Tandem Mass Spectrometric Analysis of a Complex Triterpene Saponin Mixture of *Chenopodium quinoa*

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A nano-HPLC electrospray ionization multi-stage tandem mass spectrometry (nLC-ESI-MS/MS) approach was applied to a complex crude triterpene saponin extract of *Chenopodium quinoa* seed coats. In ESI-MS/MS spectra of triterpene saponins, characteristic fragmentation reactions are observed and allow the determination of aglycones, saccharide sequences, compositions, and branching. Fragmentation of aglycones provided further structural information. The chemical complexity of the mixture was resolved by a complete profiling. Eighty-seven triterpene saponins comprising 19 reported and 68 novel components were identified and studied by MS. In addition to four reported, five novel triterpene aglycones were detected and characterized according to their fragmentation reactions in ESI-MS/MS and electron ionization mass spectrometry (EI-MS). As a novelty fragmentation pathways were proposed and analyzed based upon quantum chemical calculations using a hybrid Hartree-Fock density functional method. Accuracy of the assignment procedure was proven by isolation and structure determination of a novel compound. As the relative distribution and composition of saponins varies between different cultivars and soils, the presented strategy allows a rapid and complete analysis of *Chenopodium quinoa* saponin distribution and composition, and is particularly suitable for quality control and screening of extracts designated for pharmaceutical, agricultural, and industrial applications. (J Am Soc Mass Spectrom 2006, 17, 795–806) © 2006 American Society for Mass Spectrometry

C*henopodium quinoa* belongs to the plant family *Chenopodiaceae* and is widespread in South America [1]. The main field of usage is in nutrition, for which all parts of the plant are utilized. The seeds are outstanding because of their high protein quality and content [2, 3]. However, they comprise triterpene saponins, which require special treatment before usage for nutritional purposes. Saponins are widespread in nature and represent a family of biochemically and pharmaceutically highly interesting compounds. Investigations on the biological and pharmacological activities of *C. quinoa* saponins gave toxicity to shrimps, inhibition of fungus growth, effects against viral diseases, cholesterol lowering effects, and an enhancing of mucosal drug absorption [4–7]. Although the above properties are adverse for nutrition, saponin extracts of *C. quinoa* are used in agriculture for treatment, control, and prevention of fungal and viral diseases [4]. In pharmaceutical compositions they act as immunological and absorption adjuvants to enhance immune responses and mucosal absorption to a substance coadministered therewith [8]. These applications require quality control of the saponin extract as the relative distribution and composition of saponins varies between different cultivars and soils.

Saponins from *C. quinoa* were previously studied. Overall, four different aglycones, four monodesmosidic, and twenty-two bidesmosidic triterpene saponins are reported. The corresponding aglycones are oleanolic acid (OA), hederagenin (Hed), phytolaccagenic acid (PA), and serjanic acid (SA), all of them deriving from β-amyrin (Scheme 1). Sugars can be linked to the aglycone at the C-3 and C-28. Among the major saccharides glucose (Glc), galactose (Gal), and arabinose (Ara), glucuronic acid (GlcA) and xylose (Xyl) were found to be less common [9–16].

The recent developments in the field of mass spectrometry-related hardware, especially electrospray ionization multi-stage tandem mass spectrometry (ESI-MSⁿ) and nano-HPLC (nLC), opened up the door to natural product chemistry. The coupling of ESI-MSⁿ with nano-HPLC (nLC) allows studying saponin molecular species, aglycones, the saccharide sequence, mod-
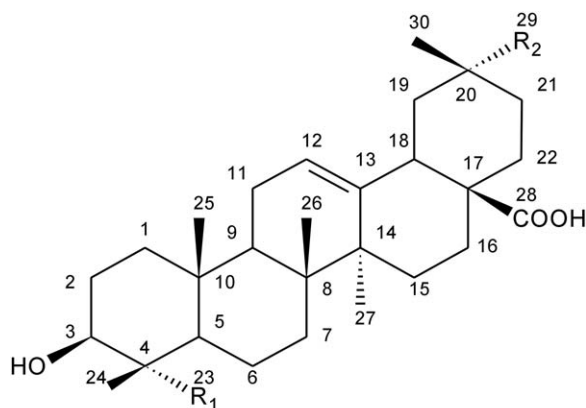
Scheme 1. Aglycones of reported *C. quinoa* triterpene saponins.

ification, branching [17–30], and deals with relative low amounts of sample, respectively. Due to the short analysis time (LC)-MS^n analysis was recommended prior the common way of natural product identification [20]. However, previous studies mainly focused on the analysis of known saponins rather than identification and complete profiling of novel compounds.

Here, we present the application of an nLC-MS/MS approach to the complex crude saponin extract of *C. quinoa* to resolve the chemical complexity by a complete profiling. Nineteen reported and more than 70 previously unknown components were identified and assigned according to their aglycones, saccharide sequences, compositions, and branching. A total number of nine different aglycones comprising the four aglycones mentioned above and five new aglycones were detected. To the best of our knowledge this is one of the most comprehensive assignments of saponins ever attempted. As an additional novelty fragmentation pathways were proposed based upon quantum chemical calculations using a hybrid Hartree-Fock density functional method. The presented strategy allows a rapid and complete analysis of saponin distribution and composition in *C. quinoa* and is particularly suitable for screening extracts designated for pharmaceutical, agricultural and industrial applications.

Experimental

Plant Material

C. quinoa seed coats were collected from *C. quinoa* plants cultivated in lower Austria (Leopold Posch, Obergrafendorf, Lower Austria, Austria).

Extraction and Purification of Chemical Constituents

Seed hulls, 20.0 g, were treated with 300 ml water/ ethanol (1:1), stirred for 3.5 h at 45 °C and centrifuged. The water/ethanol phase was decanted, the seed hulls were washed with 100 ml water/ethanol, centrifuged, decanted, and ethanol was removed under reduced pressure; 100 ml of distilled water were added to the water phase. Extraction of the saponins was performed with 100 ml 1-butanol each. The 1-butanol layers were merged, centrifuged, and lyophilized [yellowish product; yield: 9.2% (wt/wt)]. The saponins were further purified using silica gel column chromatography [150 g, chloroform/methanol/water (65/35/10, vol/vol/vol) used as eluent]. Five g crude saponin mixture obtained from several extractions under identical conditions was applied to the column. Fractions of 20 ml were collected and monitored by TLC [precoated plates, silica gel 60 F$_{254}$, 0.2 mm, Merck, Darmstadt, Germany; chloroform/ methanol/water (65/35/10, vol/vol/vol) as eluent] as well as by LC-MS. Visualization of the saponins was performed by immersion of the TLC plate into methanol/glacial acetic acid/sulphuric acid/anisaldehyde (170/20/10/1, vol/vol/vol/vol) followed by heating the TLC plate (100 °C, 10 min). Fractions containing a novel saponin were merged and the solvent was removed (yellowish residue; yield 1.30 g). This purified residue was dissolved in chloroform/methanol/water (65/35/10, vol/vol/vol), applied to the column and rechromatographed as described above. Fractions containing the compound were merged and the solvent was removed (colorless residue; yield: 180 mg). Further purification of the saponins was done with semi-preparative HPLC on a Hewlett Packard (HP) 1100 Series HPLC system equipped with a variable wavelength detector set at 220 nm (HP, Palo Alto, CA). The isocratic separation (83% water, 17% acetonitrile at 5 ml/min) was carried out on a C$_{18}$ reversed-phase (RP) column (Lichrospher 100 RP-18, 250 × 10 mm, 10 μm, Merck, Germany). The saponin of interest was eluted as a single peak, the fractions were collected manually, combined, concentrated, and lyophilized (white amorphous powder; yield: 61 mg).

Acidic Hydrolysis

Twenty mg of the crude saponin extract were placed in a 20 ml headspace vial and diluted in 15 ml HCl (1 M). The headspace vial was sealed and placed in an oven at 90 °C for 2 h, cooled to room temperature, and its seal broken. Compounds were extracted with ethyl acetate, 500 mg calcium carbonate was added, and ethyl acetate was removed. To obtain monosaccharides, the aqueous layer was evaporated to dryness. For GC/MS measurements 200 μl MSTFA were added to each of the residues and incubated at 50 °C for 1 h.

Solid Phase Extraction

The octadecyl silica stationary phase [Isolute C_{18}, 1 g/6 ml, ICT, Basel, Switzerland] was conditioned with water. Twenty mg of the samples were diluted in 5 ml of water, applied onto the column, and eluted with methanol.

Gas Chromatography Mass Spectrometry

Gas chromatography electron ionization mass spectrometry (GC/MS) was performed on a HP 6890 GC system including a HP 5973 mass spectrometer equipped with an EI source. GC parameters were as follows: initial temperature was set at 50 °C (3 min), temperature ramp was 15 °C/min to 310 °C (25 min hold), inlet was split (1:40, 40 ml/min). The separations were carried out using a HP 5MS capillary column (30 m × 250 μm, 1 μm, HP, Palo Alto, CA) with helium as carrier gas (1 ml/min, 54 kPa). Spectra were obtained over m/z 20–800. Sample preparation was done by diluting 1 mg of the trimethylsilylated sapogenin mixture in 1 ml HPLC grade methanol. Injection volume was 1 μl. System control and data evaluation was done on the HP Enhanced ChemStation software package, G1701BA Rev. B.01.00 (HP, Palo Alto, CA).

Liquid Chromatography Mass Spectrometry

HPLC separations were done on a HP 1100 Series HPLC system including a variable wavelength detector set at 220 nm (HP, Palo Alto, CA). The separations were carried out on a C_{18} RP column (Zorbax Eclipse XDB, 150 × 4.6 mm, 5 μm, HP, Palo Alto, CA) at 20 °C. The mobile phase was a nonlinear gradient (90% to 70% in 30 min, 70% to 50% in 10 min) from 90% water/acetonitrile (9:1, vol/vol), 10% acetonitrile to 50% water/acetonitrile, and 50% acetonitrile. 0.1% (vol/vol) formic acid was added to the mobile phase. Flow rate was 0.7 ml/min. System control and data evaluation was done on the HP LC/MSD Chemstation software package, Rev. A.06.05 (HP). ESI-MS was performed on a HP LC/MSD single quadrupole MS (SQMS; HP, Palo Alto, CA). The ESI-MS conditions were as follows: nitrogen was used as nebulizer/drying gas (13 l/min, 350 °C), capillary voltage was set at +4 kV and fragmentor voltage was set at 200 V. Spectra were obtained over m/z 300-1500. System control and data evaluation was done on the HP LC/MSD Chemstation software package, Rev. A.06.03 (HP). nLC was performed using an Agilent 1100 Series nanoflow LC system with a 40 μl sample loop. Sample volumes of 20 μl were injected and concentrated on a trapping column (Zorbax SB-C_{18}, 5 mm × 300 μm, 5 μm) with water containing 0.1% formic acid at 20 μl/min. After 5 min the samples were loaded onto the analytical column (Zorbax 300 SB-C_{18}, 150 mm × 75 μm, 3.5 μm), which had been equilibrated with 97% A (100% water, 0.1% formic acid) and 3% B

(95% acetonitrile, 5% water, 0.1% formic acid). Saponins were eluted using the following gradient: 0–60 min, 3–60% B; 60–65 min, 60–95% B; 65–65 min, 95% B, at flow rates of 250 nl/min. The nLC system was coupled online to an Agilent LC/MSD XCTPlus ion trap mass spectrometer (Agilent, Palo Alto, CA), equipped with a nano-electrospray ionization source (nLC-ITMS). For nano-electrospray, coated fused-silica PicoTips (New Objective, Woburn, MA) were used. The capillary voltage was set to 1500 and 1750 V. Mass spectral data were acquired over m/z 350–1800. Nitrogen was used as nebulizer gas (6 l/min, 260 °C).

Computational Details

As calculations on the entire saponins are too time consuming, density-functional calculations were performed on model compounds. Three-ring substructures of the aglycones gaining monosaccharides retained structural relevant information and allowed calculations in an acceptable amount of time (see Supplementary Material section which can be found in the electronic version of this article.) Structures were drawn, model-built and pre-optimized using the semi-empirical PM3 method implemented in HyperChem 7.1 [31]. Spin pairing was computed according to RHF. In the SCF controls the accelerate convergence was selected (convergence limit: 0.01). Optimization was performed using the Polak–Ribiere algorithm (conjugate gradient; termination condition: RMS gradient of 0.01). These geometries were used as starting geometries for the Gaussian 03 suite of programs [32], using Becke's three-parameter hybrid Hartree-Fock density functional method [33] in combination with the Lee-Yang-Parr correlation functional (B3LYP) [34] and the 6-31g(d) basis set. All structures arising from the investigated fragmentation reactions were optimized and their energies taken for the discussion. Most of the structures were characterized by frequency calculations as true minima.

NMR

NMR experiments were done on a Bruker DRX-500 spectrometer (Bruker, Rheinstetten, Germany) equipped with an HX inverse probe (^{1}H: 500 MHz; ^{13}C: 125 MHz; solvent: pyridine-d_5). ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H correlation (COSY), 1D and 2D total correlation (TOCSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum/bond coherence/correlation (HMQC/HMBC), nuclear Overhauser effect (NOESY), and selective TOCSY, selective NOESY, and selective TOCSY-NOESY spectra were recorded using standard Bruker pulse sequences. System control and data evaluation was done with the XWINNMR software package, version 2.6 (Bruker, Rheinstetten, Germany).
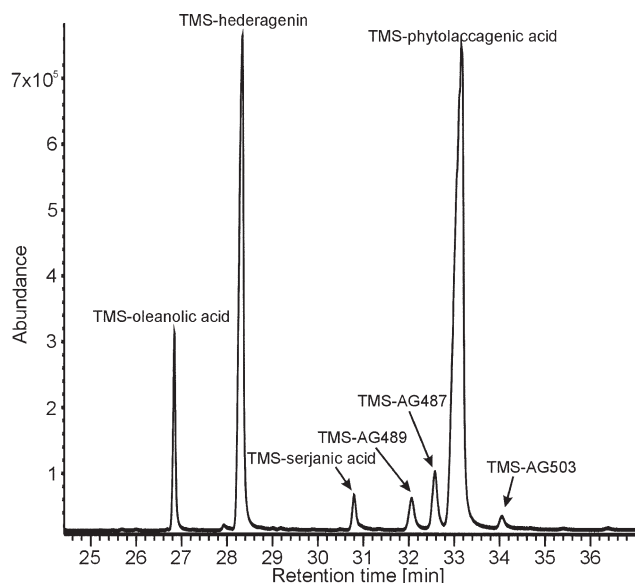
Figure 1. GC/MS chromatogram of trimethylsilylated *C. quinoa* triterpene aglycones.

Results and Discussion

Assignment Strategy

In natural product chemistry, one usually deals with highly complex and varying mixtures of compounds. As a consequence, for various applications, quality control of the extract regarding saponin composition and relative distribution is essential. In addition to this, metabolic profiling of extracts of partially or totally unknown composition gives an overview on the mixture, allows identification of the main compounds and can be followed up by directed compound isolation and structure determination. Within this study, the following assignment strategy was used to deal with a relative high number of compounds. First, the aglycones were identified by GC/MS analysis of hydrolysis products. Even though in the case of *C. quinoa* the main compounds were published in previous works, three new aglycones were detected (Figure 1). In the second step successive LC-MS and nLC-MS/MS analysis were used to identify the aglycone, to distinguish between saccharides, and to determine the oligosaccharide sequence and branching.

Assignment and Fragmentation of Reported Saponins

Separation of *C. quinoa* saponins using a modified water/acetonitrile based gradient described before [35] was adequately and allows identification a multitude of different saponins in an acceptable amount of time (Figure 2). Saponins were detected as [M + Na]$^+$ (SQMS) or [M + H]$^+$ (ITMS). All compounds comprising a sugar acid (Table 1; **E**, **F**, **K**, **R**, **S**) gave an additional signal at [M + 2Na − H]$^+$, which is probably arising from the sodium salt and can be used as an

auxiliary restraint in the assignment procedure. Assignment was started by matching the detected molecular ions to those of the previously reported compounds (Table 1). The m/z 1165 (**A**), m/z 943 (**F**), m/z 1119 (**G**), m/z 767 (**J**), and m/z 795 (**K**) corresponding to the [M + H]$^+$ ion were detected once, whereas several ions corresponding to the other compounds were found (Tables 1 and 2). These ambiguities can be resolved by tandem MS in the case that the compounds have different aglycones and/or variable sugar sequences and/or branching. However, different monosaccharide epimers cannot be distinguished. As a consequence, a differentiation of pentoses (Pent), hexose (Hex) and hexose acids (HexA) is given here. Product ion series of the oligosaccharide moiety and aglycones confirmed the latter compounds and allowed assignment of **C**, **E**, **H**, **L**, and **M**. Although [M + H]$^+$ ions of the major saponins **B**, **D**, and **I** were detected, they can be assigned as shown in Figure 2 because they represent the highest quantity within the chromatogram. No unique assignment was possible for Compounds **N**, **O**, **P**, **Q**, **R**, and **S**. For all of those, both the oligosaccharide sequence and the aglycones were identical. The most probable reasons for this observation are variations between different sugar epimers as found for Compounds **R** and **S**, where Xyl is replaced by Ara. This is emphasized by GC/MS results, which restricted the saccharide pool to Xyl, Ara, Glc, Gal, GlcA, and Glc for the C-3 and C-28 saccharide, respectively. For **N**, **O**, **P**, and **Q** up to four compounds agreeing in sugar sequences and aglycones were detected (Table 2). Saponins were found to be clustered in characteristic retention time windows according to their aglycone (Figure 3). For *C. quinoa* saponins modifications within the saccharide moiety have a small effect on retention time whereas an exchange of the aglycone shifts reten-
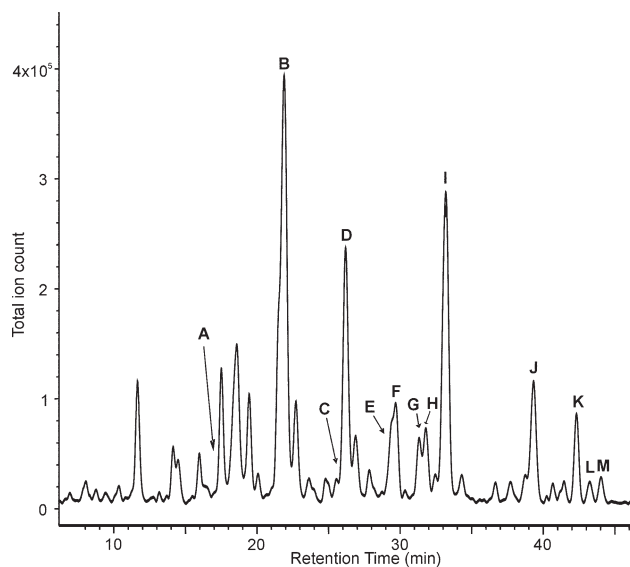
Figure 2. LC/MS chromatogram of *C. quinoa* triterpene saponins. **A** to **M** refer to the assigned literature-reported compounds.

Table 1. Assignment of reported *C. quinoa* triterpene saponins

	t_R[a]	aglycone[b]	3-*O* saccharide[c]	[M + Na]+	[M + H]+	I	II	III	IV	References
A	17.0	PA	Glc(1‴→4″)Glc(1″→4′)Glc-	1187	1165	1003	841	679	517	[42]
B	21.9	PA	Glc(1″→3′)Ara-	995	973	811	649	517		[7, 43–46]
C	25.5	Hed	Glc(1‴→4″)Glc(1″→4′)Glc-	1143	1121	959	797	635	473	[42]
D	25.1	PA	Ara-	833	811	649	517			[7, 45, 46]
E	29.2	Hed	GlcA-	833	811	649	473			[46]
F	29.6	Hed	Xyl(1″→3′)GlcA-	965/987	943	781	649	473		[46]
G	31.2	SA	Glc(1″→2′)Glc(1″3′)Ara-	1141	1119	957	795	633	501	[44, 45]
H	31.8	SA	Glc(1″→2′)Ara-	979	957	795	633	501		[44, 45]
I	33.1	Hed	Glc(1″→3′)Ara-	951	929	767	605	473		[7, 42, 44, 45]
J	39.2	Hed	Ara-	789	767	605	473			[7, 46]
K	42.3	OA	GlcA-	817/839	795	633	457			[7, 44–46]
L	43.2	OA	Glc(1‴→2″)Glc(1″→3′)Ara-	1097	1075	913	751	589	457	[45, 46]
M	44.1	OA	Glc(1″→3′)Ara-	935	913	751	589	457		[43, 44]
N	n.a.	PA	Glc(1″→3′)Gal-	1025	1003	841	679	517		[45, 46]
O	n.a.	PA	Glc(1‴→2″)Glc(1″→3′)Ara-	1157	1135	973	811	649	517	[43, 45, 46]
P	n.a.	PA	Glc(1‴→3″)Xyl(1″→2′)Glc-	1157	1135	973	811	679	517	[43]
Q	n.a.	Hed	Glc(1″→3′)Gal-	981	959	797	635	473		[45, 46]
R	n.a.	OA	Xyl(1″→3′)GlcA-	949/971	927	765	633	457		[46]
S	n.a.	OA	Ara(1″→3′)GlcA-	949/971	927	765	633	457		[43, 44]

[a]t_R: retention time; n.a. not available due to ambiguous ions with identical oligosaccharide sequence and aglycone.
[b]PA: phytolaccagenic acid, Hed: hederagenin, SA: serjanic acid, OA: oleanolic acid.
[c]In all compounds the 28-*O* linked monosaccharide was glucopyranose.
[d]I to IV refer to sequential sugar losses.

tion time significantly. Compounds **B**, **I**, and **M** coincide in their saccharide composition, branching and sequence but comprise varying aglycones. Due to the different polarity of PA, Hed, and OA, they elute at 22, 33, and 43 min, respectively, and are well separated in clusters. Within these clusters variations in saccharide composition and sequence cause minor shifts (e.g., **R** and **S**, 0.5 min).

Detailed fragmentation patterns of the four major compounds bearing different aglycones (Figure 4; **B**, **G**, **I**, **K**) are discussed by the following. Proposals were validated using quantum chemical calculations. The [M + H]+ corresponding to the Compounds **B** (m/z 973), **G** (m/z 1119), **I** (m/z 929), and **K** (m/z 795) were selected as precursor ions for MS/MS. Additional MS/MS spectra were acquired by selecting [M + H − Glc]+, which was generated at higher ionization voltages by up-front fragmentation, and these spectra provided a more complex fragmentation pattern. In all of the investigated compounds the loss of the C-28 esterified monosaccharide residue seemed to be favored. The resulting signals at [M + H − 162]+ correspond to the neutral loss of a hexose residue (Hex; Figure 4; m/z 811, 957, 767, 633 for **B**, **G**, **I**, **K**, respectively). Quantum chemical calculations using the Gaussian 03 suite of programs [32] show that the γ-H-rearrangement [21] followed by bond breaking between the C-28 carboxylic acid and the monosaccharide is energetically more favored (ΔE = 2.7 kcal/mol) compared to fragmentation at the C-3 bound saccharide. Furthermore, the carboxyl group is the place of initial ionization and therefore the starting point for fragmentation. Due to relative time consuming reaction

pattern calculations, transition-state localization of the γ-H-rearrangement was not successful. For all compounds MS/MS provided a Y type product ion series (nomenclature according to [36] that allowed sequencing of the oligosaccharide chain linked to the C-3) (Figure 4). For Compounds **B** and **I** losses of a hexose and a pentose residue were detected, which is according to the fact that with Glc-Ara- both of them have the same disaccharide linked to the C-3. Fragmentation of m/z 633 of Compound **K** resulted in m/z 457 corresponding to the [M + H − 176]+ ion, which is consistent with the loss of GlcA. These result were emphasized by the SQMS experiment, which showed a [M + Na]+ at m/z 817 and a [M + 2Na − H]+ at m/z 839, indicating the presence of a sugar acid. After the initial loss of a hexose residue the sugar sequence of Compound **G** was determined as Hex-Hex-Pent- (m/z 957→795→633→501) according to Glc-Glc-Ara- (Table 1). Fragmentation of Compounds **B**, **G**, **I**, and **K** produced product ions specific for the aglycones PA (m/z 517), SA (m/z 501), Hed (m/z 473), and OA (m/z 457), respectively. The spectra displayed in Figures 4 and 5 were surprising if one takes into account that aglycone-specific fragmentation as well as ion series representing the oligosaccharide sequence are typical for triple quadrupole instruments [24] and were not observed for ITMS. To determine whether this difference is hardware-specific, we acquired additional MS² spectra of Compound **19** on the LCQ Duo ITMS. The [M + Na]+ ions generated undergo fragmentation similar to results reported for triterpene and steroid saponins [21, 24] but do not show any product ion series specific for aglycones and the

Table 2. Assignment of novel *C. quinoa* triterpene saponins

	$t_R{}^a$	aglycone[b]	3-O saccharide[c]	[M + Na]+	[M + H]+	I	II	III	IV	V	VI
1	6.0–8.0	AG533	Pent-	849	827	665	533				
2		AG533	Hex-Pent-	1011	989	827	665	533			
3		AG533	Pent-Hex-Hex-	1173	1151	989	857	695	533		
4		AG533	Hex-Pent	1011	989	827	665	533			
5		AG533	Pent-Hex-	1011	989	827	695	533			
6	7.8	AG489	Hex-Hex-HexA-	1173/1195	1151	989	827	665	489		
7	8.7	AG489	Hex-Hex-	997	975	813	651	489			
8	9.2–11.4	AG515	Hex-Hex-Pent-	1155	1133	971	809	647	515		
9		AG515	Hex-Pent-	993	971	809	647	515			
10		AG515	Pent-	831	809	647	515				
11		AG489	Hex-Hex-Hex-	1159	1137	975	813	651	489		
12		AG489	Hex-Hex-	997	975	813	651	489			
13		AG515	Hex-Hex-Pent-	1155	1133	971	809	647	515		
14		AG515	Hex-Pent-	993	971	809	647	515			
15	11.4	AG489	Hex-Pent-Hex-	1129	1107	945	783	651	489		
16		AG489	Hex-Hex-Pent-	1129	1107	945	783	621	489		
17		AG489	Hex-HexA-	1011/1033	989	827	685	489			
18		AG489	Hex-	835	813	651	489				
19	11.8	AG489	Hex-Pent-	967	945	783	621	489			
20	11.8–13.9	AG489	Hex-Pent-Hex-	1129	1107	945	783	651	489		
21		AG503	Hex-Pent-Hex-	1143/1165	1121	959	797	665	503		
22		AG503	Hex-Hex-Pent-	1143/1165	1121	959	797	635	503		
23		AG489	Pent-Hex-Hex-	1129	1107	945	813	651	489		
24		AG489	Hex-Pent-Hex-	1129	1107	945	783	651	489		
25		AG503	Hex-Pent-Hex-	1143/1165	1121	959	797	665	503		
26		AG503	Hex-Pent-	981/1003	959	797	635	503			
27 (O)[e]		PA	Hex-Hex-Pent-	1157	1135	973	811	649	517		
28	14.0	AG489	Pent-	805	783	621	489				
29	14.3–15.0	AG489	Hex-Pent-	967	945	783	621	489			
30		AG489	Hex-Pent-	967	945	783	621	489			
31	16.4–16.7	AG489	Hex-Hex-Pent-	1129	1107	945	783	621	489		
32		PA	Hex-Pent-Pent-	1127	1105	943	781	649	517		
33 (N)[e]		PA	Hex-Hex-	1025	1003	841	679	517			
34 (N)[e]		PA	Hex-Hex-	1025	1003	841	679	517			
35 (D)[e]	16.7	PA	Pent-	833	811	649	517				
36	18.0–19.5	AG487	Hex-Hex-Pent-	1127	1105	943	781	619	487		
37		AG487	Hex-Pent-	965	943	781	619	487			
38		AG487	Hex-Hex-Pent-	1127	1105	943	781	619	487		
39 (N)[aa]		PA	Hex-Hex-	1025	1003	841	679	517			
40	19.5	PA	Hex-	863	841	679	517				
41	20.0	PA	Hex-	863	841	679	517				
42 (O)[e]	20.0–22.5	PA	Hex-Hex-Pent-	1157	1135	973	811	649	517		
43		PA	Pent-Hex-Hex-	1157	1135	973	841	679	517		
44		PA	Hex-Hex-Hex-Hex-Pent-	1481	1459	1297	1135	973	811	649	517
45 (O)[e]		PA	Hex-Hex-Pent-	1157	1135	973	811	649	517		
46 (P)[e]	20.0–22.5	PA	Hex-Pent-Hex-	1157	1135	973	811	679	517		
47		PA	Pent-Hex-Hex-	1157	1135	973	841	679	517		
48		PA	Hex-Hex-Hex-Hex-Pent-	1481	1459	1297	1135	973	811	649	517
49 (P)[e]		PA	Hex-Pent-Hex-	1157	1135	973	811	679	517		
50		PA	Pent-Hex-Hex-	1157	1135	973	841	679	517		
51 (B)[e]	22.8–26.2	PA	Hex-Pent-	995	973	811	649	517			
52		PA	Hex-Hex-Hex-Pent-	1319	1297	1135	973	811	649	517	
53 (B)[e]		PA	Hex-Pent-	995	973	811	649	517			
54 (Q)[e]	26.2–27.0	Hed	Hex-Hex-	981	959	797	635	473			
55 (Q)[e]		Hed	Hex-Hex-	981	959	797	635	473			
56		AG487	Hex-Pent-	965	943	781	619	487			
57		AG487	Pent-Hex-	965	943	781	649	487			
58 (Q)[e]	27.3–27.5	Hed	Hex-Hex-	981	959	797	635	473			
59 (Q)[e]		Hed	Hex-Hex-	981	959	797	635	473			
60 (O)[a]		PA	Hex-Hex-Pent-	1157	1135	973	811	649	517		
61		Hed	Hex-Hex-Pent-	1113	1091	929	767	605	473		

Table 2. (Continued)

	t_R[a]	aglycone[b]	3-O saccharide[c]	m/z[d]							
				[M + Na]+	[M + H]+	I	II	III	IV	V	VI
62	27.5–28.0	Hed	Pent-Hex-	951	929	767	635	473			
63		Hed	Pent-Hex-Hex-	1113	1091	929	797	635	473		
64	29.2	Hed	Hex-	819	797	635	473				
65	29.9–32.2	Hed	Hex-Hex-Pent-	1113	1091	929	767	605	473		
66		Hed	Hex-Pent-Hex-	1113	1091	929	767	635	473		
67		Hed	Pent-Hex-Hex-	1113	1091	929	797	635	473		
68 (I)[a]	30.2	Hed	Hex-Pent-	951	929	767	605	473			
69	30.6	Hed	Pent-Hex-	951	929	767	635	473			
70	36.4	OA	Hex-HexA-	979/1001	957	795	633	457			
71	37.7	OA	Hex-Hex-	965	943	781	619	457			
72	38.1–39.0	Hed	Pent-Pent-	921	899	737	605	473			
73		OA	Pent-Hex-	935	913	751	619	457			
74		OA	Pent-Hex-Hex-	1097	1075	913	781	619	457		

[a]t_R: retention time.
[b]PA: phytolaccagenic acid, Hed: hederagenin, SA: serjanic acid, OA: oleanolic acid; AG533, AG489, AG515, AG503, AG487 refer to new aglycones with a specific *m/z*.
[c]In all compounds the 28-*O* linked monosaccharide was a hexose. Hex: hexose, HexA: corresponding sugar acid, Pent: pentose. All monosaccharides were linked linear. The saccharide at the right-end of each sequence is next to the 3-*O*.
[d]I to VI refer to sequential sugar losses.
[e]Letters in brackets refer to reported compounds listed in Table 1 which agree with the novel compound in both, the oligosaccharide sequence and the aglycone. As sugar epimers cannot be distinguished by MS an unambiguous assignment was not feasible for these saponins.

oligosaccharide sequence (see Supplementary Material section online). Although the more limited fragmentation obtained may be due to the [M + Na]+ ion being more stable than the [M + H]+ ion, we think this is due to a different waveform design used in the Agilent LC/MSD XCTPlus ITMS to ramp the amplitude of the CID fragmentation voltage. As a consequence, stable product ions or product ions with low-energy fragmentation pathways can also be fragmented. The major fragmentation reactions of the aglycones consist of elimination of water, formic acid, and formaldehyde resulting in an ion series characteristic for each agly-

Figure 3. LC/MS chromatogram of *C. quinoa* saponins showing retention time clusters characteristic for saponins comprising a specific aglycone. AG533, AG489, AG515, AG503, AG487 refer to compounds comprising novel aglycones with a specific *m/z*.

cone (Figure 4). After the loss of all 28-*O* and 3-*O* linked saccharides the charge is located at the C-3 hydroxyl group. As a consequence the favored fragmentation step of the aglycone is water elimination and double-bond formation at this specific site resulting in a strong signal at [Agl + H − H_2O]+ (*m/z* 499, 455, 439, 483 for PA, Hed, OA, and SA). Quantum chemical calculations gave the lowest elimination energies (13.5 kcal/mol) between the precursor and the product ion and therefore confirm the experimental results. For PA and Hed where the aglycone comprises an additional hydroxyl group elimination of two water molecules was observed (*m/z* 481 and 437, respectively). In contrast to the latter reaction, formation of the double-bond is probably preceded by a 1,3-hydride shift. A higher energy difference between the precursor and the product ion was determined (13.7 kcal/mol), however the properties of the transition-state cannot be calculated. For all compounds water elimination was accompanied by a neutral loss of formic acid (46 u) incorporating the C-28 carboxylic acid. The double-bond formed as a result has its energetically most favored location between C-17 and C-18 with a ΔE of 16.0 kcal/mol between the precursor and the products. As the energy difference of formic acid elimination is higher than the elimination energy required for water (ΔE = 2.5 kcal/mol) the fragmentation pattern and the relative intensities of the detected peaks can be understood clearly. A third neutral loss of formaldehyde (30 u) was of minor intensity and was detected for aglycones comprising a hydroxymethyl residue. Using this information together with fragmentation of the trimethylsilylated hydrolysis products we present proposals for the new aglycones identified here. Even in cases of unknown aglycones, information on the number of hydroxyl and
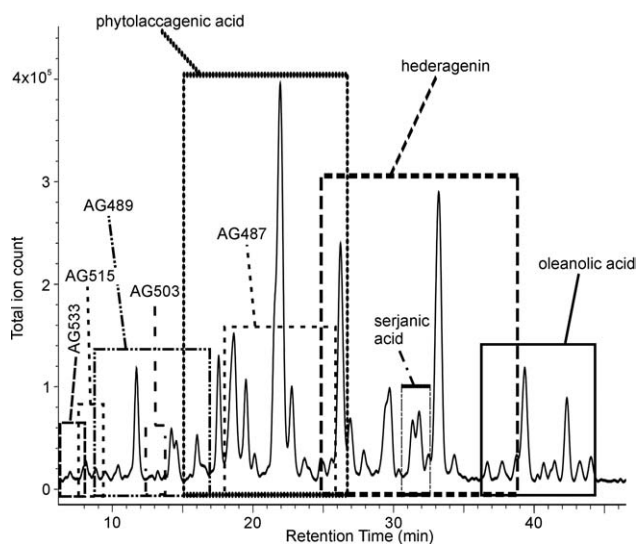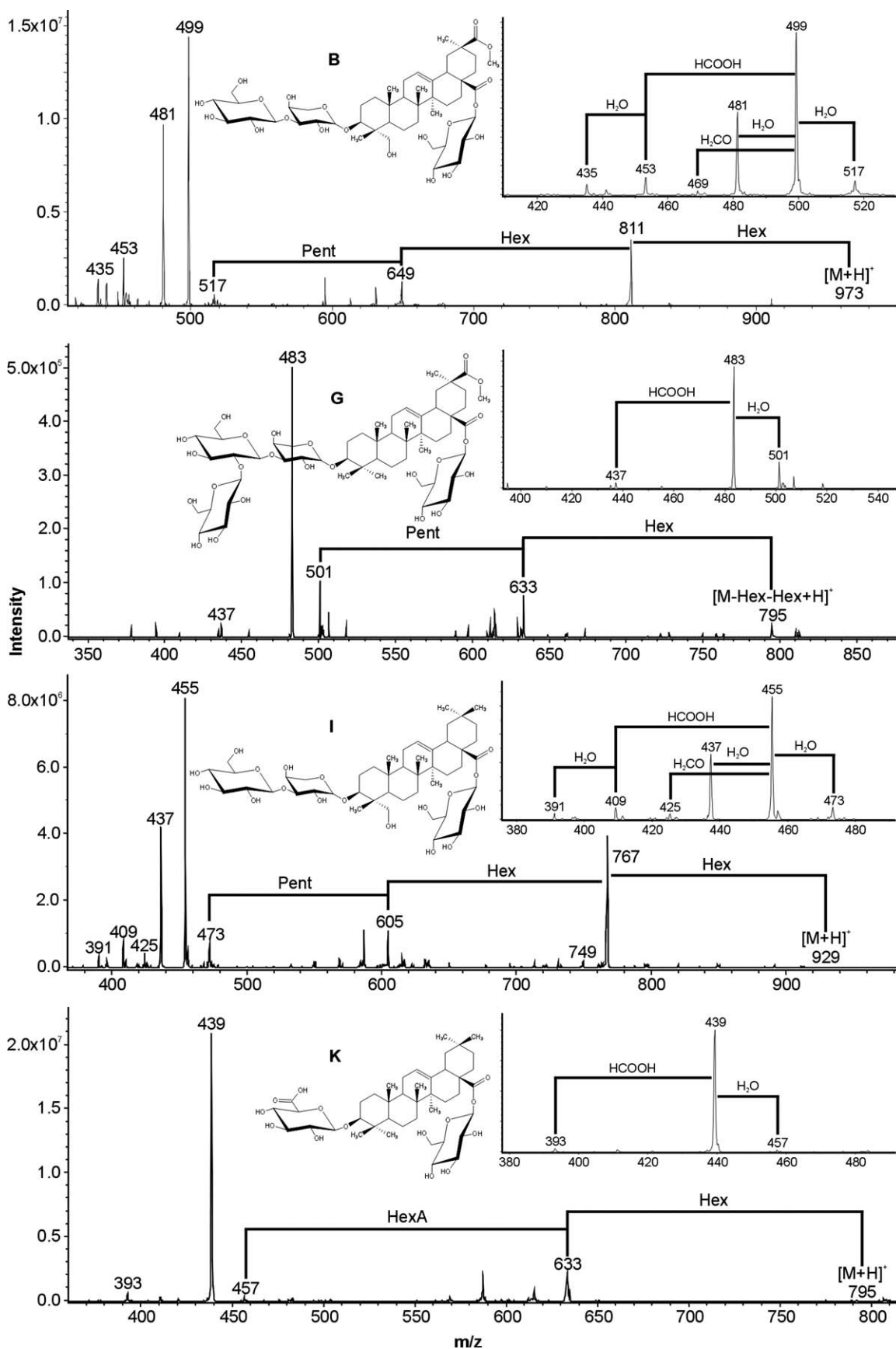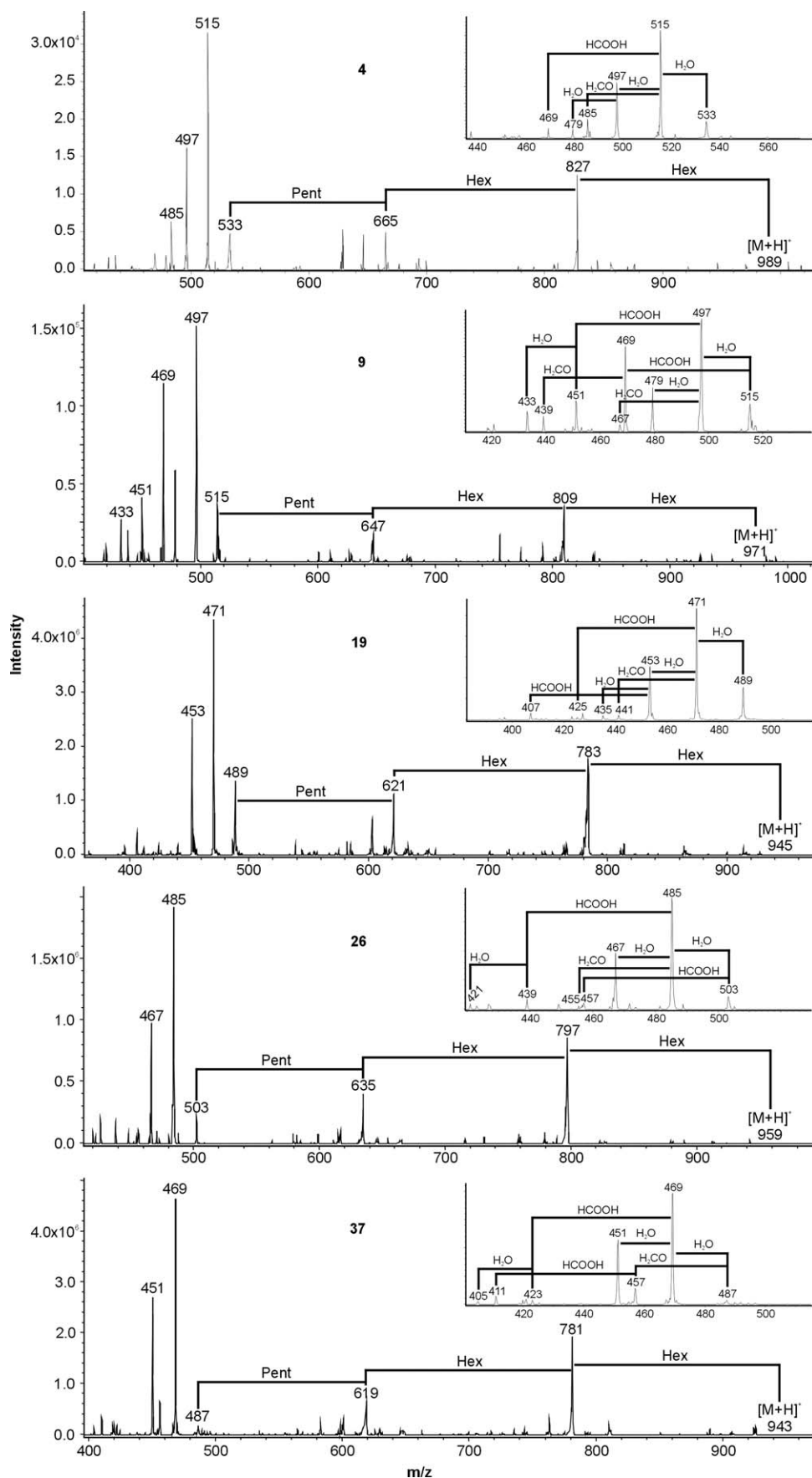
Figure 4. MS/MS spectra on the [M + H]$^+$ of the literature-reported Compounds **B** (*m/z* 973), **G** (*m/z* 1119), **I** (*m/z* 929), and **K** (*m/z* 795). [M + H − Hex]$^+$ was the higher abundant production ion for **B**, **I**, **K**, and the [M + H − 2Hex]$^+$ for **G**, respectively. Product ion series characteristic for the aglycones phytolaccagenic acid (**B**), serjanic acid (**G**), hederagenin (**I**), and oleanolic acid (**K**) are displayed in the enlarged spectra.

Figure 5. MS/MS spectra on the [M + H]$^+$ of the novel Compounds **4** (*m/z* 989), **9** (*m/z* 971), **19** (*m/z* 945), **26** (*m/z* 959), and **37** (*m/z* 943). Product ion series characteristic for the new aglycones AG533 (**4**), AG515 (**9**), AG489 (**19**), AG503 (**26**), and AG487 (**37**) are displayed in the enlarged spectra.

carboxylic acid groups can be obtained. However, stereochemical information as well as the exact structure is not available by MS.

Assignment and Fragmentation of Novel Saponins

The information obtained from the assignment of **A** to **S** was used to assign aglycone and saccharide composition of all detected compounds (Table 2). This resulted in around 70 novel saponins comprising five different aglycones which were also clustered in characteristic retention time sections (Figure 3). Fragmentation pathways of the most abundant Compounds **4**, **9**, **19**, **26**, and **37**, each representing a group of saponins comprising a novel aglycone, are discussed subsequently. Consistent with the compounds discussed above fragmentation of $[M + H]^+$ at the 28-*O*-linked hexose was favored. Sequencing of the 3-*O*-linked sugars displays neutral losses of 162 u and 132 u for all of the compounds (Figure 5) deducing a hexose linked to a pentose. This is not surprising as the most abundant compounds out of each group were taken for discussion, and it was found for PA (**B**) and Hed (**I**) that these species are favored. **4** displayed an aglycone-specific product ion at *m/z* 533. Elimination of three water molecules, formic acid, and formaldehyde point to an aglycone similar to PA but bearing an additional hydroxyl group, e.g., at the C-2 [37] or the C-16 [38]. Sugar loss of Compound **9** results in a signal detected at *m/z* 515. Fragmentation was similar to **4** except for two instead of three water losses. As, due to the minor abundance, no additional GC/MS information was available for this compound, no proposal is given. **19** (*m/z* 489) showed three water losses and fragmentation characteristic for the carboxyl moiety. GC/MS data suggest a 4-fold trimethylsilylated aglycone (*m/z* 776) with 2-fold derivatization of the retro-Diels-Alder (RDA) product ion (*m/z* 408; see Supplementary Material) [39]. Apart from the C-28 carboxyl group (118 u; HCOOTMS) this suggests an additional hydroxymethyl group at the C-29 or C-30 of that product ion (103 u; TMS, formaldehyde) and at the C-23, C-24, or C-2, respectively [40, 41]. The aglycone-specific product ion of Compound **26** was detected at *m/z* 503 and displayed two water losses in addition to formic acid and formaldehyde. GC/MS suggests a 4-fold trimethylsilylated molecular ion (*m/z* 790) with two derivatization sites at the RDA product ion (*m/z* 422). Two neutral losses of 118 u typical for carboxyl units (HCOOTMS) were detected. This suggests a structure similar to that of **19** but bearing an additional carboxyl group at the RDA product ion, most probably at the C-29 or C-30. In addition to the $[M + Na]^+$ (*m/z* 981) LC-SQMS gave an additional $[M + 2Na - H]^+$ at *m/z* 1003 characteristic for a free carboxyl group and therefore emphasizes our proposal. Apart from neutral losses detected for all aglycones **37** (*m/z* 487) undergoes only two water elimination steps. GC/MS detected a 3-fold derivatized aglycone (*m/z* 702) with the carboxyl unit being the only trimethylsilylated functional group

within the RDA product ion (*m/z* 334). A strong loss of 28 u due to elimination of ethylene from the RDA indicates a terminal ethyl group, probably at the C-29 or C-30. Based upon different polarities, these proposals are according with the elution order of their corresponding saponins within the chromatographic separation.

Isolation and Structure Determination of Compound 19

To confirm the accuracy of the assignment procedure and due to the identification of new aglycones, the most abundant novel Compound **19** was isolated and characterized by NMR (see Supplementary Material for ^{1}H and ^{13}C resonances). Using ^{1}H/^{13}C chemical shifts and absolute values of the vicinal coupling constants the configuration at the anomeric protons for arabinopyranose and glucopyranose were determined as α and β, respectively. NOESY experiments and the coupling constants of vicinal ^{1}H$-^1$H correlations were used to determine the relative stereochemistry. The novel Compound **19** was assigned as bidesmosidic triterpene saponin 3-23-30-trihydroxyolean-12-en-28-oic acid 3-*O*-[β-D-glucopyranosyl (1″→3′)-α-L-arabinopyranosyl]-28-*O*-β-D-glucopyranoside, which is emphasized by structural information obtained from GC/MS and ESI-MS/MS (Figure 5). The $[M + H - 162]^+$ at *m/z* 783 corresponds to the neutral loss of the 28-*O*-linked glucopyranose. The ion series sequencing the 3-*O*-linked moiety displayed losses of 162 u and 132 u representing the Glc (1″→3′)-Ara-disaccharide. The three neutral losses of water as well as of formic acid arise from the three hydroxyl groups at C-3, C-23, C-30, and the C-28 carboxyl moiety, respectively. The molecular ion of the trimethylsilylated aglycone (*m/z* 776) agrees with a 4-fold trimethylsilylation and rearranges to the product ion at *m/z* 408 (see Supplementary Material). Two neutral losses of 103 u (TMS, formaldehyde) and 118 u (HCOOTMS) are due to TMS groups derivatizing the C-30 hydroxyl and C-28 carboxylic acid, respectively. Our results show that although stereochemical information on the compounds is not available by MS structural information can be extracted from the spectra and proposals can be given.

Conclusions

The combination of two different mass spectrometric techniques (GC/MS and nLC-MS/MS) was proven to be a powerful tool for the assignment of reported as well as the identification and assignment of a great number of novel *Chenopodium quinoa* triterpene saponins. LC-MS/MS allowed a complete preassignment and identification of the major constituents and aglycones, respectively. Structural information on the nine different aglycones (nature, substitution) and the saccharide moieties (sequence, composition, and branch-

ing) can be extracted from the MS spectra. However, stereochemistry and the exact structure cannot be deduced from the mass spectra. Proposals for novel saponin structures can be given as demonstrated for about 70 compounds and verified by NMR spectroscopy. Fragmentation pathway analysis using quantum chemical density functional calculations is currently not established but assists in interpretation of mass spectra and will be an effective tool even for transition-state localization in the future. As this methodology allows calculation of elimination and bond-breaking energies, proposals and possibilities for different fragmentation reactions can be given and agree with the experimental results. Compared to the commonly performed and relative time consuming isolation and structure determination procedure using successive column chromatography and NMR, the MS-based assignment can be obtained in a few hours and is recommended for directed isolation and structure determination routines. Our rapid and complete approach is particularly suitable for quality control and screening of *C. quinoa* extracts designated for pharmaceutical, agricultural, and industrial applications.

Acknowledgments

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