

RESEARCH ARTICLE

Globoidnan A, rabdosiin and globoidnan B as new phenolic markers in European-sourced comfrey (*Symphytum officinale* L.) root samples

Adriana Trifan¹ | Evelyn Wolfram² | Nils Esslinger³ | Andreas Grubelnik³ |
Krystyna Skalicka-Woźniak⁴ | Mirjana Minceva⁵ | Simon Vlad Luca⁵ 

¹Department of Pharmacognosy, Grigore T. Popa University of Medicine and Pharmacy Iasi, Iasi, 700115, Romania

²Phytopharmacy and Natural Products Research Group, Zurich University of Applied Sciences, Wädenswil, 8820, Switzerland

³Alpinamed AG, 9306 Freidorf, Switzerland

⁴Independent Laboratory of Natural Products Chemistry, Department of Pharmacognosy, Medical University of Lublin, Lublin, 20-093, Poland

⁵Biothermodynamics, TUM School of Life and Food Sciences Weihenstephan, Technical University of Munich, Freising, 85354, Germany

Correspondence

Dr. Evelyn Wolfram, Phytopharmacy and Natural Products Research Group, Zurich University of Applied Sciences, 8820, Wädenswil, Switzerland.
Email: wola@zhaw.ch

Abstract

Introduction: *Symphytum officinale* L. (comfrey, Boraginaceae) is a cultivated or spontaneously growing medicinal plant that is traditionally used for the treatment of bone fractures, hematomas, muscle pains and joint pains. A wide range of topical preparations and dried roots for *ex tempore* applications are marketed in European drug stores or pharmacies.

Objective: The aim of this study was to perform the qualitative and quantitative analysis of pyrrolizidine alkaloids (PAs) and phenolic compounds in the hydroethanolic extracts of 16 commercial comfrey root batches purchased from 12 different European countries.

Methods: Liquid chromatography hyphenated with high-resolution tandem mass spectrometry (LC-HRMS/MS) was used for the profiling of PAs and phenolic compounds, whereas LC-MS/MS and liquid chromatography with diode array detection (LC-DAD) were used for their quantification.

Results: 20 PAs (i.e. intermedine, lycopsamine, acetylintermedine, acetyllycopsamine, symphytine, symphytine-*N*-oxide), 17 phenolic compounds (i.e. caffeic and rosmarinic acids, rabdosiin, globoidnan A, globoidnan B) and 9 nonphenolic compounds (sugars, organic and fatty acids) were fully or partly annotated in the analysed samples. In addition, the quantitative analyses revealed that globoidnan B, rabdosiin and globoidnan A are new phenolic markers that can be used together with rosmarinic acid and PAs for the quality control of commercial comfrey root batches.

Conclusions: This study brings new insights into the phytochemical complexity of *S. officinale*, revealing not only numerous toxic PAs, but also a significant number of valuable phenolic compounds that could contribute to the bioactivities of comfrey-based preparations.

KEYWORDS

high-resolution mass spectrometry, phenolic markers, pyrrolizidine alkaloids, rosmarinic acid, *Symphytum officinale* L

1 | INTRODUCTION

Symphytum L. (comfrey, Boraginaceae) comprises more than 35 species encountered in the spontaneous flora of Eurasia and North America or cultivated within these territories. Among them, *S. officinale* L., *S. × uplandicum* Nyman, *S. asperum* Lepech, *S. tuberosum* L. and *S. caucasicum* Bieb are commonly used for their medicinal valences.^{1,2} Ethnopharmacological studies have shown that different formulations are recommended in swellings, phlebitis, contusions, gastro-duodenal ulcers, respiratory diseases and metrorrhagia, either externally (compresses, ointments) or internally (decocts, tinctures).¹ Currently, comfrey root preparations are topically used to treat bone fractures, hematomas, muscle pains and joint pains.³ Conventionally, comfrey root is acknowledged to contain polysaccharides ($\leq 30\%$), allantoin ($\leq 4.7\%$), glycopeptides and amino acids, mono- and bidesmosidic triterpene saponin glycosides and phenolic acids, such as rosmarinic ($\leq 0.2\%$), caffeic, chlorogenic and lithospermic acids.^{2,4} Allantoin, rosmarinic acid and polysaccharides are considered to be the main bioactive components, although their molecular mechanisms of action are not fully elucidated.^{1,5} Moreover, comfrey root is also reported to contain pyrrolizidine alkaloids (PAs) with 1,2-unsaturated necine ring structures (heliotridine- or retronecine-type), usually esterified at C-7 and/or C-9 with necic acids (sarracinic, trachelantic, viridifloric, tiglic, angelic acids). In plant material, they can occur either as free bases or as mixtures of free bases and their *N*-oxidized forms.^{2,6} The most common PAs in comfrey root are intermedine, 7-acetylintermedine, 7-acetylyllopsamine and lycopsamine and their *N*-oxides.⁷ Numerous PAs have been shown to exhibit hepato-, pneumo-, cyto- and genotoxic effects.⁶ Due to their high toxicity, the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA) restricted the PA dose to $\leq 1 \mu\text{g}/\text{day}$ for maximum three years, after which the intake should be decreased to $\leq 0.35 \mu\text{g}/\text{day}$.⁸ To avoid these issues, many European manufacturers either formulate comfrey root preparations from genetically altered crops that do not biosynthesize PAs or subject them to PA-depleting processes.^{5,7} However, conventional dried roots for *ex tempore* applications and nondepleted products can still be acquired from drug stores or pharmacies.²

A wide variety of analytical procedures regarding the analysis of PA-containing plant sources such as thin-layer chromatography, gas chromatography or capillary electrophoresis were employed. Currently, liquid chromatography (LC) methods coupled with mass spectrometry (MS) or, more recently, tandem mass spectrometry (MS/MS) detectors are among the most prevailing technologies.⁹ Furthermore, different LC-MS methods were investigated, such as 2D-HPLC–diode array detector (DAD) coupled with LC–multiple-mode ionization–time-of-flight (MMI-TOF)-MS, ultrahigh pressure

LC–quadrupole-TOF (UPLC-QTOF)-MS, LC-MS/MS combined with multiple-reaction mode (MRM) or hydrophilic interaction LC (HILIC) hyphenated with an electrospray ionization (ESI)-QTOF-MS detection system.^{9–12} According to the EMA, the most common technique for PA analysis is solid-phase extraction (SPE) coupled with LC-MS.⁸

In contrast to PAs that were previously subjected to detailed phytochemical evaluations, the analysis of other categories of specialized metabolites (especially phenolic compounds) in comfrey species was less explored. Conventionally, the identification of such constituents in comfrey is performed by LC-DAD. However, the annotation of non-UV-active constituents as well as that of minor compounds is practically impossible; furthermore, a very large dataset of commercial standards is required in order to perform a proper identification. These reasons might explain why most chromatographically based phytochemical studies usually reported ubiquitous metabolites, such as caffeic, rosmarinic, *p*-hydroxybenzoic or chlorogenic acids.^{2,13,14} Similar outcomes were obtained with variations of either detector (HPLC coupled with electrochemical detection)¹⁵ or separation technique (high-performance capillary electrophoresis).¹⁶ Clearly, one of the biggest breakthroughs in natural product annotation was represented by the hyphenation of LC with MS and, especially, high-resolution accurate-mass tandem mass spectrometry (HRMS/MS) which enabled mapping convoluted plant matrices at a remarkable speed and accuracy.¹⁷ State-of-the-art HRMS/MS setups include quadrupole-orbitrap-MS (Q-orbi-MS), ion trap (IT)-QTOF-MS and QTOF-MS.¹⁸ Up to date, the use of the above advanced techniques in annotating compounds (other than PAs) in comfrey root is still very scarce. For instance, D'Ursa et al.¹⁹ analyzed by LC-ESI-Orbitrap-MSⁿ a number of 20 constituents, such as allantoin, 5-hydroxymethyl-2-furfural, three fatty acids and 14 phenolic compounds, in the hydroalcoholic extract of comfrey root. Nastic et al.²⁰ annotated by HPLC-ESI-QTOF-MS 44 metabolites in various extracts of *S. officinale* root processed by maceration, accelerated solvent extraction and supercritical fluid extraction. Interestingly, more than 50% of these compounds corresponded to fatty acids, with a reduced number of phenolic acids, such as hydroxybenzoic, caffeic and sagerinic acids, as well as salvianolic acids H/I or B.²⁰

By using a state-of-the-art LC-HRMS/MS setup, the aim of this study was to perform the simultaneous analysis of PAs and phenolic compounds in the hydroethanolic extracts of 16 commercial batches of comfrey root. In addition to the well-known phytochemical markers (such as PAs and rosmarinic acid), three caffeic acid oligomers with lignan skeleton (globoidnan B, radosiin and globoidnan A) were proposed as new phenolic markers for the quality control and intersample variability assessment.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Acetonitrile (LC grade) was purchased from J.T. Baker (Deventers, the Netherlands), while formic acid was bought from VWR International GmbH (Darmstadt, Germany). Ethanol, caffeic acid and rosmarinic acid were acquired from Sigma-Aldrich (Steinheim, Germany), whereas globoidnan A, globoidnan B and radosiin (HPLC-DAD purity >95%) were previously purified from *S. officinale*.²¹ Lycopsamine, intermedine, lycopsamine *N*-oxide, intermedine *N*-oxide, acetylyllycopsamine, acetylintermedine, acetylyllycopsamine *N*-oxide, acetylintermedine *N*-oxide and echimidine-*N*-oxide were from Phytoflan (Heidelberg, Germany).

2.2 | Plant material and extraction

A number of 16 commercial batches of comfrey root were acquired from pharmacies or local drug stores across 12 different European countries (Table S1). All samples were authenticated by one of the authors (A.T.). Voucher specimens (Table S1) were stored in the Department of Pharmacognosy, Grigore T. Popa University of Medicine and Pharmacy (Iasi, Romania). Dried powdered comfrey roots (2.5 g) were processed by ultrasound-assisted extraction (three cycles of 30 min each) with 65% ethanol (3 × 25 mL) at 60°C. The filtered and pooled extracts were concentrated under reduced pressure at 40°C, frozen and then lyophilized. The yields obtained for each sample are provided in Table S1.

2.3 | Qualitative LC-HRMS/MS analysis

Qualitative LC-HRMS/MS analysis was carried out on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1312C mobile phase delivery module, G1316A column oven, G1329B auto-sampler and a G6530B accurate-mass QTOF-MS.^{22,23} A Phenomenex Gemini C18 (2 × 100 mm, 3 μm) column and a mobile phase obtained from water (A) and acetonitrile (B), both containing 0.1% formic acid and 10mM ammonium formate, were used to perform the separation. The elution gradient was: 0–45 min – 10–60% B and 46–50 min – 90–90% B; flow-rate 0.2 mL/min; injection 10 μL. MS spectra were recorded as follows: dual-spray jet stream ESI source; full-scan high-resolution accurate-mass acquisition mode; negative and positive ion modes; range 100–1000 *m/z*; gas (N₂) flow rate 12 L/min, vaporizer temperature 350°C, nebulizer pressure 40 psi, capillary voltage 4000 V, skimmer 65 V, fragmentor 140 V, octopole radio frequency (RF) peak 750 V, collision-induced dissociation (CID) 40 V. Data acquisition was achieved with MassHunter Workstation Data Acquisition 8.0 (Agilent), whilst data analysis was performed with MassHunter Qualitative Navigator 8.0 (Agilent).

2.4 | Quantitative LC-MS/MS analysis

Quantitative LC-MS/MS analysis was performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Palo Alto, CA, USA) and coupled with a QTRAP4500 MS (AB Sciex Instruments, Framingham, MA, USA) by using a previously reported method.⁷ The quantification of intermedine, lycopsamine, intermedine *N*-oxide, lycopsamine *N*-oxide, acetylintermedine, acetylyllycopsamine, acetylintermedine *N*-oxide and acetylyllycopsamine *N*-oxide in the hydroethanolic root extracts was performed on the basis of six-point (0.2–20 ng/mL) standard calibration curves. The experiments were performed in triplicate for each standard and sample. The results were expressed as mg/g dry weight (d.w.) comfrey root. Data acquisition and analysis were achieved with Analyst 1.6.2 software (AB Sciex).

2.5 | Quantitative LC-DAD analysis

Quantitative LC-DAD analysis was performed using a Prominence HPLC system from Shimadzu (Tokyo, Japan) equipped with an LC-20AB mobile phase delivery module, DGU-20A3 degasser, SIL-20A auto-sampler and a SPD-M20A DAD. A Dionex Acclaim 120 C18 (4.6 × 250 mm, 5 μm) column and a mobile phase obtained from 0.1% formic acid in water (A) and acetonitrile (B) were used to carry out the separations. The elution gradient was: 0–5 min – 10–30% B, 15–16 min – 35–95% B, 17–17.5 min – 95–10% B; post run 10 min; flow rate 1 mL/min; injection 5 μL; detection wavelength 280 nm. The quantification of globoidnan B, radosiin, rosmarinic acid and globoidnan A in the hydroethanolic root extracts was performed on the basis of nine-point (2–100 μg/mL) standard calibration curves. Triplicate injections were performed for each standard and sample. The results were expressed as mg/g d.w. comfrey root. Data acquisition and analysis were achieved with LabSolutions 5.82 (Shimadzu).

2.6 | Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test; *p* < 0.05 was considered statistically significant.

3 | RESULTS AND DISCUSSION

In this work, 16 commercially available comfrey (*S. officinale*) root batches collected from 12 different European countries were investigated in terms of their phytochemical composition by using state-of-the-art analytical setups. The first part (Section 3.1) focusses on the LC-HRMS/MS profiling of PAs (positive ion mode) and of other categories of specialized metabolites, mainly phenolic compounds (negative ion mode). Finally, to examine the intersample variability of

the main PAs and phenolic markers present in comfrey root, quantitative LC-MS/MS and LC-DAD analyses were performed (Section 3.2). To be able to compare the outcomes, each sample was processed under the same extraction conditions: ultrasound-assisted extraction, 3 cycles of 30 min each, sample to solvent ratio 10:1 (v/w). The extraction solvent (ethanol 65%) was selected following the recommendations of the HMPC available in the *Assessment Report on Symphytum Officinale L., Radix*.⁴

3.1 | LC-HRMS/MS profiling of PAs and phenolic markers

3.1.1 | LC-HRMS/MS profiling of PAs

Comfrey toxicity is closely related to PAs which exist either as free bases or as their *N*-oxide derivatives (PANOs), with the latter being the most predominant forms; thus, intermedine, lycopsamine, acetylintermedine, acetyllycopsamine, symphytine and symlandine, alongside their corresponding *N*-oxides, were commonly identified in the roots of *Symphytum* species.^{7,24–26} The qualitative profile of the PAs present in the commercial comfrey root batches from several European countries was performed by LC-HRMS/MS analysis in positive ion mode. All investigated hydroethanolic extract samples showed similar elution and ionization profiles, with typical base peak chromatogram (BPC) and extracted ion chromatograms (EICs) provided in Figure 1. The full or partial identification of this category of metabolites was achieved by comparing individual retention times, molecular formulas, pseudo-molecular and fragment ions with those of reference standards and/or literature reports, as summarized in Table 2.

Regarding the chromatographic behaviour of PAs on the C18 reversed-phase column, some general elution patterns were observed: (i) the retention of PANOs was usually slightly higher than that of their corresponding bases (similar behaviour was also observed by Mroczek et al.²⁹ and Avula et al.²⁴); (ii) the monoester PAs were less retained compared with diester PAs. Concerning the MS behaviour of PAs, their protonated $[M + H]^+$ ions allowed the assignment of the molecular formulas with a good accuracy (mass error $\Delta < 5.90$ ppm), whereas in the case of PANOs, diagnostic dimeric $[2M + H]^+$ ions were invariably noticed. Furthermore, the MS/MS fragmentation patterns of PAs from comfrey samples indicated their retronecine type, regardless of their oxidation and esterification status, with the presence of typical ion fragments at m/z 138.0960 and 120.0867. Numerous previous studies dealt with dereplication strategies for identifying PAs in *Symphytum* species using LC-MS/MS platforms.^{11,24,25,29} Therefore, for the 20 PAs observed in the current comfrey root batches (Table 1), a brief discussion regarding their annotation is presented below. Due to several common structural and spectral features, PAs were further considered with respect to their oxidation status and the number of the ester groups attached to the retronecine skeleton.

Regarding the *monoesteric* PAs, peaks 2 and 3 ($[M + H]^+$ ions at m/z 300.1789 and 300.1788, $C_{15}H_{25}NO_5$) were assigned to intermedine and lycopsamine, respectively (Figure S1A). This assumption was also validated by using reference standards. The fragment ion at m/z 156.0970 was due to the cleavage of the ester bond with the loss of a trachelanthyl/viridifloryl group ($C_7H_{12}O_3$, 144 Da), followed by the consecutive loss of two water molecules with the resulting fragments at m/z 138.0959 and m/z 120.0867.

The main *monoesteric* PANOs identified in comfrey samples were intermedine-*N*-oxide (4) and lycopsamine-*N*-oxide (5) with $[M + H]^+$

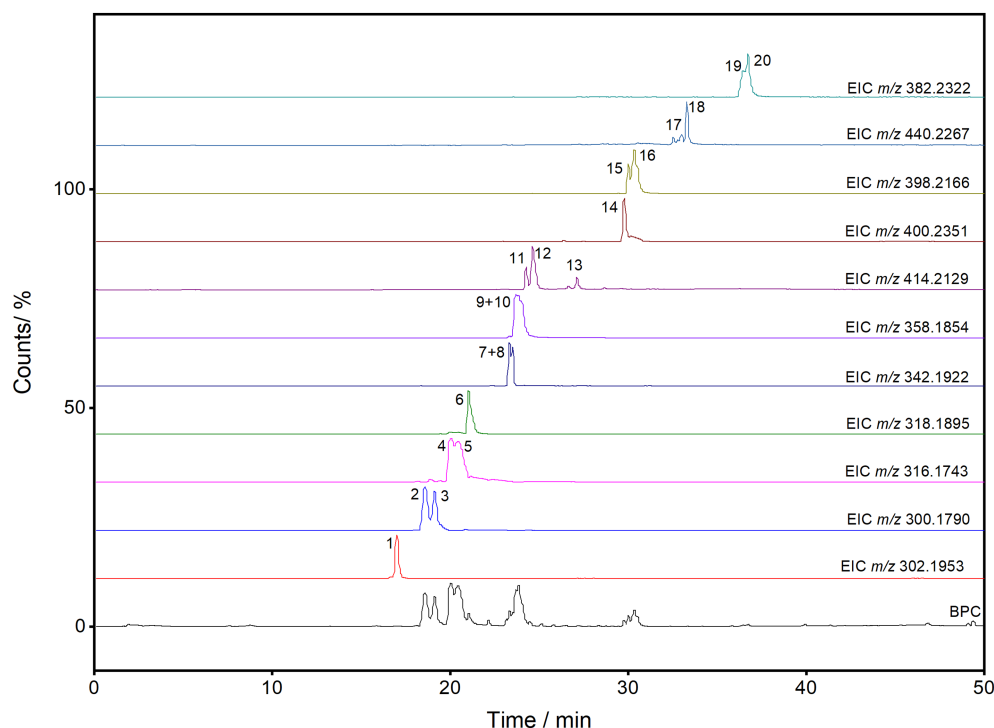


FIGURE 1 Base peak chromatogram (BPC) and extracted ion chromatograms (EICs) of pyrrolizidine alkaloids tentatively identified by liquid chromatography hyphenated with high-resolution tandem mass spectrometry (LC-HRMS/MS) (positive ion mode) in the hydroethanolic extracts obtained from commercial samples of comfrey (*Symphytum officinale* L.) root [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Chromatographic and spectral data of pyrrolizidine alkaloids tentatively identified by liquid chromatography hyphenated with high-resolution tandem mass spectrometry (LC-HRMS/MS) (positive ion mode) in the hydroethanolic extracts obtained from commercial samples of comfrey (*Symphytum officinale* L.) root

No	Proposed identity	R _t (min)	[2 M + H] ⁺ (m/z)	[M + H] ⁺ (m/z)	[M + H] ⁺ (m/z)	Δ (ppm)	MF	MS/MS (+)	Ref.
1	Unknown	17.0	605.3919	302.1953	302.1962	2.99	C ₁₅ H ₂₇ NO ₅	196.0937, 172.0932, 138.0969, 111.0748, 108.0808	-
2	Intermedine	18.6	-	300.1789	300.1805	5.51	C ₁₅ H ₂₅ NO ₅	156.0970, 138.0960, 120.0867, 112.0621, 108.0697	Std.
3	Lycopsamine	19.1	-	300.1788	300.1805	5.85	C ₁₅ H ₂₅ NO ₅	156.1089, 138.0959, 120.0896, 112.0805, 108.0826	Std.
4	Intermedine-N-oxide	20.0	631.3485	316.1753	316.1754	0.52	C ₁₅ H ₂₅ NO ₆	172.0929, 138.0889, 111.0658	Std.
5	Lycopsamine-N-oxide	20.5	631.3489	316.1767	316.1754	-3.92	C ₁₅ H ₂₅ NO ₆	172.0897, 138.0841, 111.0624	Std.
6	Dihydrointermedine-N-oxide/ dihydrolycopsamine- N-oxide (or stereoisomer)	21.0	635.3768	318.1895	318.1911	5.09	C ₁₅ H ₂₇ NO ₆	174.1021, 156.0931, 138.0850, 122.0909, 113.0741, 106.0482	27
7	7-Acetylintermedine	23.3	-	342.1921	342.1911	-2.89	C ₁₇ H ₂₇ NO ₆	198.1120, 162.0840, 138.0870, 120.0762	Std.
8	7-Acetyllycopsamine	23.5	-	342.1910	342.1911	0.33	C ₁₇ H ₂₇ NO ₆	198.1075, 138.0879, 120.0779	Std.
9	7-Acetylintermedine-N-oxide	23.7	715.3703	358.1876	358.1860	-4.40	C ₁₇ H ₂₇ NO ₇	298.1359, 214.0980, 180.0913, 154.0790, 137.0748, 120.0757, 111.0635	Std.
10	7-Acetyllycopsamine-N-oxide	23.9	715.3684	358.1865	358.1860	-1.32	C ₁₇ H ₂₇ NO ₇	298.1441, 214.1101, 180.1028, 154.0886, 137.0845, 120.0846, 111.0706	Std.
11	7-SarracinyI-9- trachelanthylretronecine-N- oxide (or stereoisomer)	24.2	827.4224	414.2132	414.2122	-2.32	C ₂₀ H ₃₁ NO ₈	270.1361, 236.1322, 220.1434, 172.0893, 137.0791, 120.0766, 118.0617, 106.0639	27
12	7-SarracinyI-9-viridiflorylretronecine- N-oxide (or stereoisomer)	24.6	827.4260	414.2140	414.2122	-4.25	C ₂₀ H ₃₁ NO ₈	270.1365, 236.1169, 220.1180, 172.0958, 154.0776, 138.0835, 136.0636, 120.0766, 106.0639	27
13	Echimidine N-oxide	27.1	827.4281	414.2133	414.2122	-2.65	C ₂₀ H ₃₁ NO ₈	254.1422, 220.1297, 172.0933, 154.0913, 136.0721, 120.0750, 106.0595	Std.
14	Unknown	29.8	799.4615	400.2351	400.2330	-5.31	C ₂₀ H ₃₃ NO ₇	298.1579, 256.1140, 172.0914, 138.0867, 111.0620	-
15	Symphytine-N-oxide (or stereoisomer)	30.0	795.4346	398.2187	398.2173	-3.51	C ₂₀ H ₃₁ NO ₇	254.1336, 220.1361, 172.0981, 154.0800, 137.0817, 136.0714, 120.0784, 106.0640	25
16	Symlandine-N-oxide (or stereoisomer)	30.3	795.4291	398.2170	398.2173	-0.75	C ₂₀ H ₃₁ NO ₇	254.1357, 220.1268, 172.0956, 154.0844, 137.0820, 136.0708, 120.0789, 106.0639	25
17	3'-Acetylsymphytine-N-oxide (or stereoisomer)	32.9	879.4467	440.2256	440.2279	5.22	C ₂₂ H ₃₃ NO ₈	398.2071, 254.1148, 220.1228, 180.0985, 172.0878, 154.0757, 138.0779, 136.0738, 120.0806, 118.0614	5
18	3'-Acetylsymlandine-N-oxide (or stereoisomer)	33.3	879.4464	440.2267	440.2279	2.72	C ₂₂ H ₃₃ NO ₈	398.2123, 254.1130, 220.1262, 180.0985, 172.0796, 137.0751, 136.0694, 120.0724, 118.0526	5
19	Symphytine (or stereoisomer)	36.5	-	382.2232	382.2224	-2.06	C ₂₀ H ₃₁ NO ₆	238.1401, 156.1035, 138.0817, 120.0767	25
20	Symlandine (or stereoisomer)	36.7	-	382.2207	382.2224	4.44	C ₂₀ H ₃₁ NO ₆	238.1454, 156.1060, 138.0803, 120.0740	25

MF, molecular formula; MS, mass spectra; R_t, retention time; Std., standard; Δ, mass error.

ions at m/z 316.1753 and 316.1767, respectively (Table 1, Figure S1B); their presence was also confirmed by standard injection. Both compounds gave similar MS/MS ion fragments, with a prominent ion at m/z 172.0929, typical for the retronecine-*N*-oxide that resulted from the cleavage of the monoester bond with the loss of trachelanthyl and viridifloryl moieties, respectively. Further, peak **6**, with $[M + H]^+$ at m/z 318.1895 was tentatively annotated as dihydrointermediate-*N*-oxide (Figure S1C) by comparison with Mroczek et al.²⁹ This peak presented a similar fragmentation pattern to intermediate-*N*-oxide but yielded fragment ions at m/z values of 2 Da higher; thus, the loss of a trachelanthyl/viridifloryl moiety (144 Da) gave the main fragment ion at m/z 174.1021, which subsequently underwent consecutive losses of two water molecules, generating the ions at m/z 156.0931 and 138.0850.

Four *diesteric* PAs were annotated in the investigated samples. Peaks **7** and **8** showing $[M + H]^+$ ions at m/z 342.1921 and m/z 342.1910, respectively, were assigned to 7-acetylintermediate and 7-acetyllycopsamine (Figure S1D) by comparison with authentic standards. Furthermore, these two peaks exhibited specific fragment ions at m/z 198.1120 (loss of trachelanthyl and viridifloryl groups, respectively) and m/z 138.0870 [loss of acetic acid ($C_2H_4O_2$, 60 Da)]. Peaks **19** and **20** were tentatively identified as symphytine and symlandine (Figure S1J) by comparison of their chromatographic and mass spectral data with those reported by Kim et al.²⁵ Both compounds showed similar $[M + H]^+$ ions at m/z 382.2232 and m/z 382.2197, respectively, and their MS/MS spectra displayed typical fragment ions at m/z 238.1401 and m/z 156.1035, resulting from the consecutive loss of viridifloryl and tiglyl/angelyl moieties (C_5H_6O , 82 Da).²⁵

Diesteric PANOs were the most predominant PAs found in the comfrey root samples. All of them shared a retronecine-*N*-oxide core, consistent with the presence in the MS/MS spectra of a characteristic ion at m/z 172.0933, alongside its derived fragment ions at m/z 154.0913, 138.0835/136.0636 and 120.0766/118.0614. Thus, by analysing their MS/MS spectra and comparing them with those of commercial standards, peaks **9** and **10** were annotated as 7-acetylintermediate-*N*-oxide and 7-acetyllycopsamine-*N*-oxide (Figure S1E), respectively. Peaks **15** and **16** shared similar $[M + H]^+$ ions at m/z 398.2187 and 398.2170, respectively, which were 16 Da greater than those of symphytine and symlandine. Both gave MS/MS fragments at m/z 254.1336 $[M-viridifloryl+H]^+$ and 172.0796 $[M-viridifloryl-tiglyl/angelyl+H]^+$. Taking into the account the MS data and literature reports,²⁵ compounds **15** and **16** were tentatively assigned to symphytine-*N*-oxide and symlandine-*N*-oxide, respectively (Figure S1H). Peaks **11** ($[M + H]^+$ at m/z 414.2132), **12** ($[M + H]^+$ at m/z 414.2140) and **13** ($[M + H]^+$ at m/z 414.2133) shared the same molecular formula ($C_{20}H_{31}NO_8$) but yielded different fragment ions in the MS/MS spectra (Table 1). Compounds **11** and **12** displayed similar fragmentation pathways, with a fragment ion at m/z 270.1361, consistent with the loss of trachelanthyl/viridifloryl moiety (144 Da), followed by the loss of a sarraciny group ($C_5H_6O_2$, 98 Da), thus yielding the fragment ion at m/z 172.0893. Therefore, by compiling the MS/MS data and comparing them with those from Mroczek et al.,²⁹ peaks **11** and **12** were putatively identified as 7-sarraciny-

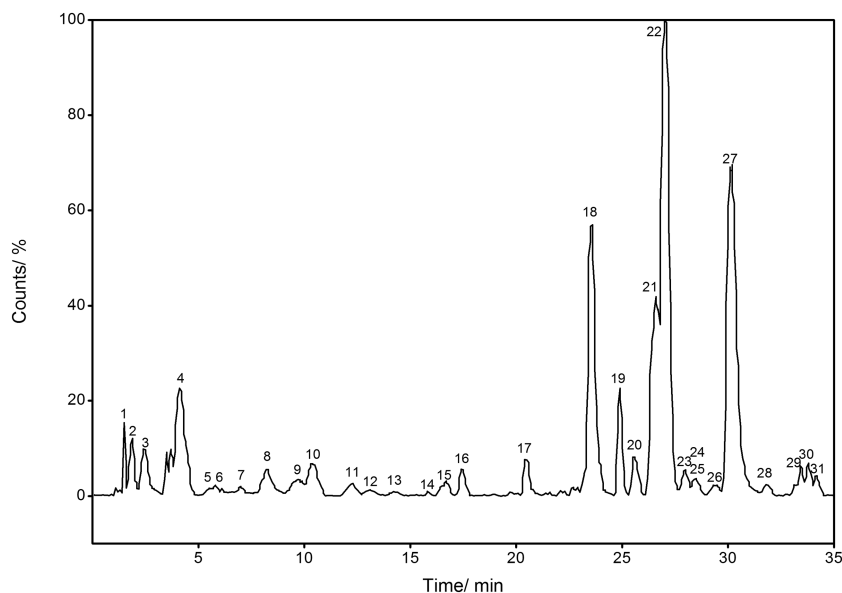
9-trachelanthylretronecine-*N*-oxide and 7-sarraciny-9-viridiflorylretronecine-*N*-oxide, respectively (Figure S1F). Showing a different fragmentation pattern, compound **13** was annotated as echimidine-*N*-oxide (Figure S1G) by comparison with the reference standard. Its MS/MS spectra showed fragment ions at m/z 254.1422 and 172.0914 corresponding to the consecutive loss of echimidinyl ($C_7H_{12}O_4$, 160 Da) and angelyl (82 Da) moieties from the protonated ion. The mass spectra of peaks **17** ($[M + H]^+$ at m/z 440.2256) and **18** ($[M + H]^+$ at m/z 440.2267) indicated the same molecular formula ($C_{22}H_{33}NO_8$). Their main MS/MS fragment ions were at m/z 398.2017 (loss of acetyl, 42 Da), 254.1148 (further loss of viridifloryl, 144 Da) and 172.0878 (further loss of tiglyl/angelyl, 82 Da). As compared with the MS data of symphytine/symlandine-*N*-oxides, an additional acetyl group was implied, which may be located on the viridifloryl/trachelanthyl ester side chain; therefore, compounds **17** and **18** were tentatively assigned to 3'-acetylsymphytine-*N*-oxide and 3'-acetylsymlandine-*N*-oxide, respectively (Figure S1I).⁵ Two peaks remained unsolved, namely peak **1** with $[M + H]^+$ at m/z 302.1953 ($C_{15}H_{27}NO_5$) and peak **14** with $[M + H]^+$ at m/z 400.2351 ($C_{20}H_{33}NO_7$). Their MS/MS fragment ions were similar to the above PAs (Table 1); moreover, the presence of fragment ions at m/z 172.0932 and 138.0969 is indicative of a retronecine-*N*-oxide core structure.

As expected, the LC-HRMS/MS profiling of the hydroethanolic comfrey root extracts obtained from batches sold on the European market revealed a significant number of PAs, with most of them, such as intermediate (**2**), lycopsamine (**3**), intermediate-*N*-oxide (**4**), lycopsamine-*N*-oxide (**5**), 7-acetylintermediate (**7**), 7-acetyllycopsamine (**8**), 7-acetylintermediate-*N*-oxide (**9**) and 7-acetyllycopsamine-*N*-oxide (**10**), being previously reported in *S. officinale*.⁶ However, it is worth mentioning that four derivatives, namely 7-sarraciny-9-trachelanthylretronecine-*N*-oxide (**11**), 7-sarraciny-9-viridiflorylretronecine-*N*-oxide (**12**), 3'-acetylsymphytine-*N*-oxide (**17**) and 3'-acetylsymlandine-*N*-oxide (**18**), were proposed as PAs with new putative structures. In addition to their uncontested recognition as toxicity markers, PAs can also serve as chemical markers for the quality control of comfrey roots, their presence certifying sample authenticity.

3.1.2 | LC-HRMS/MS profiling of phenolic markers

Since many of the biological activities of *S. officinale* preparations are linked to the presence of phenolic compounds, the phytochemical analysis of the European-marketed comfrey root batches was dubbed by the LC-HRMS/MS metabolite profiling in negative ion mode. The untargeted qualitative profile of the analysed hydroethanolic extracts is provided in Figure 2, all samples having similar elution/ionization profiles. A number of 31 peaks were annotated, with their retention times, molecular formulas, pseudo-molecular and fragment ions given in Table 2. Of these, five peaks were unidentified, whereas nine peaks were assigned to nonphenolic compounds. For instance, two sugars, sucrose (**1**) and gluconic acid (**2**), were tentatively identified by

FIGURE 2 Representative base peak chromatogram (BPC) of compounds tentatively identified by liquid chromatography hyphenated with high-resolution tandem mass spectrometry (LC-HRMS/MS) (negative ion mode) in the hydroethanolic extracts obtained from commercial samples of comfrey (*Symphytum officinale* L.) root



comparing their chromatographic and spectral data with those reported by Ammar et al.²⁷ Comfrey roots are acknowledged as a rich source of monosaccharides, oligosaccharides and, especially, polysaccharides, with the latter category reported as possessing important antioxidant, immune-modulatory, antidiabetic or antilipidemic activities.³¹ It is most likely that sucrose and gluconic acid were directly extracted from the plant material, but they could have also emerged through hydrolysis from higher-molecular-weight sugars during the extraction step. The negative ion mode LC-HRMS/MS profiling further revealed the presence of several organic acids, potentially belonging to citric acid (**3**), viridifloric acid (**7**), sarracinic acid (**8**) and trachelanthic acid (**9**). Based on their deprotonated ions $[M-H]^-$ (m/z 161.0827 and 161.0824), the same molecular formula ($C_7H_{14}O_4$) was suggested for peaks **7** and **9**, respectively. The fragment ions at m/z 135.0578 $[M-CO-H]^-$ and 117.055 $[M-CO_2-H]^-$ indicated the presence of a carboxylic group in their structure. Their tentative labelling was based on the fact that intermedine and lycopsamine are structurally retronecine esters of trachelanthic and viridifloric acids, respectively.²⁹ A similar approach was proposed for annotating sarracinic acid (**8**, $[M-H]^-$ at m/z 115.0397), a necic acid included in the structure of several PAs, such as 7-sarracinyl-9-viridiflorylretronecine.²⁹ Viridifloric, trachelanthic and sarracinic acids are reported for the first time in comfrey root; their presence as free acids could be partly explained via de-esterification from the corresponding PAs during storage or extraction steps. Roseoside (**15**, $[M-H]^-$ at m/z 385.1872), a cyclohexenone hexoside, was putatively identified by comparing its fragment ions (at m/z 223.1533 $[M-glucosyl-H]^-$, 179.0559 $[glucose-H]^-$ and 161.0420 $[glucosyl-H]^-$) with those reported by Sun et al.³⁰ Finally, two fatty acids were found in the nonpolar region of the chromatogram, namely compounds **30** ($[M-H]^-$ at m/z 329.2330) and **31** ($[M-H]^-$ at m/z 313.2380). By comparing their mass spectral data with those reported by Nastic et al.,²⁰ they were tentatively assigned as trihydroxy-octadecenoic and dihydroxy-octadecenoic acids,

respectively. These polyunsaturated fatty acids were previously reported to be present in comfrey root extracts.^{19,20}

Eventually, a number of 17 phenolic compounds were fully or partly annotated. Interestingly, all of them contained one or more carboxylic functions; in terms of their MS/MS analysis, this feature is recognized by the invariable neutral loss of CO_2 (44 Da) groups. Thus, danshensu (**6**), vanillic (**5**), hydroxybenzoic (**10**), caffeic (**16**) and rosmarinic (**22**) acids were easily annotated by comparing their chromatographic and spectral data with those reported in the literature.^{20,27,28} Furthermore, the presence of caffeic and rosmarinic acids, which are already very well-known constituents of comfrey root,⁷ was confirmed by standard injection. Three hexoside derivatives of hydroxybenzoic (**4**), caffeic (**14**) and rosmarinic (**19**) acids were also tentatively identified (Figure S2A-D). In addition to the decarboxylated fragment ions derived from the corresponding free acids, the loss of glucosyl (162 Da) group was specifically observed in the MS/MS spectra of these three derivatives (Table 2).

Next, a series of caffeic acid oligomers with lignan skeleton were unequivocally identified as globoidnan B (**18**), radosiin (**21**) and globoidnan A (**27**) by comparing their chromatographic and spectral data with those obtained after standard injections and with available literature reports.^{19,28,32} These derivatives showed similar fragmentation patterns, summarized as follows: after the neutral losses of caffeic acid (180 Da) or danshensu (198 Da), the lignan-based fragment ions underwent sequential elimination of the corresponding number of carboxyl groups present in their structures. For instance, globoidnan B (**18**, $[M-H]^-$ at m/z 537.1018, $C_{27}H_{22}O_{12}$) afforded fragment ions at m/z 493.1152 $[M-CO_2-H]^-$, 357.0512 $[M-C_9H_8O_4-H]^-$, 339.0492 $[M-C_9H_{10}O_5-H]^-$, 313.0703 $[M-C_9H_8O_4-CO_2-H]^-$, 295.0547 $[M-C_9H_{10}O_5-CO_2-H]^-$ and 269.0791 $[M-C_9H_8O_4-2 \times CO_2-H]^-$ (Figure S2F). As compared with globoidnan B, the $[M-H]^-$ ion at m/z 717.1474 of compound **21** (radosiin, $C_{36}H_{30}O_{16}$) indicated the

TABLE 2 Chromatographic and spectral data of compounds tentatively identified by liquid chromatography hyphenated with high-resolution tandem mass spectrometry (LC-HRMS/MS) (negative ion mode) in hydroethanolic extracts obtained from commercial samples of comfrey (*Symphytum officinale* L.) root

No	Proposed identity	R _t (min)	[M-H] ⁻ _{exp} (m/z)	[M-H] ⁻ _{calcd} (m/z)	Δ (ppm)	MF	MS/MS (-) (m/z)	Ref
1	Sucrose	1.5	341.1105	341.1089	-4.57	C ₁₂ H ₂₂ O ₁₁	179.0582, 119.0329	27
2	Gluconic acid	1.9	195.0515	195.0510	-2.42	C ₆ H ₁₂ O ₇	129.0531	27
3	Citric acid	2.4	191.0199	191.0197	-0.91	C ₆ H ₈ O ₇	129.0198, 111.0100	20
4	Hydroxybenzoic acid hexoside	4.1	299.0765	299.0772	2.47	C ₁₃ H ₁₆ O ₈	239.0432, 209.0216, 179.0240, 137.0283	20
5	Vanillic acid	5.4	167.0348	167.0350	1.08	C ₈ H ₈ O ₄	149.0250, 123.0479, 108.0203	27
6	Danshensu	5.8	197.0449	197.0455	3.27	C ₉ H ₁₀ O ₅	179.0377, 135.0449, 123.0463	28
7	Viridifloric acid	8.2	161.0827	161.0819	-4.74	C ₇ H ₁₄ O ₄	135.0578, 117.055	29
8	Sarracinic acid	7.0	115.0397	115.0401	3.17	C ₅ H ₈ O ₃	-	29
9	Trachelanthic acid	9.7	161.0824	161.0819	-2.88	C ₇ H ₁₄ O ₄	135.0578, 117.055	29
10	Hydroxybenzoic acid	10.3	137.0249	137.0244	-3.49	C ₇ H ₆ O ₃	111.0023	20
11	Unknown	12.2	323.1343	323.1348	1.41	C ₁₃ H ₂₄ O ₉	257.1031, 179.0505, 119.0387	-
12	Unknown	13.2	365.1260	365.1242	-4.94	C ₁₈ H ₂₂ O ₈	347.1115, 203.0370, 177.0210, 161.0785	-
13	Unknown	14.3	365.1256	365.1242	-3.85	C ₁₈ H ₂₂ O ₈	347.1115, 203.0370, 177.0210, 161.0785	-
14	Caffeic acid hexoside	15.7	341.0885	341.0878	-2.03	C ₁₅ H ₁₈ O ₉	179.0347, 135.0478	27
15	Roseoside	16.8	385.1872	385.1868	-1.06	C ₁₉ H ₃₀ O ₈	223.1533, 179.0559, 161.0420, 153.0928	30
16	Caffeic acid	17.4	179.0343	179.0350	3.79	C ₉ H ₈ O ₄	161.0484, 135.0435	Std.
17	Unknown	20.4	436.2228	-	-	-	316.1627, 259.1278, 245.1291, 119.0475	-
18	Globoidnan B	23.5	537.1018	537.1038	3.81	C ₂₇ H ₂₂ O ₁₂	493.1152, 357.0512, 339.0492, 313.0703, 295.0547, 269.0791, 197.0415, 179.0317	Std.
19	Rosmarinic acid hexoside	24.9	521.1315	521.1301	-2.75	C ₂₄ H ₂₆ O ₁₃	359.0952, 197.0465, 161.0465, 135.0475	-
20	3-Carboxy-6,7-dihydroxy-1-(3',4'-dihydroxyphenyl)-naphthalene	25.6	311.0557	311.0561	1.32	C ₁₇ H ₁₂ O ₆	267.0640, 239.0684, 209.0650, 197.0589, 175.0500, 159.0393	19
21	Rabdosiin	26.6	717.1474	717.1461	-1.81	C ₃₆ H ₃₀ O ₁₆	537.1003, 519.0935, 475.1023, 365.0681, 339.0518, 243.0283, 197.0435	Std.
22	Rosmarinic acid	27.0	359.0780	359.0772	-2.11	C ₁₈ H ₁₆ O ₈	197.0454; 179.0344; 161.0248; 135.0398	Std.
23	Rabdosiin isomer	28.0	717.1459	717.1461	0.29	C ₃₆ H ₃₀ O ₁₆	537.1003, 519.0935, 475.1023, 365.0681, 339.0518, 243.0283, 197.0435	-
24	Dihydrogloboidnan A	28.5	493.1132	493.1140	1.66	C ₂₆ H ₂₂ O ₁₀	295.0616, 185.0269	-
25	Dihydroxarabdoisin	28.6	719.1619	719.1618	-0.21	C ₃₆ H ₃₂ O ₁₆	539.1140, 359.0687, 297.0795, 271.1022, 243.0620, 197.0450, 161.0562	-
26	Dehydrorabdosiin	29.4	715.1308	715.1305	1.62	C ₃₆ H ₂₈ O ₁₆	517.0991, 337.0390	-
27	Globoidnan A	30.1	491.1001	491.0984	-3.51	C ₂₆ H ₂₀ O ₁₀	311.0562, 267.0663, 197.0468, 179.0317, 135.0461	Std.
28	Hydroxygloboidnan A	31.8	507.0947	507.0933	-2.78	C ₂₆ H ₂₀ O ₁₁	327.0507, 309.0373, 283.0615, 253.0494, 197.0434	-

TABLE 2 (Continued)

No	Proposed identity	R _t (min)	[M-H] ⁻ _{exp} (m/z)	[M-H] ⁻ _{calcd} (m/z)	Δ (ppm)	MF	MS/MS (-) (m/z)	Ref
29	Unknown	33.5	417.2130	417.2130	0.01	C ₂₀ H ₃₄ O ₉	345.1555, 327.1457, 217.1075	-
30	Trihydroxy-octadecenoic acid	33.8	329.2330	329.2333	1.05	C ₁₈ H ₃₄ O ₅	229.1437; 211.1333; 199.1259	20
31	Dihydroxy-octadecenoic acid	34.8	313.2380	313.2384	1.28	C ₁₈ H ₃₂ O ₄	201.1187; 127.1105	20

MF, molecular formula; MS, mass spectra; R_t, retention time; Std., standard; Δ, mass error.

presence of an additional danshensu unit, whereas that at *m/z* 491.1001 of compound **27** (globoidnan A, C₂₆H₂₀O₁₀) pointed out the lack of a carboxyl group (Figure S2G). Based on the above observations, all the remaining phenolic compounds were tentatively annotated as congeners of these lignan derivatives (Figure S2E, G-L). In comparison with rabdosiin, the molecular formula (C₃₆H₃₂O₁₆) of peak **25** ([M-H]⁻ at *m/z* 719.1619) indicated a lower double-bond equivalent (DBE) value (DBE-1) that could appear after a formal hydrogen addition, possibly leading to a dihydrorabdosiin-based structure (Figure S2H). On the contrary, compound **26** ([M-H]⁻ at *m/z* 715.1308, C₃₆H₂₈O₁₆) with DBE+1 (as compared with rabdosiin) suggested an additional carbon-carbon double bond in the lignan structural unit, tentatively labelled as dehydrorabdosiin (Figure S2I). Similarly, two derivatives of globoidnan A were proposed. Peak **24** ([M-H]⁻ at *m/z* 493.1132, C₂₆H₂₂O₁₆) with DBE-1 was annotated as dihydrogloboidnan A (Figure S2J). Finally, the molecular formula (C₂₆H₂₀O₁₆) of peak **28** ([M-H]⁻ at *m/z* 507.0947) indicated the additional presence of a hydroxyl group attached to the structure of globoidnan A (Figure S2L). Interestingly, the fragment ions observed in MS/MS spectra of the last four lignan derivatives showed typical fragmentation patterns as those described for the three congeners confirmed by standards.

However, it is worth mentioning that the molecular formulas and spectral data of the above caffeic acid oligomers resemble those of several salvianolic acids. For example, globoidnan B shares the same mass and MS/MS fragment ion patterns as lithospermic acid A or salvianolic acids H and I, while rabdosiin is isomer with salvianolic acid B.^{20,28} In the absence of appropriate standards, these compounds can be mislabelled, as the experimental MS conditions do not allow an efficient dereplication. Nevertheless, despite the above technique-related limitations, *S. officinale* can be considered an important source of phenolic compounds.

3.2 | Quantification of PAs and phenolic markers

3.2.1 | LC-MS/MS quantification of PAs

To investigate the quantitative profile of the PAs among the 16 comfrey root samples, LC-MS/MS analyses were next performed. As presented in Table 3, intermedine, lycopsamine and their *N*-oxides alongside acetylintermedine, acetyllycopsamine and their *N*-oxides were included as quantitative markers for all comfrey batches. PANOs were found in a higher amount than their free base forms. Intermedine *N*-oxide and lycopsamine *N*-oxide showed a similar variability profile. Thus, SO15 displayed the lowest contents of intermedine *N*-oxide (0.200 mg/g d.w. plant material) and lycopsamine *N*-oxide (0.241 mg/g); meanwhile, the highest amounts of the two PANOs were found in SO1 (1.692 mg/g and 1.873 mg/g, respectively). Acetylintermedine *N*-oxide and acetyllycopsamine *N*-oxide (quantified as sum of the two stereoisomers) ranged between 0.121 mg/g (SO9) and 1.899 mg/g (SO15). Intermedine and lycopsamine were found in traces, displaying comparable values; the content of intermedine

TABLE 3 Contents of the four major pyrrolizidine alkaloids markers in commercial samples of comfrey (*Symphytum officinale* L.) root

Sample	Content (mg/g d.w. comfrey root)						Σ_{PAs}
	Intermedine	Lycopsamine	Acetylintermedine + acetylyllopsamine ^a	Intermedine-N-oxide	Lycopsamine-N-oxide	Acetylintermedine-N-oxide + acetylyllopsamine-N-oxide ^a	
SO1	0.058 ± 0.003 ^a	0.053 ± 0.010 ^a	0.067 ± 0.017 ^a	1.692 ± 0.295 ^a	1.873 ± 0.346 ^a	1.272 ± 0.215 ^a	5.015 ± 0.877 ^a
SO2	0.030 ± 0.002 ^b	0.028 ± 0.001 ^b	0.061 ± 0.002 ^a	0.806 ± 0.031 ^b	1.205 ± 0.036 ^b	1.723 ± 0.032 ^b	3.852 ± 0.063 ^b
SO3	0.019 ± 0.001 ^c	0.019 ± 0.001 ^c	0.089 ± 0.002 ^b	0.406 ± 0.017 ^c	0.576 ± 0.024 ^c	1.494 ± 0.012 ^c	2.603 ± 0.039 ^{c,e}
SO4	0.023 ± 0.002 ^c	0.030 ± 0.002 ^b	0.057 ± 0.001 ^a	0.399 ± 0.007 ^c	0.747 ± 0.016 ^c	0.967 ± 0.023 ^d	2.223 ± 0.016 ^c
SO5	0.039 ± 0.001 ^d	0.033 ± 0.002 ^b	0.023 ± 0.001 ^c	0.498 ± 0.011 ^{c,d}	0.699 ± 0.021 ^c	0.326 ± 0.006 ^e	1.618 ± 0.014 ^d
SO6	0.010 ± 0.001 ^e	0.006 ± 0.001 ^d	0.053 ± 0.002 ^a	0.368 ± 0.015 ^c	0.333 ± 0.008 ^d	1.172 ± 0.028 ^a	1.942 ± 0.042 ^{d,e}
SO7	0.020 ± 0.001 ^c	0.015 ± 0.001 ^c	0.040 ± 0.001 ^d	0.444 ± 0.021 ^c	0.528 ± 0.016 ^c	0.477 ± 0.016 ^e	1.524 ± 0.039 ^d
SO8	0.033 ± 0.001 ^b	0.024 ± 0.001 ^b	0.055 ± 0.001 ^a	0.669 ± 0.024 ^{b,d}	0.759 ± 0.087 ^c	0.751 ± 0.091 ^f	2.292 ± 0.175 ^{c,e}
SO9	0.089 ± 0.003 ^f	0.118 ± 0.004 ^e	0.019 ± 0.001 ^c	0.624 ± 0.036 ^{b,d}	1.132 ± 0.013 ^b	0.121 ± 0.004 ^g	2.103 ± 0.055 ^{c,d,e}
SO10	0.040 ± 0.001 ^d	0.003 ± 0.001 ^b	0.037 ± 0.001 ^d	0.730 ± 0.030 ^b	0.803 ± 0.022 ^{c,e}	0.550 ± 0.004 ^e	2.191 ± 0.035 ^{c,d,e}
SO11	0.008 ± 0.001 ^e	0.015 ± 0.001 ^c	0.033 ± 0.001 ^d	0.320 ± 0.010 ^c	0.868 ± 0.038 ^c	0.982 ± 0.023 ^d	2.227 ± 0.048 ^{c,d,e}
SO12	0.015 ± 0.001 ^g	0.015 ± 0.001 ^c	0.122 ± 0.003 ^f	0.421 ± 0.008 ^c	0.586 ± 0.030 ^c	2.673 ± 0.040 ^h	3.831 ± 0.015 ^b
SO13	0.100 ± 0.002 ^h	0.054 ± 0.001 ^a	0.069 ± 0.002 ^a	1.266 ± 0.029 ^e	0.987 ± 0.031 ^{b,e}	0.819 ± 0.021 ^d	3.294 ± 0.051 ^{b,f}
SO14	0.047 ± 0.003 ⁱ	0.045 ± 0.003 ^f	0.059 ± 0.003 ^a	0.804 ± 0.014 ^b	1.129 ± 0.043 ^b	1.090 ± 0.020 ^a	3.174 ± 0.044 ^f
SO15	0.009 ± 0.001 ^e	0.008 ± 0.001 ^d	0.151 ± 0.003 ^g	0.200 ± 0.005 ^f	0.241 ± 0.006 ^d	1.899 ± 0.052 ^b	2.507 ± 0.005 ^{c,d,e}
SO16	0.020 ± 0.001 ^c	0.022 ± 0.001 ^b	0.082 ± 0.001 ^b	0.443 ± 0.010 ^c	0.799 ± 0.019 ^c	1.822 ± 0.017 ^b	3.187 ± 0.002 ^f

Data are presented as mean ± standard deviation (SD) of three determinations; values sharing different superscripts within columns are significantly different at $p < 0.05$ (Tukey's test).

^a Quantified as sum of stereoisomers; PAs, pyrrolizidine alkaloids.

varied between 0.009 mg/g (SO15) and 0.100 mg/g (SO13), whereas that of lycopsamine ranged from 0.003 mg/g (SO10) to 0.118 mg/g (SO9). The amounts of acetylintermedine and acetyllycopsamine (quantified as sum of the two stereoisomers) varied from 0.019 mg/g (SO9) up to 0.151 mg/g (SO15). Overall, the total PA content (obtained by adding the values of the individual PAs) showed a great variability: three samples (SO5, SO6 and SO7) contained below 2.0 mg/g, seven samples (SO3, SO4, SO8–SO11 and SO15) between 2.0 and 3.0 mg/g, five samples (SO2, SO12–SO14 and SO16) between 3.0 and 4.0 mg/g and one sample (SO1) had values higher than 5.0 mg/g.

Our results are in agreement with previous reports on PA content in *S. officinale*. By using the same analytical method, we have recently shown that total PAs accounted for 2.4 mg/g in a Swiss commercial comfrey root batch, with acetylintermedine (0.954 mg/g) followed by acetyllycopsamine (0.793 mg/g), acetylintermedine-*N*-oxide (0.254 mg/g), acetyllycopsamine-*N*-oxide (0.271 mg/g), intermedine (0.037 mg/g), lycopsamine (0.037 mg/g), lycopsamine-*N*-oxide (0.008 mg/g) and intermedine-*N*-oxide (0.007 mg/g).⁷ The levels of intermedine and lycopsamine in a German root specimen varied between 0.218 and 0.483 mg/g and 0.096 and 0.249 mg/g, respectively, depending on the extraction temperature (50–125°C) and additive (phosphoric acid, ammonia, sulfuric acid, acetic acid, formic acid) included in the aqueous extraction solvent.³³ The same two PA markers were quantified by Avula et al.,²⁴ with their levels up to maximum 0.015 mg/g. Liu et al.²⁶ showed that lycopsamine levels in a Colombian comfrey root specimen were between 0.010 and 0.014 mg/g in a pressurized hot water extract and between 0.029 and 0.032 mg/g in a methanol/water (1/1) extract obtained under reflux.

3.2.2 | LC-DAD quantification of major phenolic markers

To investigate the variability profile of the phenolic compounds among the 16 comfrey root samples, quantitative LC-DAD analyses were next performed. As depicted in Figure 3, all analysed samples were invariably characterized by the presence of globoidnan B ($t_R = 8.7$ min), rabdosiin ($t_R = 9.7$ min), rosmarinic acid ($t_R = 11.2$ min) and globoidnan A ($t_R = 13.3$ min) as the major markers. The amounts of globoidnan B varied from 0.135 mg/g d.w. comfrey root (SO9) to 0.994 mg/g (SO13) (Table 4). Comparable values ranging from 0.142 mg/g (SO9) to 0.887 mg/g (SO12) were obtained for rabdosiin. Rosmarinic acid and globoidnan A showed even higher-variability profiles; the lowest concentrations of these two markers were observed in SO9, with 0.261 mg/g and 0.129 mg/g, respectively (Table 4). However, rosmarinic acid reached its highest concentrations in SO12 and SO4, with 1.945 mg/g and 1.944 mg/g, respectively, whereas the highest amount of globoidnan A was attained in SO2 (1.935 mg/g). After adding the total amounts of these four major phenolic markers, it was observed that SO2, SO3, SO4, SO12 and SO13 showed very high values (>4.4 mg/g), whilst SO7 and SO9 exhibited values below 1.0 mg/g.

The high variability of the concentration of rosmarinic acid in the analysed samples makes the comparison with previous literature data quite difficult. Trifan et al.⁷ reported 1.77 mg rosmarinic acid/g in a comfrey root batch commercialized in Switzerland and extracted with 65% ethanol. Sowa et al.¹³ showed a higher concentration (1.85 mg/g) in the roots of specimens collected from a Polish botanical garden and extracted with 50% ethanol. However, when comfrey roots obtained from the same botanical garden were extracted with decreasing concentrations of methanol (100–60%), rosmarinic acid

FIGURE 3 Liquid chromatography–diode array detector (LC-DAD) chromatograms of major phenolic markers quantified in the hydroethanolic extracts obtained from commercial samples of comfrey (*Symphytum officinale* L.) root. Phenolic markers: Globoidnan B (GB, $t_R = 8.7$ min), rabdosiin (RB, $t_R = 9.7$ min), rosmarinic acid (RA, $t_R = 11.2$ min) and globoidnan a (GA, $t_R = 13.3$ min) [Colour figure can be viewed at wileyonlinelibrary.com]

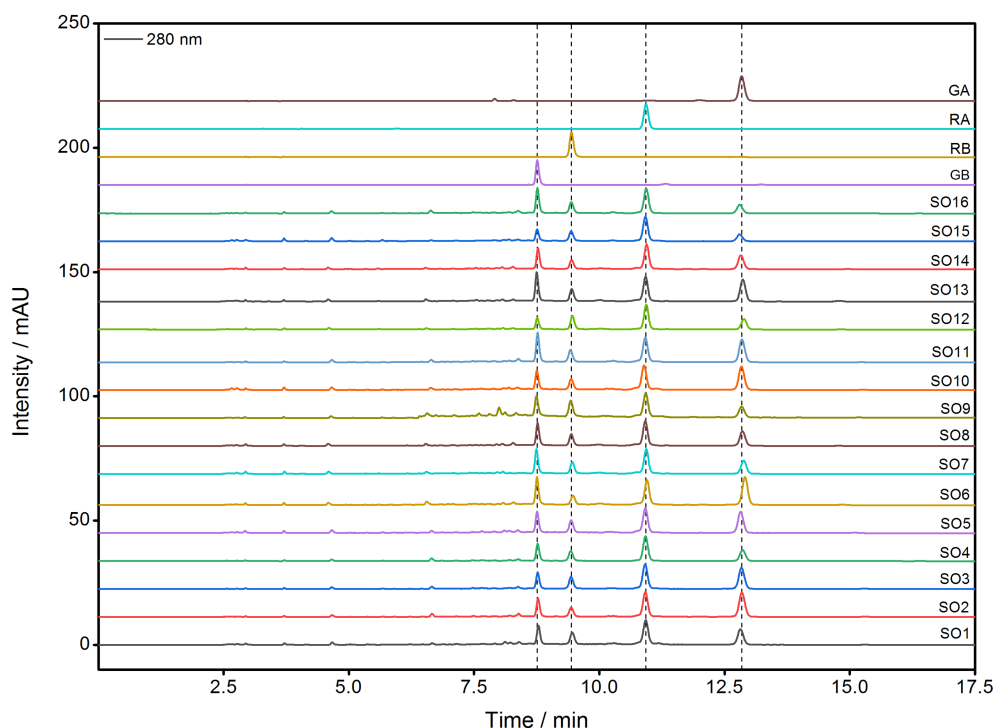


TABLE 4 Contents of the four major phenolic markers in commercial samples of comfrey (*Symphytum officinale* L.) root

Sample	Content (mg/g d.w. comfrey root)				
	Globoidnan B	Rabdosiin	Rosmarinic acid	Globoidnan A	ΣPhenolics
SO1	0.520 ± 0.008 ^a	0.466 ± 0.008 ^a	1.133 ± 0.008 ^a	0.864 ± 0.043 ^a	2.983 ± 0.048 ^a
SO2	0.720 ± 0.024 ^b	0.508 ± 0.014 ^b	1.599 ± 0.058 ^b	1.935 ± 0.086 ^b	4.763 ± 0.164 ^b
SO3	0.599 ± 0.064 ^a	0.663 ± 0.011 ^c	1.650 ± 0.041 ^b	1.650 ± 0.026 ^c	4.561 ± 0.030 ^c
SO4	0.843 ± 0.051 ^c	0.676 ± 0.019 ^c	1.944 ± 0.026 ^c	1.027 ± 0.022 ^d	4.491 ± 0.019 ^c
SO5	0.325 ± 0.020 ^d	0.275 ± 0.007 ^d	0.681 ± 0.007 ^d	0.691 ± 0.040 ^e	1.973 ± 0.034 ^d
SO6	0.351 ± 0.015 ^{d,f}	0.156 ± 0.002 ^e	0.539 ± 0.024 ^e	0.707 ± 0.026 ^e	2.139 ± 0.019 ^e
SO7	0.180 ± 0.014 ^e	0.149 ± 0.002 ^e	0.393 ± 0.010 ^f	0.267 ± 0.019 ^f	0.988 ± 0.007 ^f
SO8	0.438 ± 0.010 ^{a,f}	0.292 ± 0.008 ^d	0.797 ± 0.031 ^g	0.564 ± 0.040 ^e	2.091 ± 0.031 ^{d,e}
SO9	0.135 ± 0.003 ^e	0.142 ± 0.007 ^e	0.261 ± 0.017 ^h	0.129 ± 0.005 ^f	0.668 ± 0.011 ^g
SO10	0.301 ± 0.005 ^d	0.239 ± 0.006 ^f	0.673 ± 0.027 ^d	0.749 ± 0.029 ^{a,e}	1.961 ± 0.057 ^d
SO11	0.466 ± 0.055 ^a	0.291 ± 0.002 ^d	0.723 ± 0.004 ^d	0.858 ± 0.082 ^{a,e}	2.338 ± 0.025 ^h
SO12	0.549 ± 0.014 ^a	0.887 ± 0.003 ^g	1.945 ± 0.006 ^c	1.027 ± 0.058 ^d	4.409 ± 0.044 ^c
SO13	0.994 ± 0.009 ^g	0.551 ± 0.011 ^h	1.447 ± 0.047 ⁱ	1.457 ± 0.049 ^g	4.449 ± 0.005 ^c
SO14	0.505 ± 0.013 ^a	0.293 ± 0.008 ^d	1.001 ± 0.004 ^j	0.672 ± 0.025 ^e	2.417 ± 0.018 ^h
SO15	0.192 ± 0.025 ^e	0.255 ± 0.006 ^{d,f}	0.750 ± 0.003 ^{d,g}	0.288 ± 0.048 ^f	1.484 ± 0.002 ⁱ
SO16	0.589 ± 0.070 ^a	0.390 ± 0.008 ⁱ	1.059 ± 0.030 ^{a,j}	0.523 ± 0.095 ^e	2.561 ± 0.059 ^h

Data are presented as mean ± standard deviation (SD) of three determinations; values sharing different superscripts within columns are significantly different at $p < 0.05$ (Tukey's test)

was found in even higher amounts (7.10 mg/g).¹⁶ A concentration of 74.77 µg/mL (equivalent to 0.37 mg/g plant material) was shown in a water/glycerol/ethanol 1/1/1 (v/v/v) extract obtained from the roots of a comfrey specimen collected from the spontaneous flora of Romania.¹⁴ Savic et al.³⁴ reported 12.80% rosmarinic acid (corresponding to 8.06 mg/g plant material) in the water extract of roots provided by a Serbian institute for medicinal plants.

In contrast to rosmarinic acid, which is a well-acknowledged comfrey root constituent, globoidnan B, rabdosiin and globoidnan A were scarcely previously reported in *S. officinale*.^{16,21} Since they were invariably shown in significant amounts in all comfrey root samples commercialized within several European countries, they are proposed as new phenolic markers for their quality control. These three caffeic acid oligomers were found to be endowed with interesting biological activities. In addition to their recently proven anti-inflammatory potential,^{19,21,32} rabdosiin, globoidnans A and B were already claimed as possessing antioxidant,³⁵ neuroprotective³⁶ or anti-HIV^{37,38} activities. Nevertheless, due to the presence of toxic PAs, the use of comfrey root for novel bioactivities still remains questionable. PA-depleted extracts (which might become mandatory in all European comfrey pharmaceutical/food products) or preparations containing purified constituents, alone or combined in bioactive formulas, could represent a starting point for extending the valences of *S. officinale*, a traditional anti-inflammatory plant remedy, to new pharmacological applications.

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ORCID

Simon Vlad Luca  <https://orcid.org/0000-0003-0904-0060>

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