosic acid	96.0	$C_{20}H_{28}O_4$	332.43	15.63–250	14.71	6.90	$y = 0.00153x + 0.02517$	0.9880
13	Ursolic acid	97.0	$C_{30}H_{48}O_3$	456.70	3.91–250	4.00	1.31	$y = 0.01537x + 0.01792$	0.9923
LOQ = limits of quantification, LOD = limits of detection.									

present in the extracts remain unidentified since no compound was identified within the mass spectral databases that corresponded to the compounds detected in the sage and rosemary extracts. These compounds, which numbered 65 in sage and 54 in rosemary, were found in higher intensities with a clear peak area and fragmentation. Most of these compounds were found to be the derivatives or dimers or trimers of luteolin, rosmarinic acid, caffeic acid, rosmanol, rosmadial, and carnosol, matching their fragmentation with their original molecule. However, there is a need to study these unknown molecules using further advanced techniques like NMR spectroscopy for identification and confirmation of these unknown molecules. Thus, the untargeted dimension of this method has contributed to the elucidation of the phytochemical composition of sage and rosemary leaf extracts by defining the mass spectra of the majority of compounds present in these extracts and deducing the tentative identities of many of these by comparison with mass spectral libraries. This sets the stage for additional research identifying the structures and functions of the currently unnamed compounds within these extracts, leading to a complete polyphenol profile of these herbal extracts from which an herbal fingerprint could be developed and used for efficient and accurate routine quality control. It should be noted that the model set forth in this paper should be roughly applicable to create quality control tools for any other herbal material.

A thorough and complete polyphenol profiling of sage and rosemary was made possible by using an ionization technique that was consistently applied to a wide range of molecular structures. We used mass spectral library searching to identify non-targeted compounds based on their mass spectral fragmentation patterns. This strategy of identifying compounds based on their molecular fragmentation fingerprint is very useful and very powerful but is limited to the compounds present in the MS/MS libraries available. This method brought to light a large number of compounds that were not represented in any of the mass spectral libraries available at this time. Thus, they remain unknown in structure and function, although the QTOF analysis did provide a highly accurate molecular mass. These compounds, which numbered 65 in sage and 54 in rosemary, represent an area of great interest for further study. As the research in this field grows, new compounds are added to the databases by the researchers, and the libraries will thus expand. This growing database information will undoubtedly help imminent researchers to effectively use the data to avoid many false-positive results. This strategy can be further developed into a comprehensive methodology for defining a fingerprint of the queried sample that can be used as a quality control indicator, which can then be used for assessing the quality of sage or rosemary plant material or extracts. This analytical metabolomic strategy of the unknown identification using compound databases will help researchers for putative compound class identification of plant-specific metabolites through non-targeted screening, reducing the economic burden of procuring many stock standards. There is considerable potential for identifying unknown novel compounds in the extracts using this approach.

Materials and Methods

Chemicals

LCMS grade methanol and acetonitrile were procured from Honeywell and formic acid and glacial acetic acid were purchased from Merck. Reference standards, apigenin, luteolin, rutin, chlorogenic acid, hesperidin, and hesperetin were obtained from Sigma-Aldrich. Caffeic acid, carnosic acid, luteolin-7-*O*-glucoside, ursolic acid, and rosmarinic acid were procured from Toronto Research Chemicals. Narirutin and carnosol were from Cayman Chemical. Ultrapure water produced using a Milli-Q A10 water purification system (Millipore Sigma) was used throughout.

Sample collection and extraction

S. officinalis (voucher no. ISC-454696) and *R. officinalis* (voucher no. ISC-454695) leaves were collected from the Regenerative Organic Farm, Maharishi University of Management, Fairfield, Iowa, USA, during the year 2018. Sage seeds for the experiment were collected from Ohio Heirloom Seeds, and rosemary seeds were collected

from Everwilde Farms, Sand Creek, WI, USA. Sage seeds were started in the greenhouse in March, and 45-day-old seedlings were transplanted into the main field in the first week of May. Rosemary seedlings were started in November, and 6-week-old seedlings were transplanted into pots and then into the main field in May. Sage leaves were harvested 3 months after planting (in September 2018), and rosemary was harvested 10 months after planting (in October 2018). Freshly harvested leaves were spread over muslin cloth and shade dried for 10 days, then were powdered for extraction and further chromatographic analysis. Next, 5 g of dry leaf powder were extracted ultrasonically in 50 mL LCMS grade methanol for 1 h 30 min with a frequency of 40 kHz in a Bransonic-52 ultrasonic bath unit from Branson. Finally, 10 mL of the extract were centrifuged, and 9 mL of supernatant were lyophilized and stored in the dark at -20 °C until analysis.

UHPLC-QTOF- MS/MS conditions

Chromatographic analysis was performed on a Shimadzu Nexera *X2* UHPLC system (Shimadzu Corporation) equipped with an LC-30AD binary pump, SIL-30AC autosampler, and a CTO- 30A column oven. The column was a Kinetex XB C18 (1 mm × 50 mm, 2.6 m, 100 Å pore size; Phenomenex) with a KrudKatcher UHPLC in-line filter (0.5 μm), maintained at 45 °C. A mixture of MilliQ water (A) and 0.1 % acetic acid in acetonitrile (B) was used as a mobile phase with a flow rate of 0.25 mL/min using a multi-step linear gradient elution: 0 min, 10 % B; 10 min, 90 % B; 12.5 min, 90 % B; 15 min, 10 % B; 20 min, 10 % B. The total run time was 20 min, including time for re-equilibration to initial conditions. The sample autosampler injection volume was 10 μL.

Mass spectrometry was performed using a Triple TOF-5600 (AB SCIEX), a hybrid triple QTOF mass spectrometer equipped with an ESI source, with the mass range set at *m/z* 50–1000. ESI was set in the negative mode with an ion spray needle voltage set at 4500 V and ion source gas-1 and gas-2 each set at 50 psi. The curtain gas was 35 psi and the collision gas pressure was 20 psi with a source temperature of 600 °C. The declustering potential was -50 eV in TOF-MS and MS/MS experiments, whereas the collision energy values for TOF-MS were 5 eV, and for MS/MS experiments, 25 eV with a spread of 15 eV.

For SWATH-MS^2 acquisition in the collision cell (Q2), nitrogen gas was used for the fragmentation of the precursor ions. In the SWATH-MS^2 acquisition, a variable SWATH window was used to cover the mass range of *m/z* 50–1000 in 16 segments (15 × 48.5 msec), yielding a cycle time of 0.8268 sec, which included one 50 msec TOF-MS scan. A variable window of the SWATH-MS^2 generates complex MS/MS spectra that are a composite of the spectra for all of the analytes that elute in that window.

Sample analysis

Sage and rosemary leaf extracts were dissolved in 4.5 mL of 10 % acetonitrile and 0.1 % acetic acid. Samples were further diluted in 10 % acetonitrile (1/200 to 1/40000) to bring the levels of analytes within the linear range of the standard calibration curve, thereby avoiding MS signal saturation. Of diluted sample, 190 μL were transferred to an autosampler vial along with 10 μL of internal standard (chloramphenicol) before analysis. Chloramphenicol was used to correct the instrument variability between injections, normalize the signal intensities, and check the instruments' response over the analytical run. Chloramphenicol, a small molecule, ionizes well in the negative mode of ionization and gives good sensitivity due to the presence of terminal hydroxyl groups. With the ESI interface, like the majority of polyphenols, chloramphenicol forms single charged ions [M - H]¯ at *m/z* 321.0154. Chloramphenicol chromatography was well studied in our lab and found to be suitable for any C18 column with a generic gradient with long run times. The standard calibration curves for apigenin, luteolin, luteolin-7-*O*-glucoside, hesperidin, hesperetin, rutin, narirutin, caffeic acid, chlorogenic acid, rosmarinic acid, carnosol, carnosic acid, and ursolic acid were constructed for quantification of those compounds using chloramphenicol as the internal standard. ▶ **Table 4** represents calibration parameters, including LOQ, LOD, calibration range, regression equations, and slopes. The sensitivity, accuracy, and precision of the method were also determined. The mobile phase constituents and the chromatographic gradient were adopted from Gifford et al. with slight modifications, which, together with optimized mass spectrometric conditions, maximized sensitivity and linearity [54]. All extractions and analyses were made in six replications, and the results of polyphenol quantification are expressed as the mean ± standard deviation.

Unknown polyphenolic compounds and flavonoids were identified based on their accurate mass (*m/z*) and molecular (*m/z*) ion fragmentation pattern using Peak View Software, ver.2.2, Master View, Library View (all from AB SCIEX), and several mass spectral libraries such as NIST - 2017, AOI (All-in-One) HR-MS/MS Spectral Library 2.0 (Sciex), MassBank of North America (MoNA), and HILIC library database from University of California, Davis.

Supporting Information

Preparation of standard cocktail tables and fragments of standard compounds are presented in the Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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