Quinoa seed coats as an expanding and sustainable source of bioactive compounds: An investigation of genotypic diversity in saponin profiles

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Karina B. Ruiz[a,1], Bekzod Khakimov[b,c,1], Søren B. Engelsen[c], Søren Bak[b], Stefania Biondi[a], Sven-Erik Jacobsen[b,*]

[a] *Dipartmento di Scienze Biologiche, Geologiche e Ambientali (BiGeA), Università degli Studi di Bologna, Via Irnerio 42, 40126, Bologna, Italy*
[b] *Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Hoejbakkegaard Alle 13, DK, 2630 Taastrup, Denmark*
[c] *Department of Food Science, University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg, Denmark*

A R T I C L E I N F O

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A B S T R A C T

Saponins (SAPs) are a diverse family of plant secondary metabolites and due to their biological activities, SAPs can be utilised as biopesticides and as therapeutic compounds. Given their widespread industrial use, a search for alternative sources of SAPs is a priority. Quinoa (*Chenopodium quinoa* Willd) is a valuable food source that is gaining importance worldwide for its nutritional and nutraceutical properties. SAPs from quinoa seed coats could represent a new sustainable source to obtain these compounds in high quantities due to the increasing production and worldwide expansion of the crop. This research aims to characterise saponins of seed coat waste products from six different quinoa varieties for their potential use as a saponin source. Gas chromatography (GC)- and Liquid chromatography (LC)- with mass spectrometry (MS) were applied for qualitative and relative quantitative analysis of saponins. GC–MS led to the identification of three main aglycones, oleanolic acid (Ole), hederagenin (Hed), and a phytolaccagenic acid (Phy), while LC–MS enabled characterization of 24 SAPs with varying sugar moieties. Hed was the most abundant aglycone, followed by Phy and Oledepending on the genotype. Saponin distribution and relative abundances are discussed in the light of genotype provenance and agronomic features. Improved knowledge on the phytochemicals present in quinoa varieties might help in finding valuable and sustainable uses for quinoa SAPs in agroindustry as biopesticides as well as in the production of food and pharmaceuticals.

1. Introduction

Currently, the major industrial source of SAPs is the bark of quillay (*Quillaja saponaria* Molina) accounting for 80% of the world market (San Martín, 2000). Unfortunately, the increasing commercial use of quillay SAPs is causing damage to Chilean forests (San Martín et al., 2000; Schlotterbeck et al., 2015). In the pharmaceutical industry, SAPs are used in the synthesis of hormones, contraceptives, anti inflammatories, expectorants, and diuretics (Vincken et al., 2007; Augustin et al., 2011). In addition, SAPs have found wide application in food and cosmetic production (Price et al., 1987; Sparg et al., 2004). In agriculture, SAPs have potential as biopesticides and growth promoters (Andresen and Cedergreen, 2010; Andresen et al., 2015). For example, molluscicidal and vermicidal activity has been reported in complex mixtures of SAP derivatives obtained from alkali treated quinoa seed coats (San Martín et al., 2008) and from SAP rich by products of tea oil production (Potter et al., 2010). Increased consumer demand for natural products with beneficial physico chemical (e.g., surfactant) and biological (e.g., biocidal, antimicrobial) properties makes steroidal and triterpenoid SAPs promising compounds for industrial applications.

Triterpenoid SAPs are a diverse group of compounds characterized by the presence of a triterpenoid aglycone backbone ($C_{30}H_{48}$) with one or more sugar moieties attached to them via glycosidic and/or ester bonds (Khakimov et al., 2016b). Triterpenoid SAPs are ubiquitous in the plant kingdom (Sparg et al., 2004; Vincken et al., 2007). Triterpe noid aglycones found in plants include dammaranes, tirucallanes, oleananes, lupanes, hopanes, ursanes, taraxasteranes, cycloartanes, lanostanes, cucurbitanes, and steroids (Vincken et al., 2007). Biosynth esis and heterogeneity of SAPs depend on plant species and tissue type (Augustin et al., 2011). Moreover, environmental, agronomic factors as well as post harvest treatments, such as storage and processing seem to influence SAP composition of plants (Fenwick et al., 1991; Yoshiki

Abbreviations: SAPs, saponins; Ole, oleanolic acid; Phy, phytolaccagenic acid; Hed, hederagenin; GC, Gas Chromatography; LC, Liquid Chromatography; MS, Mass Spectrometry

* Corresponding author.
E-mail address: seja@plen.ku.dk (S.-E. Jacobsen). [1] These authors contributed equally to this paper.

et al., 1998; Szakiel et al., 2011). Even though some research has been devoted to reveal the relationship between biological properties and chemical structure (Dini et al., 2001a,b; Francis et al., 2001; Güclü Üstündağ and Mazza, 2007; Khakimov et al., 2012), this aspect deserves further investigation.

In foods, SAPs may have a bitter and astringent taste depending on chemical structure, and consequently SAP levels in food products are reduced to decrease bitterness (Price et al., 1987; Gómez Caravaca et al., 2014). This is done through dehulling and washing of seeds, or by using so called sweet varieties with low SAP levels as in the case of quinoa (Ward, 2000; Dini et al., 2002). Quinoa (*Chenopodium quinoa* Willd) has become an extremely popular food in the last 20 years, especially in Europe and North America, due to the increased interest in vegetarian diets, its high nutritional quality, and the increasing number of people with gluten intolerance and celiac disorder (Rubio Tapia et al., 2009; Abugoch, 2009; Vega Gálvez et al., 2010).

Quinoa cultivation continues to expand worldwide, and the potential of quinoa in the food and healthcare sector is huge (Bazile et al., 2016; Jacobsen, 2003). Therefore, it is of high relevance to gain further knowledge on the SAP profiles of various quinoa varieties. It has been shown that SAP content of quinoa is mainly genotype dependent (Ward, 2000; Miranda et al., 2014). The major part of SAPs are present in the outer layers of the seed, and protect it from pests and herbivores (birds and insects) and microorganisms (Ridout et al., 1991; Abugoch, 2008). When properly handled to remove the bitter SAPs, which are the major anti nutritional factors, quinoa seeds have a mild flavour (Maradini et al., 2015). Compared to wheat, quinoa seed has higher protein content, but especially a more favourable amino acid profile (Repo Carrasco et al., 2003; Stikic et al., 2012). Quinoa has higher levels of energy, calcium, phosphorus, iron, dietary fiber and B vitamins than cereals (Abugoch, 2009). In addition to its nutritional profile, new targets in research are focused on quinoa by products in order to add value to its secondary metabolites, such as the SAPs that are removed prior to consumption and are, therefore, a waste product.

Bitter varieties of quinoa contain more SAPs (4.7 11.3 g kg^{-1} dry matter) than sweet varieties (0.2 0.4 g kg^{-1} dry matter) (Mastebroek et al., 2000), thus they are more widely cultivated (San Martín et al., 2008; Stuardo and San Martín, 2008). The coat represents about 8 12%w/w of the quinoa seed and is the main storage tissue of SAPs (up to 86% of the total amount in seeds) (Ando et al., 2002). In total more than 30 SAPs have been identified from quinoa plants that possess mainly five different triterpenoid aglycones including Ole, Hed, Phy, and Serjanic acid (SA) (Mastebroek et al., 2000; Dini et al., 2001a,b, 2002; Zhu et al., 2002; Kuljanabhagavad et al., 2008; Kuljanabhagavad and Wink, 2009). The major sugar moieties of SAPs are glucose, arabinose, galactose, glucuronic acid, xylose, and rhamnose (Woldemichael and Wink, 2001; Zhu et al., 2002). Recently published genome sequencing of quinoa allowed the identification of the tran scription factor that is high likely to control the triterpenoid saponins synthesis in seeds (Jarvis et al., 2017). The finding is expected to ease future research on selection for sweet varieties of quinoa. In general, the high genetic variability in quinoa represents a precious resource, which can be exploited for selecting and breeding cultivars adapted to the most diverse soil and climatic conditions (Zurita Silva et al., 2014).

The present study reports, for the first time, the results of a comparative analysis of SAP profiles in powdered seed coats, rather than whole seeds of six different varieties of quinoa originating from Bolivia or selected and bred in Denmark. The main objective of the study was to screen a complexity and relative amounts of SAPs as well as their corresponding triterpenoid aglycones backbones from the quinoa seed coat waste products using comprehensive GC MS and LC MS/MS analysis.

Table 1
An overview of quinoa seed samples, harvested in 2014, investigated in this study.

Variety	Acronym	Origin	Place of Cultivation (Lat;Long)	Quality	Seed colour
Sample 1	P1	Bolivia	Uyuni (BO)	bitter	white
Sample 2	P2	(BO)	20°28′12″S;	bitter	white
Real	QR		66°48′50″W	bitter	white
Titicaca	Ti	Denmark (DK)	Taastrup (DK)	bitter	light brown
Puno	Pu		55°40'13.2"N;	bitter	light yellow
Vikinga	Q124		12°18'13.4"E	sweet	light brown

2. Materials and methods

2.1. Seed material

Seeds were collected in 2014 from six different varieties of quinoa (Table 1). QR is a *Real* type variety originating from Bolivia; P1 and P2 are mixed samples of Bolivian material. Titicaca (Ti) and Puno (Pu) are registered varieties that were selected and bred in Denmark from varieties originated in southern Chile and Peru (Jacobsen, 1998; Adolf et al., 2012). Q124 is a Danish variety bred for low SAP content, now registered under the name *Vikinga*. The seed coats powder of *Real* varieties was obtained from seeds harvested in Bolivia, while Danish varieties were obtained from seeds harvested in Denmark.

2.2. Sample preparation

Twenty five mg DW of the outer layer of quinoa seeds (obtained after mechanical dehulling using a mill) were powdered and extracted following the procedure described by Khakimov et al. (2016a,b) with some modifications, using 80% methanol in the ratio of 25:1 (w/v). The mixture was vortexed for 30 s, incubated at 70 °C for 3 h with agitation at 1400 rpm using a ThermoMixer F2.0 (Eppendorf, Hørsholm, Denmark), and cooled to room temperature. Extracts were then vortexed for 30 s, and centrifuged at 16k g for 10 min. In order to identify the triterpenoid aglycones and characterise sugar moieties of SAPs, the supernatant was separated into aliquots as follows: 0.1 mL for LC MS/MS analysis; 0.37 mL for HCl (acidic) hydrolysis and 0.37 mL for basic (NaOH) hydrolysis based GC MS analysis. Basic hydrolysis was applied prior to GC MS in order to cleave sugar moieties linked via ether bonds, while the acidic hydrolysis removed sugar moieties linked via both ether and glycosidic bonds. The general procedure for SAP extraction is summarized in Fig. 1.

Aliquots consisting of 0.37 mL of supernatants were completely dried in a ScanVac (Labogene, Lynge, Denmark) operating at 45 °C and 200 g for 2 3 h. For the acidic hydrolysis, 0.5 mL of 2 M HCl was added, the mixture was vortexed for 30 s and then incubated at 100 °C for 1.5 h in the ThermoMixer (1400 rpm). The mixture was cooled to room temperature. For basic hydrolysis, 0.5 mL of 2 M NaOH was added to the dried aliquots of 0.37 mL supernatant; the mixture was vortexed for 30 s and incubated at 25 °C for 1.5 h with mixing at 1400 rpm as described above. Thereafter, 0.1 mL of 12 M HCl was added to adjust the pH $\leq$ 2. Both acidic and basic extracts were then transferred to fresh 15 mL Falcon tubes. Free aglycones, released by hydrolysis, were extracted by addition of diethyl ether at an extract:ether ratio of 1:4 (v/v). This procedure was repeated twice and the combined ether fractions were washed with MilliQ water (ether:water, 2:1, v/v) in order to remove the residual acid or base. The washed ether fraction was transferred into a new 15 mL tube, dried under fume hood and re suspended in 0.25 mL of 80% methanol. After centrifugation (16k g for 3 min) of the final methanol extracts, 0.1 mL aliquot was used for LC MS/MS and 0.05 mL was used for GC MS. Prior to GC MS, samples

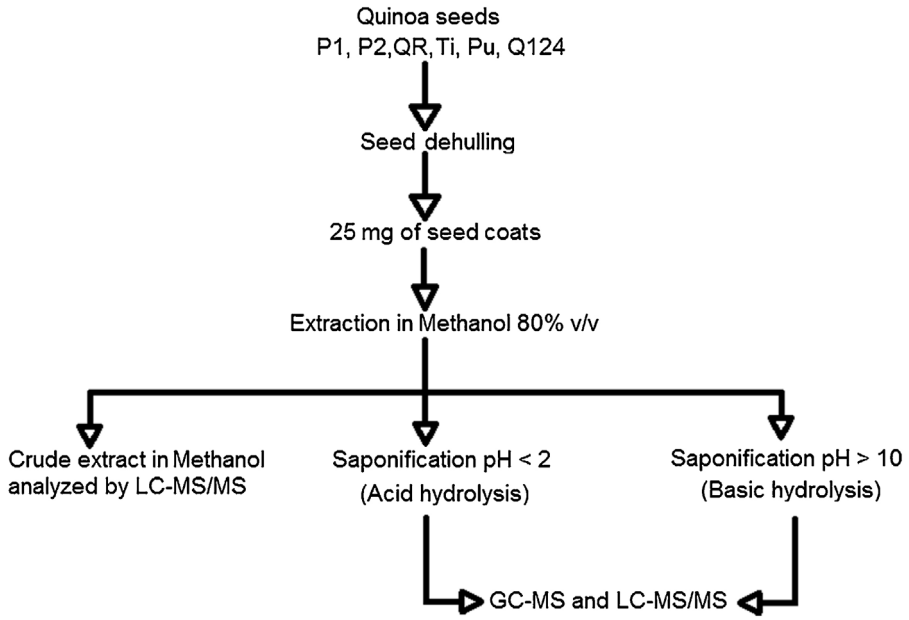

Fig. 1. Schematic protocol to determine SAP contents by two different chromatographic approaches after acidic and basic hydrolysis of powdered quinoa seed coats.

were dried in 0.2 mL glass inserts using a ScanVac (2.5 h at 40 °C, 100 rpm). Dried samples were stored at −20 °C until derivatization.

2.3. GC MS analysis of samples

Dried samples were derivatized using trimethylsilylation as described by Khakimov et al. (2013). Thirty µL of trimethylsilyl cyanide (TMSCN, Sigma Aldrich, St. Louis, USA) were added to each sample and agitated at 750 rpm for 50 min at 45 °C. All steps involving sample derivatization and injection were automated using a MultiPurpose Sampler (Gerstel, Mülheim an der Ruhr, Germany). The GC MS system consisted of an Agilent 7890A GC and an Agilent 5975C series MSD. GC separation was performed on a Phenomenex ZB 5MSi column (30 m × 250 µm × 0.25 µm). A derivatized sample volume of 1 µL was injected into a split/splitless inlet at 320 °C using a pulsed splitless mode at an injection pulse pressure of 20 psi until 2 min of splitless time; purge flow to split vent after splitless time and septum purge flow were set at 15 and 3 mL min^{-1}, respectively. Hydrogen was used as carrier gas, at a constant flow rate of 1.2 mL min^{-1}. The GC oven program was set as follows: initial temperature 40 °C, equilibration time 2.0 min, heat up to 270 °C at a rate of 12 °C min^{-1}, then heat at a rate of 6 °C min^{-1} until 320 °C and hold for 5 min. Mass spectra were recorded in the range of 50 500 *m/z* with a scanning frequency of 3.2 scans s^{-1}, and the MS detector was switched off during the 6 min of solvent delay time. The transfer line, ion source, and quadrupole temperatures were set at 290, 230 and 150 °C, respectively. The mass spectrometer was tuned according to the manufacturer's recommenda tion by using perfluorotributylamine (PFTBA). The aglycones were identified by comparing retention time (RT) and Electron Impact Mass Spectrum (EI MS) of authentic standards of Hed and Ole (Fluka, Sigma Aldrich).

2.4. LC MS/MS analysis of samples

In order to determine SAP levels, LC MS/MS analysis was performed as described by Augustin et al. (2012) using an Agilent 1100 Series LC device (Agilent Technologies, Inc., USA), equipped with a Gemini NX column, 150 mm length and 3.5 mm diameter (Phenomen ex, Torrance, USA) operated at 35 °C and coupled to a Bruker HCT Ultra ion trap mass spectrometer (Bruker Daltonics). The samples (0.1 mL each) were transferred into glass vials and diluted to 1.5 mL with 85% methanol for LC MS analysis. The mobile phases consisted of two eluents: water with 0.1% (v/v) formic acid (A), and acetonitrile with 0.1% (v/v) formic acid (B). The gradient was programmed as follows: 0 1 min, isocratic 12% B; 1 33 min, linear gradient 12% 80% B; 33 35 min, linear gradient 80% 99% B; 35 38 min, isocratic 99% B; 38 45 min, isocratic 12% B at a constant flow rate of 0.2 mL min^{-1}. The Bruker HCT Ultra ion trap mass spectrometer (Bruker Daltonics) was operated using electrospray ionization and *m/z* ions were detected in a negative mode using tandem mass spectrometry. Qualitative analysis and visualization of LC MS/MS data were performed using the DataAnalysis 4.0 (Bruker Daltonics) software.

2.5. Data analysis

In order to calculate the GC MS peak areas of aglycones (relative concentrations), the raw GC MS data were processed by the PARAFAC2 as described previously (Khakimov et al., 2012). Relative concentrations of SAPs were calculated from the total ion current (TIC) chromatograms of the raw LC MS data. Prior to SAP profile comparison, the data was normalized using the 1 norm (division of each peak area by the sum of areas of all peaks detected in the same sample).

3. Results

A total of six different quinoa seed coat samples of varying origins and genetic backgrounds were analysed for their SAP profiles using GC MS and LC MS/MS prior to and after acid or basic hydrolysis.

3.1. Identification of triterpenoid aglycones by GC MS

GC MS analysis of acid hydrolysed samples revealed three major aglycones: Ole, Hed, and Phy. Serjanic acid (SA) was only detected in a neglectable amount in all quinoa varieties, thus it is outside the scope this paper to discuss it further. Ole and Hed were identified at level 1 (Sumner et al., 2007) using authentic standards, while Phy and SA were identified at level 2 using mass spectral and retention index comparison with data reported in the literature (Fig. 2A) (Gómez Caravaca et al., 2012; Medina Meza et al., 2016). Electron Ionization Mass Spectrum (EI MS) and Retention Indices [RIs] of all four triterpenoid aglycones are illustrated in Supplementary Fig. 1. The relative abundance of aglycones in each variety showed that Hed was the most abundant

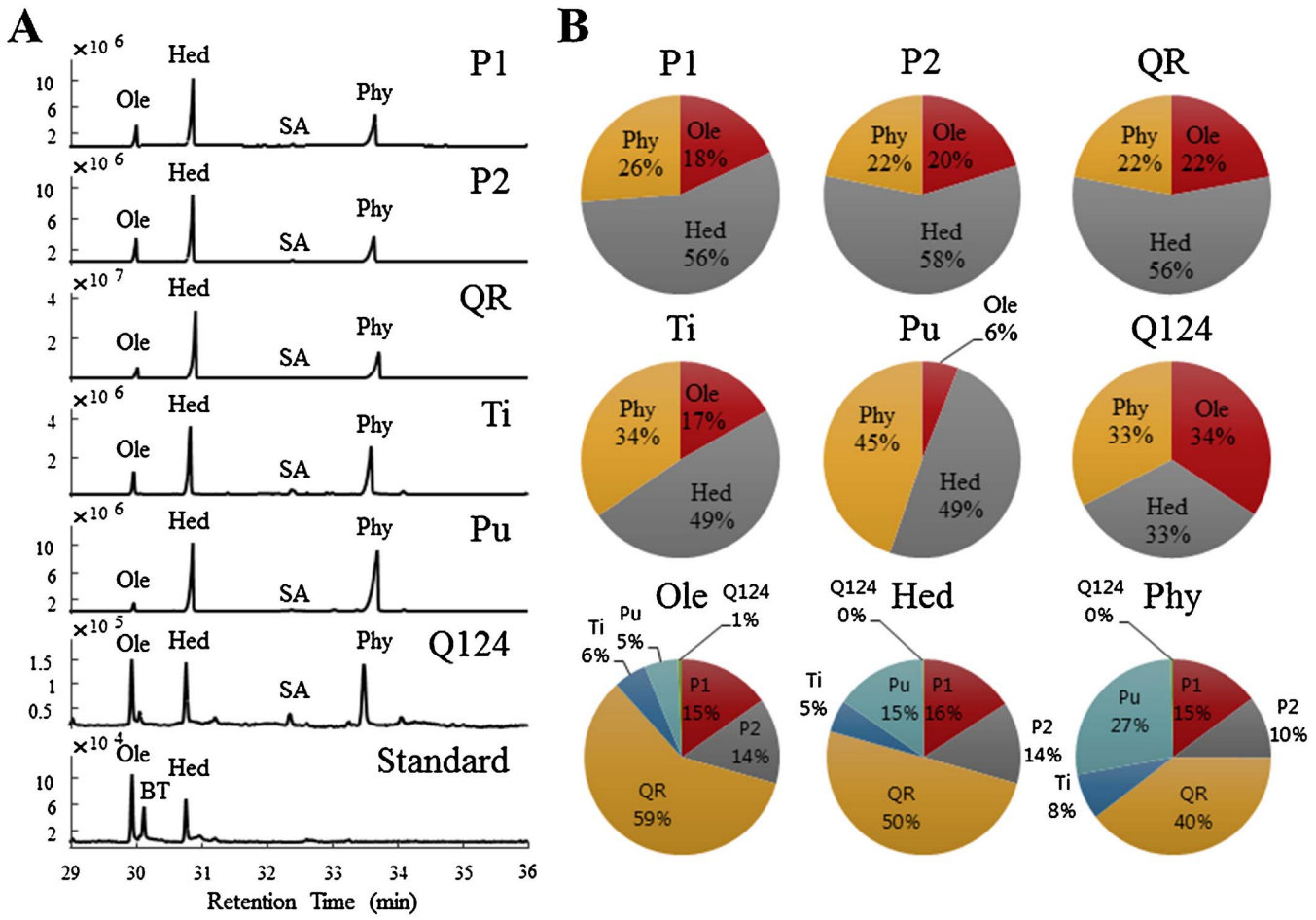

Fig. 2. Total ion current (TIC) chromatograms of the GC–MS data obtained on acidic hydrolysed extracts of seed coats of six quinoa genotypes (**A**). *Ole: oleanolic acid, BT: betulinic acid, Hed: hederagenin, SA: serjanic acid, Phy: phytolacccagenic acid. Relative concentrations of the three major triterpenoid aglycones, Ole, Hed and Phy, within and across quinoa varieties (**B**).

triterpenoid aglycone in all six quinoa samples, amounting to ca. 50% of the triterpenes detected, except in Q124 where all three major sapogenins reached similar percentages (Fig. 2B). Relative concentrations of Ole and Phy were similar in P1, P2, and QR and ranged from 18 to 26% of the total aglycones (Fig. 2B). Phy increased to twice the amount of Ole (17%) in Ti. In Pu, the percentage of Ole was detected at the lowest relative amount (6%).

When the six quinoa samples were compared with respect to each of the three identified aglycones, QR exhibited the highest amounts (relative to the other varieties) of all three major aglycones, whereas Q124 had the lowest amounts (Fig. 2C). P1 and P2 showed 14 and 15% relative concentrations of Ole, respectively, while Pu and Ti contained only 5 6%; Q124 contained the lowest relative amount of this SAP (Fig. 2C). The relative concentration of Hed was similar in P1, P2 and Pu (14 16%), whereas Ti (5%) and Q124 (< 1%) showed lower amounts of this aglycone. The relative concentration of Phy in QR was 40%, followed by Pu with 27%, P1, P2 and Ti with 15, 10 and 8%, respectively; in Q124, this SAP was below detection threshold.

GC MS analysis of the same quinoa extracts using basic hydrolysis showed a notable decrease in the amounts of Ole and Hed in P1, P2, QR and Pu (data not shown). This suggests that these quinoa samples mostly contain Ole and Hed based SAPs where sugar moieties are linked to the aglycones via a glycosidic bond.

3.2. Saponin characterization by LC MS/MS analysis

A total of 24 SAPs were tentatively characterized by LC MS/MS in the six quinoa seed coat samples (Table 2). The relative amounts of these SAPs in each sample are shown in Fig. 3. Tandem mass spectrometry data allowed us to interpret fragmentation patterns of SAPs, where mainly loss of a hexose (162 Da) and loss of an unknown adduct ion with a mass of 172 Da were observed. It is speculated that loss of the latter adduct ion from SAPs may derive from the acyl moiety (acyl H_2O), 3,5 dihydroxy 6 methyloctanoic acid, which is possibly conju gated to a hydroxyl group of the aglycone. Several saponins of *Quillaja saponaria* based on quillaic acid indeed possess 3,5 dihydroxy 6 methy loctanoic acid and their MS/MS spectra exhibit the loss of the same 172 Da adduct (Sun et al., 2003; Qin et al., 2006).

Three major SAPs, with three to four sugar moieties, showed an aglycone molecular weight (MW) of 472 Da, which is in agreement with the GC MS data in which Hed (472 Da) was found to be the most abundant aglycone in all quinoa samples. Seven putative SAPs, with two to four sugar moieties, showed an aglycone MW of 456 Da, in agreement with the GC MS data which identified Ole (456 Da) as one of the three main triterpenoids. Four SAPs, all containing three sugar moieties, showed an aglycone with MW of 500 Da, which was also in agreement with the GC MS data that allowed identification of SA with MW of 500 Da. Although it was not possible to determine the MW of Phy (516 Da) from the MS/MS experiment, it is speculated that the last seven putative SAPs, with two to three sugar moieties, correspond to Phy aglycone. However interpretation of such a fragmentation pattern that may result in *m/z* ion at 440 Da remains scarce (Table 2). More over, two SAPs with an aglycone MW of 470 Da were detected and showed loss of three sugar moieties.

The genotype with the highest diversity in SAPs was Pu (20 peaks) followed by P1 > P2 > QR > Ti (16 15 peaks) > Q124 (10

Table 2
A list of 24 putative triterpenoid saponins from six quinoa varieties identified by LC–MS/MS. Molecular weight (MW) of aglycones and number of sugar moieties attached to the aglycones were determined from ESI–MS fragmentation patterns. In order to examine the positions where sugar moieties are attached to aglycones, each sample was also analysed after acidic and basic hydrolysis.

Peak No.	RT (min)	Aglycone MW (Da)	Mass spectral fragmentation pattern*	Quinoa genotypes	Detection after hydrolysis**	
					Acidic	Basic
1	13.3	472	967-162-162-172 = 471	P1; P2; QR; Ti; Pu; Q124	nd	reduced
2	13.6	456	805-162-172 = 455	P1; Ti; Pu	nd	reduced
3	14.0	472	967-162-162-172 = 471	QR; Pu	nd	reduced
4	14.2	456	805-162-172 = 455	P2; QR; Ti; Pu	nd	nd
5	14.5	472	1129-3(162)-172 = 471	P1; P2; QR; Ti; Pu; Q124	nd	reduced
6	15.1	470	1127-3(162)-172 = 469	P1; P2; QR; Ti; Pu; Q124	nd	reduced
7	15.5	500	995-162-162-172 = 499	Pu	nd	reduced
8	16.0	500	995-162-162-172 = 499	P1; P2; QR; Ti; Pu; Q124	nd	nd
9	17.1	500	1005-2(172)-162 = 499	P1; P2; QR; Ti; Pu; Q124	nd	nd
10	18.1	456	951-2(162)-172 = 455	P1; P2; QR; Ti; Pu; Q124	nd	nd
11	18.5	470	965-2(162)-172 = 469	Pu	nd	reduced
12	18.8	470	995-162-162-172 = 499	Ti; Pu; Q124	nd	nd
13	19.3	456	789-162-172 = 455	P1; P2; QR; Ti; Pu; Q124	nd	reduced
14	19.4	500	833-2(172)-162 = 499	Ti; Pu	nd	nd
15	20.3	456	988-3(162)-2(46) = 455	P1; P2; QR; Ti; Pu	nd	nd
16	20.6	456	988-3(162)-2(46) = 455	P1; P2; QR; Ti; Pu	nd	nd
17	21.1	456	988-3(162)-2(46) = 455	P1; P2; QR; Ti; Pu; Q124	nd	reduced
18[a]	21.8	440	972-3(162)-2(46) = 439	P1; P2; QR	nd	nd
19[a]	22.4	440	972-3(162)-2(46) = 439	P1; P2; QR; Pu	nd	nd
20[a]	22.9	440	972-3(162)-2(46) = 439	Ti; Pu	nd	nd
21[a]	23.1	440	972-3(162)-2(46) = 439	P1; P2; QR	nd	nd
22[a]	23.5	440	972-3(162)-2(46) = 439	Pu; Q124	nd	nd
23[a]	24.2	440	972-3(162)-2(46) = 439	P1; P2; QR	nd	reduced
24[a]	25.5	440	949-3(162)-1(46) = 439	P1; P2; QR; Ti	nd	nd

*Mass spectra were recorded in negative mode, thus proposed aglycone masses correspond to [M 1]. Fragmentation patterns from negative mode LC–MS data correspond to MW of aglycone [M H] anions. 46 Da may correspond to loss of either two sodium ions or some other unknown adduct ion as a breakdown product of a molecule during ionization. 162 Da corresponds to loss of hexose; 172 Da may corresponds to loss of a putative acyl moiety (Acyl-H_2O): 3,5-dihydroxy-6-methyloctanoic acid as described in (Sun et al., 2003; Qin et al., 2006). Aglycones with a MW of 456, 472, and 500 Da correspond to Ole, Hed, and SA, respectively. [a]Identification of MW of Phy remains unclear from LC–MS/MS and it is speculated that an observed *m/z* ion 440 Da in MS/MS experiment may in fact correspond to Phy. However interpretation of such a fragmentation of the aglycone remains scarce. An ion at *m/z* 470 also remains unknown although a number of aglycones with the same MW (e.g., 4-OH-epi-hederagenin, glycyrrhetin, and goreishic) have been previously identified (Khakimov et al., 2012, 2013). ** nd: not detected after hydrolysis.

peaks). As shown in Table 2, putative SAPs 1, 5, 6, 8, 9 and 13 were found in all six quinoa genotypes. Peaks 8 and 9 displayed the highest relative percentage in all samples with Q124 showing the highest relative abundance in these two peaks. Peaks 18, 21 and 23 were detected only in samples P1, P2, and QR, while peak 7 and 11 were exclusively observed in Pu. Quinoa variety Q124 was the most different than other five varieties and lacked peaks 15 and 16 (Table 2; Fig. 3).

The LC MS/MS analysis of methanol extracts after acid or basic hydrolysis revealed a dramatic reduction in both the number and intensity of the putative SAP peaks (Table 2). This pattern was observed in all six samples. The hydrolytic process, in which the sugar moieties of SAPs are cleaved off, causes a loss of polarity, resulting in decreased ionization in ESI. Most of the SAP peaks disappeared after acid hydrolysis, while fewer peaks disappeared or were only reduced after mild basic hydrolysis (Table 2). This suggests that the investigated quinoa genotypes possess SAPs decorated with sugar moieties (e.g., glucose, cellobioside) attached to the triterpenoid aglycones either by glucosidic bond (e.g., C3 or C23 positions in Hed) or by ester (e.g., C28 positions in Hed) bond.

4. Discussion

This study reports the relative concentrations of aglycones (after saponification) and SAP profiles obtained by GC MS and LC MS/MS, respectively, from the seed coat powders of six different quinoa varieties. Three major aglycones identified in all six genotypes were Ole, Hed, and Phy. Several studies have identified these three aglycones as the major triterpenoid backbones in both bitter and sweet quinoa seeds (Mizui et al., 1990; Mastebroek et al., 2000; Woldemichael and Wink, 2001; Dini et al., 2001a,b, 2002; Zhu et al., 2002; Madl et al., 2006; Verza et al., 2012). Similar to this study several authors have identified a fourth aglycone, SA, in seeds and other tissues of quinoa as being less abundant than the other triterpenoid aglycones (Dini et al., 2001a,b, 2002; Madl et al., 2006; Kuljanabhagavad et al., 2008; Medina Meza et al., 2016).

The most abundant aglycone detected in the six genotypes was Hed, followed by Phy, and Ole. As previously reported by Kuljanabhagavad et al. (2008), Hed is indeed present in quinoa seed, seed coats, flowers and fruits in high quantities. The relative proportions of triterpenoids changed depending on genotype. The samples consisting of *Real* type varieties (P1, P2, and QR) generally contained higher relative amounts of Hed, Ole, and Phy than other varieties which may account for their strong bitterness. In the Danish varieties Ti and Pu, Phy represented a higher percentage than in other varieties. Phy and Ole were reported to be the most abundant aglycones in seeds of *Ch. berlandieri* and Phy was more concentrated in the seed coat than Ole (Lazo Vélez et al., 2016). While Pu and Ti had distinctly less Ole than other varieties, QR had the highest relative amount of this as well as of the other two aglycones. This difference may be associated with the strongly contrasting origins of these varieties, with QR belonging to the *salares* ecotype (from high altitudes and stressful environments in Bolivia) and the day length neutral varieties, bred in Denmark using Chilean Peruvian material, being better adapted to European environments (Christiansen et al., 2010; Jacobsen, 2015; Ruiz et al., 2016).

Bitterness of quinoa whole seed was associated to a high content of Phy (Medina Meza et al., 2016). Likewise, in this study we found Phy in the seed coat powder of all bitter varieties of quinoa, except in the sweet variety Q124. Moreover, relative amounts of Phy were much higher in Bolivian bitter varieties. In plants, Ole based SAPs may function as defence compounds against herbivores or pathogens

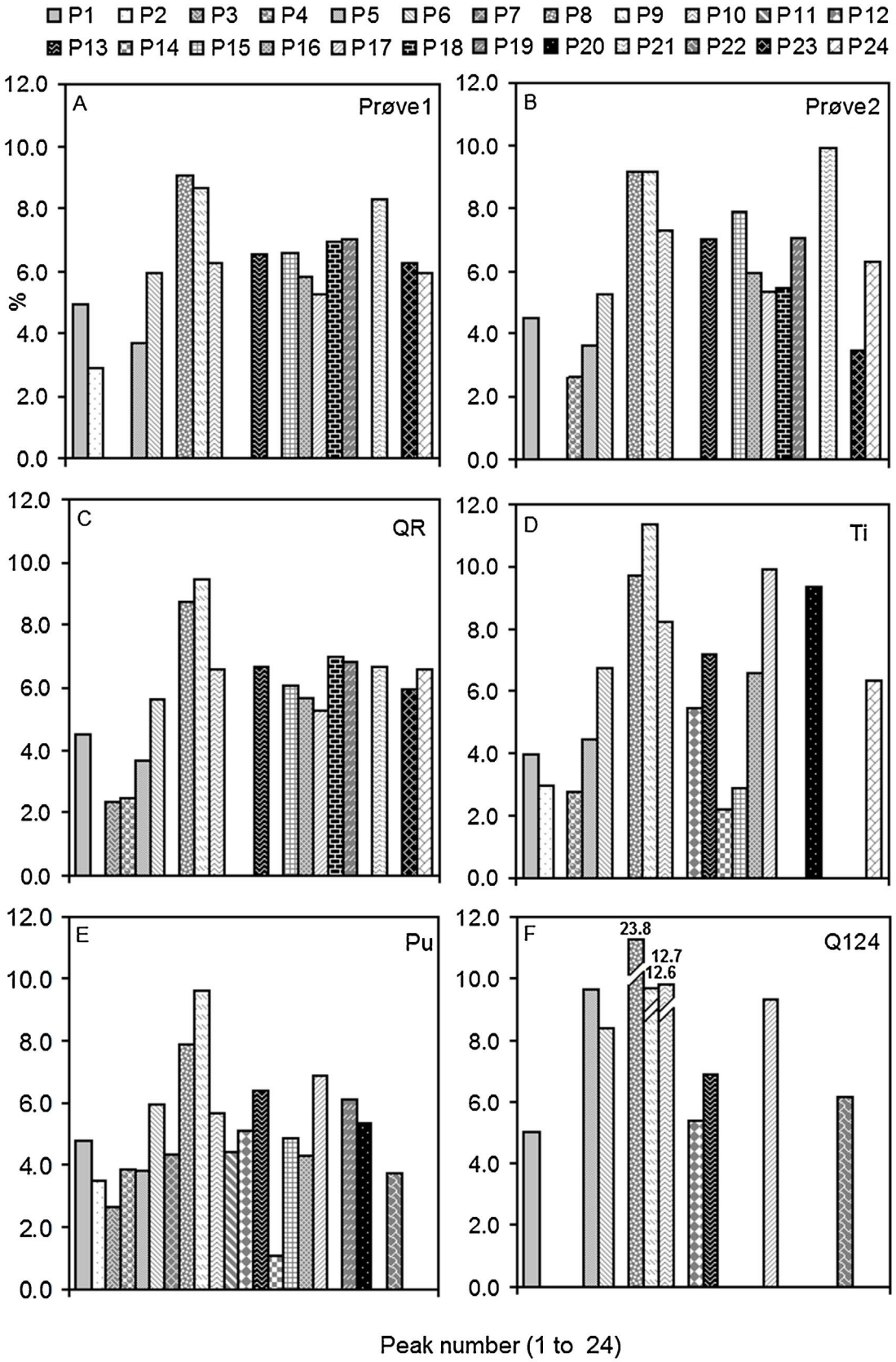

Fig. 3. Relative percentage concentrations of the 24 SAPs tentatively identified by LC–MS/MS in the methanolic extract of seed coats of six different varieties of quinoa. (**A**) P1, (**B**) P2, (**C**) QR, (**D**) Ti, (**E**) Pu, and (**F**) Q124.

(Augustin et al., 2012), or as allelopathic agents (Szakiel et al., 2003; Pollier and Goossens, 2012). Free Ole can be also found in the epicuticular waxes of plants, preventing water loss and serving as a first defence barrier against pathogens (Kubo and Matsumoto, 1984). Woldemichael and Wink (2001) showed that glycosides of Ole and Hed from quinoa seed exhibited antifungal activity against *Candida albicans*. Stuardo and San Martín (2008) found high antifungal activity against *Botrytis cinerea*, one of the most important diseases of table grapes, in alkali treated quinoa saponins based on Phy (the most abundant), Hed and Ole.

A total of 24 putative SAPs were detected from seed coats of six quinoa varieties using LC MS/MS that depicted unique fragmentation patterns. Kuljanabhagavad et al. (2008) identified 20 triterpene SAPs from flowers, fruits, seed coats, and seeds of quinoa, but the presence of at least 80 triterpenoid compounds is predicted (Madl et al., 2006). Other *Chenopodium* species, although closely related, do not contain such a wide variety of SAP structures (*Ch. album*, Lavaud et al., 2000; *Ch. ficifolium*, Gohar et al., 2002). Seeds of varieties belonging to the coastal lowlands ecotype of quinoa (Chilean origin) are considered bitter, like the *Real* varieties (*salares* ecotype), and they may thus be expected to display an equally high diversity of SAPs (Ward 2000). Indeed, Pu, which exhibited the highest SAP diversity, is a Danish bred variety containing Chilean and Peruvian genetic material. On the other hand, Q124 showed the lowest number of peaks in the SAP profile; this relatively lower SAP diversity may partially account for its sweet feature, possibly due to the absence of SAPs responsible for the bitter taste (Ng et al., 1994). Moreover, Q124 had a 1:1 Ole:Hed ratio, while all bitter varieties had higher relative amount of Hed than Ole. A higher Ole:Hed ratio ($>$ ca. 2.5) is associated with the sweet trait and a low ratio ($<$ 2.5) with bitterness (Mastebroek et al., 2000). Meyer et al. (1990) reported that compounds responsible for bitterness in quinoa appear to be a mixture of SAPs whose acidic hydrolysis gave Ole and Hed as the only detectable aglycones by GC/MS analysis; a tetraglycoside of Hed, named quinoside A, was identified as one of these SAPs. Kuljanabhagavad et al. (2008) and Kuljanabhagavad and Wink (2009) reviewed the chemical structures and biological activities of quinoa SAPs, describing the strong relationship between chemical structure of SAPs with their biological activities. Since knowledge regarding the link between biological properties and phytochemistry is, however, still scarce, breeding for quinoas with specific SAP compositions and quantitative structure activity relationship (QSAR) studies will contribute towards understanding this complex issue.

5. Final remarks

Quinoa seeds are a highly nutritional food source that is gaining importance worldwide. This has greatly stimulated its cultivation in recent decades and the trend is likely to continue. In addition, there is an urgent need to discover new SAP sources from annual plant species. Quinoa offers a great potential due to the increasing demand for its seeds, combined with the fact that SAPs, present in the seed coat, are discarded and can be regarded as a waste product. The level of SAPs in quinoa is higher than most other species and by considering quinoa SAPs as a waste product with commercial value, we can overcome the problems associated with forest depletion caused by the use of quillay SAPs and with the suboptimal rheological and biocidal properties of triterpene SAPs from other sources. The broad genotypic diversity of quinoa (Ruiz et al., 2014) and, consequently, the diversity in its SAP composition suggests that optimizing the pharmacological, industrial, and agricultural uses of quinoa seed coat SAPs requires a broader and appropriate selection of genotypes. In this study, the diversity and distribution of agro economically important SAPs from an industrial waste material, quinoa seed coats, is reported for the first time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.04.007.

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