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Grau de Química

Treball Final de Grau

Identification of bioactive compounds in quinoa seeds following a metabolomics approach.

Identificació de compostos bioactius en llavors de quinoa seguint una estratègia metabolòmica.

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M’agradaria dedicar un parell de línies a aquelles persones que han fet possible, d’una manera o altra, la realització d’aquest treball.

A la Laura, per dedicar bona part del seu temps personal a guiar-me i orientar-me en cada pas de la realització d’aquest treball, tant en la part experimental com en la redacció d’aquesta memòria. A la Rocío per la seva amabilitat a l’hora d’ajudar-me sempre que ho he necessitat. A totes dues per estar sempre disposades a resoldre tots els dubtes que m’han sorgit durant aquest procés, moltes gràcies. Als meus pares, germà i parella per donar-me suport dia a dia i empènyer-me a aconseguir tot allò que desitjo.

Moltes gràcies a tots!

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The 2030 Agenda is a plan of action for people, planet, and prosperity developed by the United Nations (UN). It involves the 17 Sustainable Development Goals that incorporate social, economic, and environmental challenges for the sustainable development of humanity [1]. These goals can be divided in the 5P's: People, Prosperity, Planet, Peace, and Partnership.

This work could be included in the People's P and Prosperity's P. This works pretends to improve the knowledge in the quinoa bioactivity in order to find valuable and sustainable uses for quinoa seeds in the agroindustry, as well as to produce functional foods and nutraceuticals. Currently, there are numerous studies reporting the great benefits of quinoa consumption for human health, and this work aims to continue with this line of research.

Figure 1: ODS main goal of this work [1].

Ensuring healthy lives and promoting well-being at all ages is essential for a sustainable development. Among the 17 Sustainable Development Goals, this work covers the number 3: good health and well-being. Despite there is no specific indicator that covers the objective of this work within goal number 3, the realization of this work would be undoubtedly included in the promotion of good health and well-being of society, since it is intended to expand the knowledge about the bioactive compounds of quinoa that offer physiological benefits such as antioxidant, anti-inflammatory, and anticancer bioactivities, among others. In addition, quinoa also provides a glucose-free diet alternative for people with celiac disease.

CONTENTS

1. SUMMARY

Many studies reveal that the intake of food with a high content of bioactive compounds provides physiological benefits to consumers. Quinoa (*Chenopodium quinoa* Willd.) has been the subject of many of these recent studies, since it has been progressively introduced in Western countries, where it is sold as a protein-rich and gluten-free food, which means an excellent alternative for people with celiac disease. In this work, an untargeted metabolomics approach based on liquid chromatography-mass spectrometry (LC-MS) was applied for the identification of low molecular mass bioactive compounds in six colored quinoa seed accessions from different regions of Peru (two white (W), two grey (G), and two pink (P)). After an exhaustive literature search, the creation of an extensive database, and the application of a suitable LC-MS workflow, 111 low molecular mass bioactive compounds were identified (considering all the quinoa accessions). Most of these bioactive compounds belonged to the family of saponins, a type of terpenoids, which possess antioxidant and anticancer activity, among other physiological bioactivities. Flavonoids, phenolic acids, and organic acids were also identified. In order to find metabolomic differences between the accessions, the areas of the low molecular mass bioactive compounds identified in the quinoa accessions were considered for multivariate data analysis using principal component analysis, PCA, followed by partial least squares-discriminant analysis, PLS-DA. After that, a total of 73 out of the 111 identified bioactive compounds were found to be important for discriminating between W, G, and P accessions, 32 of them belonging to the family of saponins.

Keywords: Bioactive compounds, metabolomics approach, PCA, PLS-DA, quinoa

2. RESUM

Molts estudis revelen que la ingesta d'aliments amb un alt contingut de compostos bioactius proporciona beneficis fisiològics per als consumidors. La quinoa (*Chenopodium quinoa* Willd.) ha estat objecte de molts d'aquests estudis recents, ja que s'ha anat introduint progressivament als països occidentals, on es ven com un aliment ric en proteïnes i sense gluten, la qual cosa suposa una excel·lent alternativa per a persones amb malaltia celíaca. En aquest treball, es va aplicar una estratègia metabolòmica no dirigida basada en la cromatografia de líquids acoblada a l'espectrometria de masses (LC-MS) per a la identificació de compostos bioactius de massa molecular baixa en sis accessions de llavors de quinoa de diferent color i de diferents regions del Perú (dues blanques (W), dues grises (G) i dues roses (P)). Després d'una recerca exhaustiva en la literatura, la creació d'una base de dades extensa i l'aplicació d'un flux de treball de LC-MS adequat, es van identificar 111 compostos bioactius de massa molecular baixa (tenint en compte totes les accessions de quinoa). La majoria d'aquests compostos bioactius pertanyen a la família de les saponines, un tipus de terpenoides, que posseeixen activitat antioxidant i anticancerígena, entre d'altres bioactivitats. També es van identificar flavonoides, àcids fenòlics i àcids orgànics. A més, per tal de trobar diferències a nivell metabolòmic entre les accessions, es van considerar les àrees dels compostos identificats per portar a terme una anàlisi de dades multivariant mitjançant l'anàlisi de components principals, PCA, seguida de l'anàlisi discriminant de mínims quadrats parcial, PLS-DA. Després de la seva aplicació, es va trobar que 73 dels 111 compostos bioactius identificats eren importants per discriminar entre les accessions de diferent color (W, G i P), 32 d'ells pertanyents a la família de les saponines.

Paraules clau: Compostos bioactius, estratègia metabolòmica, PCA, PLS-DA, quinoa.

3. INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd.) is an Andean grain recognized for its exceptional nutritional properties and its ability to adapt to very diverse agroecological conditions [2]. Quinoa has been progressively introduced in western countries, where it is sold as a gluten-free protein-rich food with a broad amino acid spectrum, presenting higher levels of lysine, methionine, and cysteine than conventional cereals and legumes [3-6]. Nowadays, there are up to 6,000 quinoa ecotypes [7], which can be classified by their origin and color (e.g., white, black, grey, yellow, red, and pink, etc.), among other properties [8]. Despite the large number of existing quinoa varieties, all of them present, in a greater or lesser amount, low molecular mass compounds, namely bioactive compounds, with a wide range of health-promoting effects and potential bioactivities, fact that makes quinoa products one of the most significant healthy alternatives at these times [7].

Quinoa bioactive compounds can be mainly classified as flavonoids, terpenoids (including saponins), phenolic acids, and organic acids [9]. All of them have been reported to exert numerous bioactivities, including antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, and anticancer, among others [10].

Flavonoids

Flavonoids are polyphenolic compounds responsible for the color and some pharmacological activities of quinoa [11]. Their structure consists of two aromatic (benzene) rings joined together by a 3-carbon chain cyclized through an oxygen atom [12][13]. According to their chemical nature, flavonoids can be divided into isoflavonoids, flavanones, flavanols, flavonols, flavones, and anthocyanidins [14]. A study carried out in 2020 revealed that, among the 6 possible types of flavonoids in quinoa, flavonols are the most abundant, especially quercetin, kaempferol, and their respective derivatives [15]. Despite it has not been possible to determine the specific mechanisms that allow flavonoids to remove free radicals, regulate cell metabolism, and prevent stress-related diseases, it is known that these activities are related to the anticancer bioactivity of quinoa [14].

Terpenoids (saponins)

Terpenoids are metabolites that come from modified terpenes, containing oxygen molecules that are constructed via biochemical modifications (removal or addition of methyl groups) [16]. It has been reported that terpenoids reduce reactive oxygen species through their anti-inflammatory and antioxidant properties and increase the activity of superoxide dismutase in radical scavenging [17]. Terpenoids in quinoa mainly include monoterpenoids and triterpenoids, which are biosynthesized through the isoprenoid metabolic pathway [18]. Monoterpenoids usually play functions as allelochemicals in quinoa [18]. Triterpenoids are present in the seed coats and have a characteristic bitter or astringent taste to protect it from birds and insects. Specifically, saponins are an important group of triterpenoids [19]. They are made up of hydrocarbon chains bound to phenol, alcohol, nitrogen, or sulfur groups (these groups are known as aglycones) [20]. Despite saponins are responsible for the characteristic bitter taste of quinoa, the saponin content decreases in some stages of the processing (e.g., cooking), which significantly reduces its bitterness [20]. Furthermore, several studies of quinoa have reported their numerous bioactivities, including anti-inflammatory, antibacterial, antioxidant, anti-obesity, and neuroprotective effects [17].

Phenolic acids

Phenolic acids are metabolites that contain a phenolic ring and a carboxylic group [21]. Vanillic acid, gallic acid, ferulic acid, and p-hydroxybenzoic acid, have been found in different colored quinoa varieties [11]. Various studies have shown that phenolic acids exert a protective role against cardiovascular diseases, diabetes, and inflammation [10]. Additionally, due to the donation of hydrogen groups, phenolic acids have been reported to present a high antioxidant capacity. Notwithstanding, it is also believed that the high reactivity of the phenol group could be somehow related to this antioxidant activity [7]. Phenolic acids also stand out for their capacity to inhibit α-glucosidase and α-amylase [12]. Inhibitors of these two enzymes are capable of lowering glucose levels after meals, since they decrease the absorption of carbohydrates by the digestive tract [13].

Organic acids

Although the classification of organic acids can be quite complex, in this work, compounds classified as organic acids are mostly linear chain acids without phenolic groups and without or containing few benzene rings [22]. Even though organic acids are reported to be critical nutrients

to determine the quality of different quinoa varieties, they are less studied than, for example, terpenoids, flavonoids, and phenolic acids [23]. Specifically, a study carried out in 2019 [4] determined that the most abundant organic acids in 39 different quinoa varieties (including red, black, and white quinoa) were oxalic acid and citric acid.

Despite the widely recognized beneficial effects of these bioactive compounds in quinoa seeds, their abundance may differ between varieties, mainly depending on the type of farm (e.g., conventional or organic), color, environmental conditions, and growth factors, among others. In this work, in order to reliably characterize six quinoa seed accessions from different regions of Peru, which have never been studied before, an untargeted metabolomics approach based on the obtention of liquid chromatography-mass spectrometry (LC-MS) fingerprints of quinoa bioactive compounds has been applied. After database searching and, due to the high amount of complex multidimensional data generated, multivariate data analysis tools, such as principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), were used to assess metabolomic differences between the accessions. Improved knowledge in the quinoa bioactivity might help in finding valuable and sustainable uses for quinoa seeds in the agroindustry, as well as for the production of functional foods and nutraceuticals.

4. OBJECTIVES

The aim of this work was to apply an untargeted metabolomics approach based on LC-MS for the identification of low molecular mass bioactive compounds (flavonoids, terpenoids (including saponins), phenolic acids, and organic acids) in six colored quinoa seed accessions from different regions of Peru (two white (W), two grey (G), and two pink (P) quinoas). Special emphasis was made on the creation of a comprehensive database of quinoa bioactive compounds, the application of an appropriate workflow for accurate database searching, as well as on the use of multivariate data analysis tools (PCA and PLS-DA) to assess metabolomic differences between the accessions.

5. EXPERIMENTAL SECTION

5.1. SAMPLES

Six different quinoa seed accessions were kindly provided by the National Institute of Agricultural Innovation (INIA), an organization belonging to the Ministry of Agriculture of Peru. These six accessions belong to the INIA germplasm bank. Despite the seeds were cultivated in different regions of Peru (information not provided), all of them are preserved in the Agricultural Experimental Station-ILLPA-PUNO. Table 1 shows the code for the six accessions (also with the official PER code), which are classified by their color (white (W), grey (G), and pink (P)).

Table 1. Codes and classification (by color) for the six quinoa seed accessions studied in this work.

Accession code	Color	PER code
W1	White	PER003423
W2	White	PER004153
G1	Grey	PER004449
G2	Grey	PER003967
P1	Pink	PER004442
P2	Pink	PER004200

5.2. SAMPLE TREATMENT

The extraction of low molecular mass bioactive compounds from the six quinoa seed accessions (W1, W2, G1, G2, P1, and P2) was carried out as described elsewhere with some modifications [24]. It is worth mentioning that this extraction procedure was already done by the Bioanalysis group of the University of Barcelona.

Briefly, quinoa seeds for each accession were ground to a fine powder. Fifty mg of this fine powder were then homogenized with 2.5 mL of a solution of methanol/H_2O (4:1, v/v) with 0.1%

(v/v) of HCOOH and allowed to stand at room temperature for 20 minutes. The mixture was centrifuged at 1000 x g for 20 minutes, keeping the temperature at 4°C. The supernatant was removed, and a second extraction was carried out from the solid residue. In this case, the solid was homogenized with 1 mL of methanol/H_2O (4:1, v/v) with 0.1% (v/v) of HCOOH. The rest of the process was carried out in the same manner as in the first extraction. The two supernatant solutions were mixed and filtered through 0.20 µm nylon filters before analysis by LC-MS.

5.3. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

Quinoa extracts from the six seed accessions (W1, W2, G1, G2, P1, and P2) were analyzed in triplicate by LC-MS (in total, 18 injections). It is also worth mentioning that the LC-MS analysis was already done by the Bioanalysis group of the University of Barcelona following a procedure described in [25].

Briefly, LC-MS experiments were performed in a 1260 Infinity liquid chromatograph coupled to a 6546 LC/QTOF mass spectrometer with an orthogonal electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). For the separation, a Zorbax SB-C18 column (150 mm total length (LT) × 2.1 mm internal diameter (ID), 5 µm particle size, 90 Å pore diameter, Agilent Technologies) was used. Experiments were carried out at a flow rate of 350 µL/min. Mobile phase solvents were (A) water and (B) acetonitrile (both with 0.1 % (v/v) of HCOOH). The optimized elution gradient of solvent B was: 5 % (v/v) for 1 min and from 5 % to 95 % (v/v) in 15 min, followed by cleaning and reequilibration steps at 95 % (v/v) for 2 min, from 95 % to 5 % (v/v) in 2 min, and finally 5 % (v/v) for 5 min. The injection volume was 5 µL. The mass spectrometer was operated in negative ESI mode using the following parameters: capillary voltage 3500 V, drying gas temperature 350°C, flow rate 8 L/min, nebulizer gas 30 psi, fragmentor voltage 150 V, skimmer voltage 60 V, and OCT 1 RF Vpp voltage 300 V. MassHunter software version B.10.0 (Agilent Technologies) was used for instrument control, data acquisition, and data processing.

5.4. DATA PROCESSING

5.4.1. Creation of a database of quinoa bioactive compounds

A comprehensive database of low molecular mass bioactive compounds from quinoa was created after an exhaustive search in the literature [26-82]. This database included information about the molecular formula and the theoretical molecular mass of a total of 565 quinoa bioactive

compounds, which belong to different classes: flavonoids (156 compounds), terpenoids (151 compounds, including 52 saponins), phenolic acids (127 compounds), organic acids (105 compounds), lignans and coumarins (16 compounds), and monosaccharides/disaccharides (10 compounds). Despite lignans, coumarins, and monosaccharides/disaccharides have not been widely studied, they were included in the database, as some publications have reported its presence in quinoa [83].

5.4.2. Identification of bioactive compounds through a database search

Raw LC-MS data files from the quinoa extracts (six accessions per triplicate, in total, 18 injections) were crossed against the database of low molecular mass quinoa bioactive compounds (which needed to be in a specific .csv format) using the Molecular Feature Extraction (MFE) tool of the MassHunter software (version B.10.0, Agilent Technologies). This tool performs mass spectra deconvolution among the chromatographic peaks, allowing to identify compounds (called molecular features) and compare them with those found in suitable databases. MFE has the particularity that locates ions that are covariant in the LC-MS dataset and that are logically related by charge-state envelope, isotopic distribution, and/or presence of adducts. Using this approach, the MFE algorithm can distinguish multiple co-eluting compounds that would appear as a single peak in a chromatogram. The most important parameters of the MFE workflow are as follows:

-Studied time range: from 0 to 18 min (according to the gradient shown in section 5.3)

-Mass error: 20 ppm

-Adducts: hydrogen (-H)

-Charges: from 1 to 2 ($[M-H]^-$ and $[M-2H]^{2-}$ molecular ions, respectively)

-Mas score: 100

-Isotope abundance score: 60

-Isotope spacing score: 50

-Expected MS variation: 2.0 mDa + 5.6 ppm

After using the MFE tool, a list of molecular features was provided for each quinoa sample, including information about the compound name, the retention time (t_r), the experimental molecular mass, and the experimental m/z of the $[M-H]^-$ and/or $[M-2H]^{2-}$ molecular ions. In order

to increase the reliability of the identifications, only features that appear in two out of the three replicates were selected for each quinoa accession.

Peak areas for the selected molecular features were carefully recovered from the full scan LC-MS raw data, taking as a reference the m/z value and the t_r provided by the MFE tool. These areas were used to build a data matrix containing the area of each molecular feature (i.e., bioactive compound) in every sample.

5.4.3. Multivariate data analysis

The areas of the low molecular mass bioactive compounds identified in the different quinoa samples (18 in total) were considered for multivariate data analysis using PCA followed by PLS-DA. PCA was first used for the unsupervised identification of trends and clustering of the data, as well as for the detection of outliers [84]. PLS-DA was then applied to build a classification model with improved class separation [85] and to reveal the importance of the different quinoa bioactive compounds considering their variable importance in the projection (VIP) scores [86]. A leave-one-out cross validation of the PLS-DA model was carried out during model optimization, as well as an auto scale normalization [86]. PCA and PLS-DA were performed using SOLO (Version 9.2.1, Eigenvector Research Incorporated, Wenatchee, WA, USA).

6. IDENTIFICATION OF BIOACTIVE COMPOUNDS

As previously explained, the MFE tool of the Agilent MassHunter software was used in order to cross the raw LC-MS files from the quinoa extracts (18 injections, previously analyzed by the Bioanalysis group) against the database of low molecular mass quinoa bioactive compounds created after an exhaustive search in the literature [26-82]. After applying the MFE tool, a list of molecular features was provided for each quinoa sample, including information about the compound name, the t_r, the experimental molecular mass, and the experimental m/z of the molecular ions. In order to increase the reliability of the identifications, only features that appear in two out of the three replicates were selected for each quinoa accession. After manually checking all the selected features in the full scan LC-MS raw data (taking as a reference the m/z value and the tr provided by the MFE tool), a total of 111 low molecular mass bioactive compounds were identified considering the six quinoa seed accessions (W1, W2, G1, G2, P1, and P2).

Supplementary Table 1, presented as an Annex, shows the peak number (from 1 to 111), the t_r (with the percentage of relative standard deviation, %RSD), the theoretical m/z of the $[M-H]^-$ and/or $[M-2H]^{2-}$ molecular ions, the mass error (in ppm), the compound name, the compound class, and the presence or not (+ and -, respectively) of the identified bioactive compounds in the six quinoa seed accessions. As can be seen in the table, the %RSD in t_r was adequate for all the identified compounds, with values ranging from 0 to 7.0%. Mass errors were also excellent, between 0.1 and 5.1 ppm, confirming the reliability of the identifications. However, it can be observed that some of the identified compounds eluted at different t_r (e.g., licoricesaponin B2 **(63)** and licoricesaponin B2 **(83)**, t_r of 11.03 and 11.57, respectively). This is because, at this point, and taking advantage of the highly-accurate experimental molecular masses provided by the QTOF mass spectrometer in full scan mode, identifications were solely based on the measured experimental molecular masses. In the future, tandem-mass spectrometry (MS/MS) data will be used in order to improve the reliability of the identity assignments, as well as unequivocally confirm the structure of the proposed compounds.

Table 2 shows the relationship between the number of identified low molecular mass bioactive compounds in the six quinoa seed accessions. As can be seen, a similar total number of

compounds were identified in W1, W2, G2, P1, and P2 (47, 54, 58, 47, and 48 compounds, respectively). However, this number was higher in G1, with a total of 70 bioactive compounds identified. Despite the information provided by INIA only allows classifying the seed accessions by their color, variations in the number of identified compounds could also be explained due to differences in the regions where they have been cultivated and farming type, among other properties. Table 2 also shows the relationship between the number of identified low molecular mass bioactive compounds regarding its classification into terpenoids (including saponins), flavonoids, organic acids, phenolic acids, and monosaccharides/disaccharides.

Table 2. Relationship between the number of identified low molecular mass bioactive compounds (and compound classes) in the six quinoa seed accessions.

Sample	Total compounds	Terpenoids (Saponins)	Flavonoids	Organic acids	Phenolic acids	Monosaccharides/ Disaccharides
W1	**47**	29 (18)	9	5	2	2
W2	**54**	31 (23)	8	9	2	4
G1	**70**	41 (33)	11	9	5	4
G2	**58**	30 (24)	10	10	4	4
P1	**47**	18 (14)	13	7	5	4
P2	**48**	25 (18)	11	7	1	4

As it is shown, among the bioactive compounds identified in the six accessions, the higher number of them could be classified as terpenoids, mainly saponins (18, 23, 33, 24, 14, and 18 identified saponins in W1, W2, G1, G2, P1, and P2, respectively). This is because the samples that have been studied in this project are seeds that contain the pericarp (edible tissue around the seeds), where it has been described that saponins are highly-abundant [87]. Flavonoids are the second class with a higher number of identified compounds (between 8 and 13), followed by organic acids (between 5 and 10), phenolic acids (between 1 and 5), and, finally, monosaccharides/disaccharides (between 2 and 4).

Figure 2 shows, as an example, the base peak chromatograms (BPCs) obtained by LC-MS for the quinoa extracts of a white (W1), a grey (G1), and a pink (P1) sample. As it can be seen, the LC-MS profile for the three quinoa seed accessions was very similar at naked eye, and, in general, compound classes followed the same order of elution: organic acids and monosaccharides/disaccharides (t_r from 1.06 to 1.87 min), phenolic acids (t_r from 3.93 to 7.97 min, no peaks are observed in the BPC due to their low intensity), flavonoids (t_r from 8.24 to 9.41 min), and terpenoids (including saponins) (t_r from 9.58 to 14.22 min). However, despite the similarities, we could see a slight difference in the intensity of the three BPCs (see y-axes, higher intensity for W1, followed by G1, and finally, P1), which could be related to a lower concentration of the identified bioactive compounds in G1, and, especially, P1.

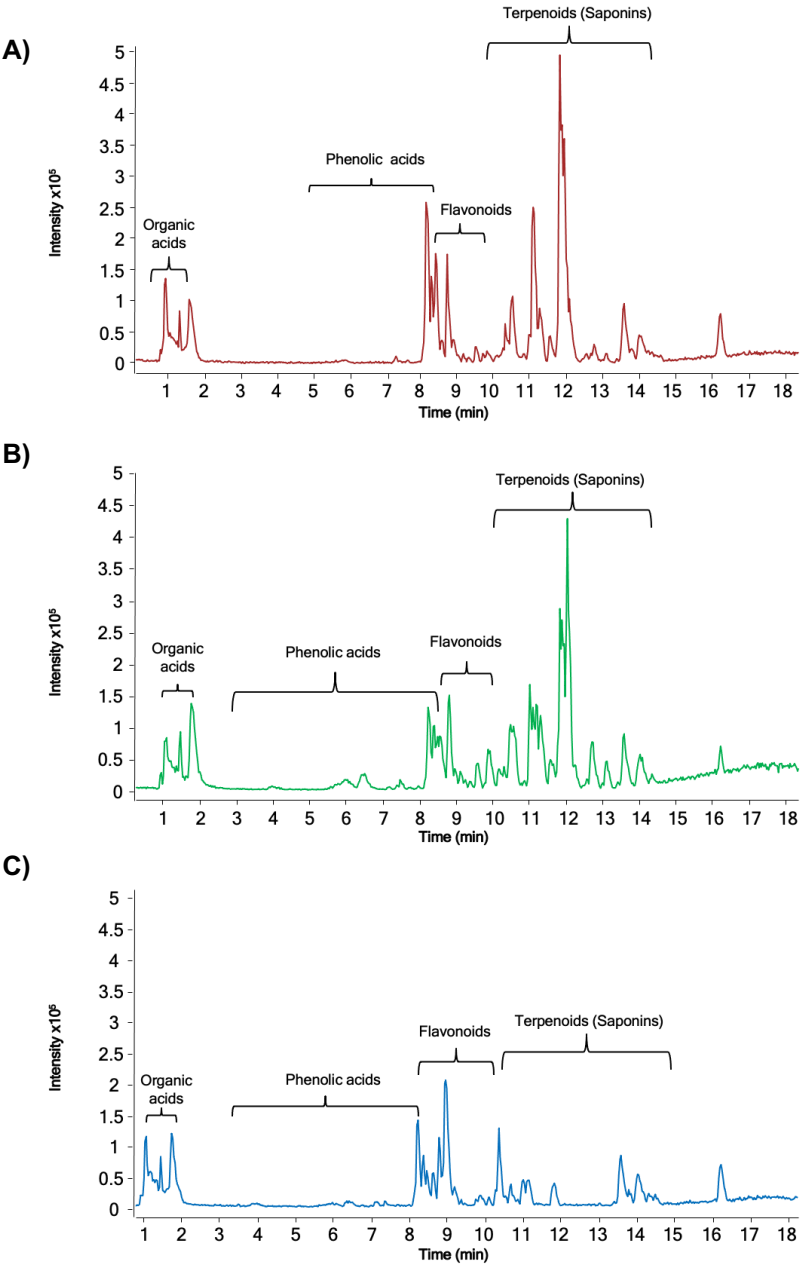

Figure 2. Base peak chromatograms (BPCs) obtained by LC-MS for the quinoa extracts of a (A) white (W1), (B) grey (G1), and (C) pink (P1) sample.

7. MULTIVARIATE DATA ANALYSIS

Despite the LC-MS profile and the number of identified bioactive compounds was similar between the six quinoa seed accessions (regardless of the compound class), it was necessary to consider differences at the concentration level for a more confident discrimination. For this purpose, a matrix containing the areas of the identified compounds (111 columns) in each sample (6 seed accessions per triplicate, in total, 18 rows) was built and subjected to multivariate data analysis, i.e., PCA and PLS-DA.

7.1. PCA

First, we explored the data with PCA for the unsupervised identification of trends and clustering of the data, as well as for the detection of outliers. Two principal components (PCs) allowed explaining 42.1% of the variance (Figure 3). Two PCs were selected because we observed that a 3 PCs model did not improve class separation. As can be observed in the scores plot, PC1 (23.1% of the explained variance) allowed a slight separation between W and G samples (P1 and P2 samples were also clearly separated by PC1), while PC2 (19.0% of the explained variance) slightly separated W and P from G quinoa samples. It is worth mentioning that all the replicates (_1, _2, and _3) from a quinoa seed accession were clustered together, suggesting the excellent repeatability of the extraction procedure and the LC-MS analysis. In addition, all the samples were clustered inside the 95% confidence ellipse, confirming the absence of outliers. As can be seen in Figure 3, W samples were grouped together (W1 and W2 per triplicate, in total, 6 samples), as well as G samples (G1 and G2 per triplicate, in total, 6 samples). However, P samples (P1 and P2) were clearly separated by PC1, probably due to differences in the regions where they have been cultivated and farming type, among other properties (information not provided by INIA).

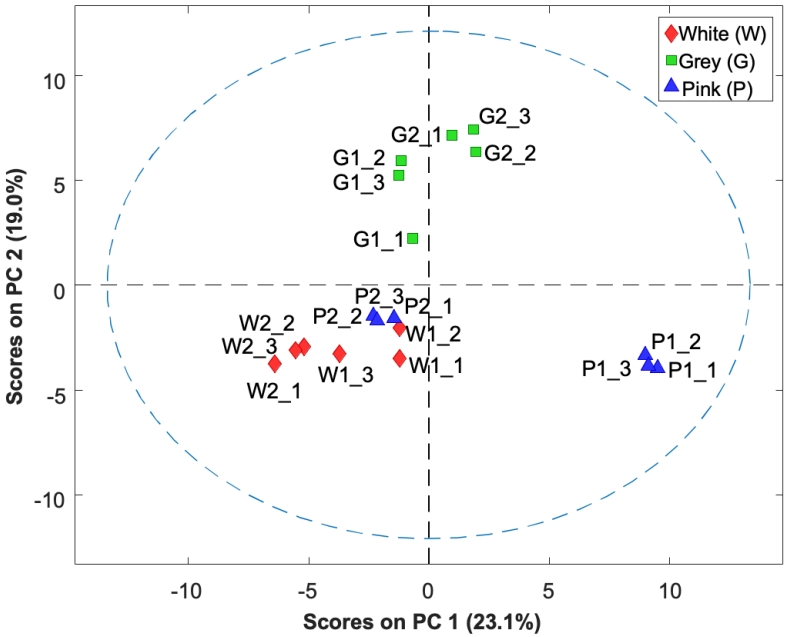

Figure 3. Scores plot of the PCA model applied to the 18 quinoa samples using the areas of the 111 identified low molecular mass compounds.

Once the data was explored by PCA, three classes were defined (i.e., W, G, and P) to build a PLS-DA model with improved class separation and to reveal the importance of the different low molecular mass bioactive compounds for discrimination between the quinoa classes.

7.2. PLS-DA

We built a PLS-DA model with 2 latent variables (LVs) (39.5% of X-variance and 89.3% of Y-variance explained) that allowed a proper discrimination between the three quinoa classes (W, G, and P). Sensitivity, specificity, and classification error in the calibration and leave-one-out cross-validation were excellent. As can be seen in the scores plot (Figure 4), LV1 (19.1% of the explained variance) allowed separating W and P from G quinoa samples, whereas LV2 (20.4% of the explained variance) allowed discriminating W and G from P quinoa samples. In this case, P1 and P2 samples were slightly separated by LV2 (not as much as in PCA), suggesting again differences in the farming region or type.

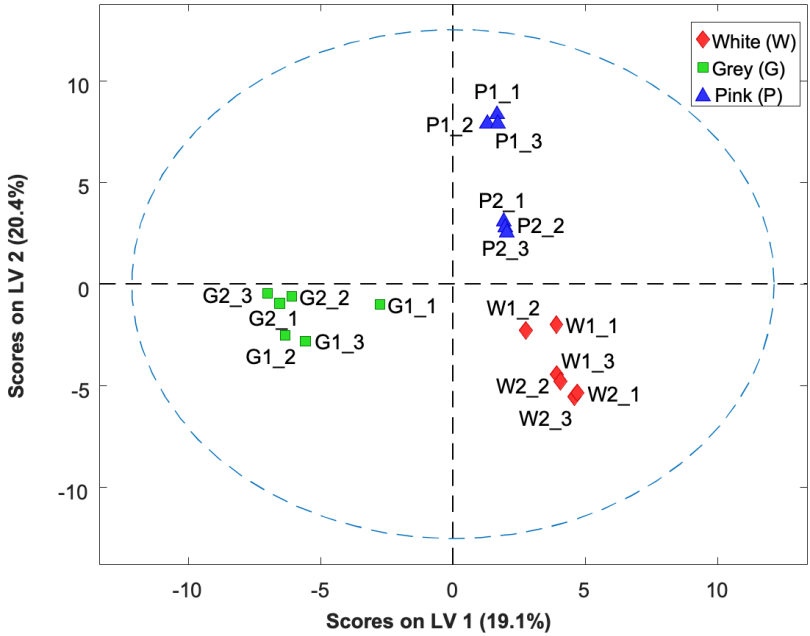

Figure 4. Scores plot of the PLS-DA model applied to the 18 quinoa samples using the areas of the 111 identified low molecular mass compounds.

The contribution of the different variables (low molecular mass bioactive compounds) to the LVs can be observed in the loadings plot of Figure 5. As can be seen in this plot, variables related to W quinoa were grouped in the right lower quadrant (LV1 between 0 and 0.2, and LV2 between 0 and -0.2), variables related to G quinoa were clustered in the left lower quadrant (LV1 between 0 and -0.2, and LV2 between 0 and -0.2), and, finally, variables related to P quinoa were mostly grouped in the upper quadrant (LV1 between -0.2 and 0.2, and LV2 between 0 and 0.2).

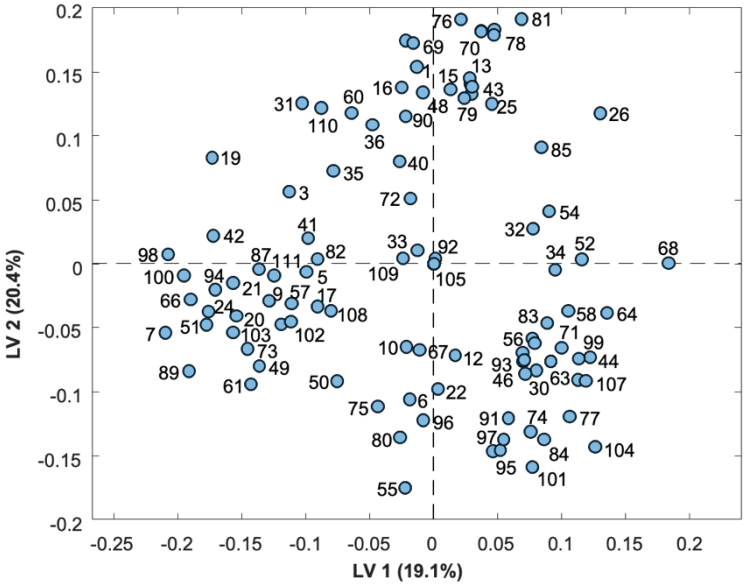

Figure 5. Loadings plot of the PLS-DA model applied to the 18 quinoa samples using the areas of the 111 identified low molecular mass compounds.

In contrast to PCA, the VIP scores in the PLS-DA model allowed to quantify the influence of the different variables on the separation between the quinoa classes. The bar plots of Figure 6 show the VIP scores of the different variables when considering separation of W (A), G (B), and P (C) quinoa samples from the rest of classes, respectively. VIP scores estimated the importance of the low molecular mass bioactive compounds in the projection and only those with a VIP score over 1 were considered important for discrimination [81].

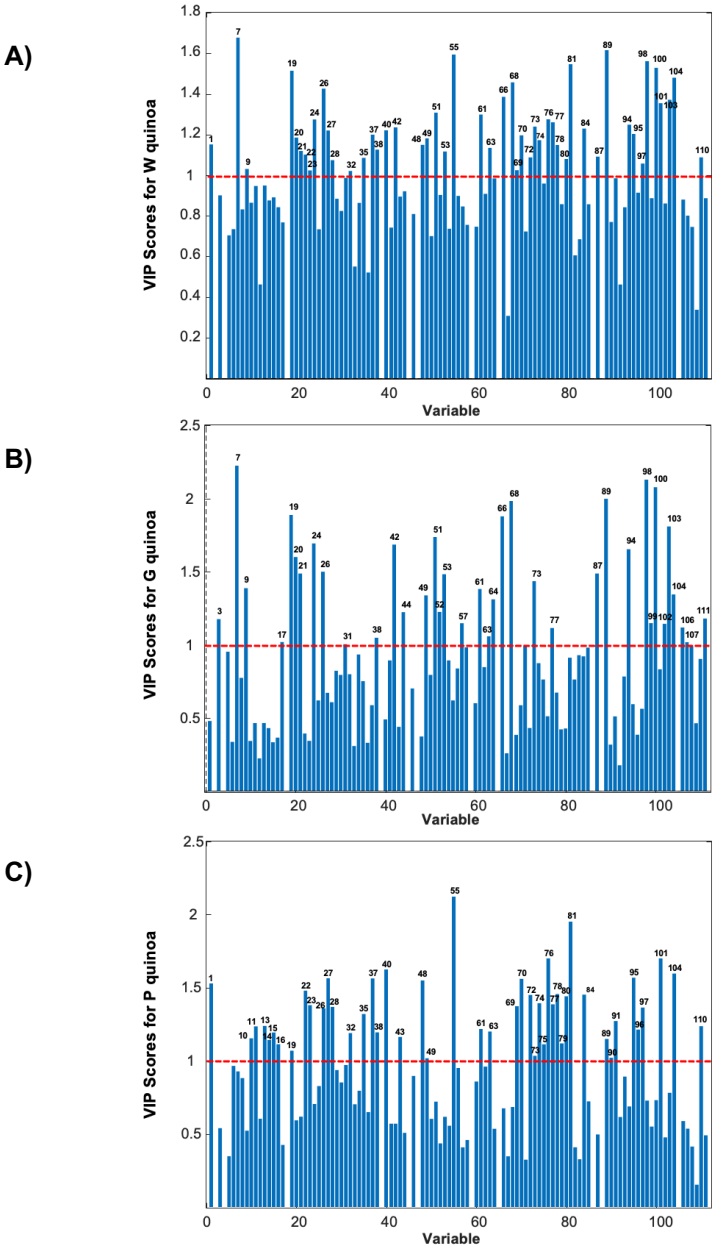

Figure 6. VIP scores of the different variables when considering the separation of (A) W, (B) G, and (C) P samples from the rest of classes. Low molecular mass bioactive compounds with a VIP score value higher than 1 are numbered (see also Table 3).

As can be observed in Figure 6, 49, 37, and 46 out of the 111 identified bioactive compounds were important for discriminating W, G, and P quinoa from the rest of classes, respectively, whereas 38 out of the 111 identified bioactive compounds were non-critical for differentiation. Table 3 shows the VIP scores values higher than 1 for the 73 identified bioactive compounds that were important for discrimination (considering the three classes, W, G, and P). Among them, we found 42 terpenoids (including 32 saponins), 12 flavonoids, 10 organic acids, 6 phenolic acids, and 3 monosaccharides/disaccharides. The higher number of bioactive compounds critical for discrimination between classes were, as expected, saponins. This fact is logical, considering that they represented the highest percentage of identified compounds in each quinoa seed accession (see Table 2).

Table 3. Peak number and compound class of the identified low molecular mass compounds used as PLS-DA variables with their corresponding VIP scores values (for discrimination between W, G, and P samples).

PLS-DA bioactive compounds variables				
Peak number	**Compound class**	**VIP Scores**		
		W	**G**	**P**
1	Monosaccharide	1.15	-	1.53
3	Disaccharide	-	1.18	-
7	Organic acid	1.68	2.22	-
9	Organic acid	1.03	1.39	-
10	Organic acid	-	-	1.15
11	Organic acid	-	-	1.23
13	Disaccharide	-	-	1.24
14	Phenolic acid	-	-	1.14
15	Phenolic acid	-	-	1.19
16	Phenolic acid	-	-	1.11

17	Phenolic acid	-	1.02	-
19	Flavonoid	1.51	1.88	1.07
20	Flavonoid	1.19	1.60	-
21	Organic acid	1.12	1.48	-
22	Flavonoid	1.10	-	1.48
23	Flavonoid	1.02	-	1.38
24	Phenolic acid	1.27	1.70	-
26	Flavonoid	1.43	1.50	1.36
27	Flavonoid	1.22	-	1.56
28	Flavonoid	1.07	-	1.37
31	Flavonoid	-	1.01	-
32	Flavonoid	1.02	-	1.19
35	Flavonoid	1.09	-	1.32
37	Organic acid	1.20	-	1.56
38	Flavonoid	1.13	1.05	1.19
40	Flavonoid	1.22	-	1.62
42	Organic acid	1.23	1.68	-
43	Terpenoid (saponin)	-	-	1.16
44	Terpenoid (saponin)	-	1.22	-
48	Terpenoid	1.15	-	1.55
49	Terpenoid (saponin)	1.18	1.33	1.02
51	Terpenoid (saponin)	1.31	1.73	-

52	Terpenoid (saponin)	-	1.22	-
53	Terpenoid	1.12	1.48	-
55	Terpenoid (saponin)	1.59	-	2.12
57	Terpenoid (saponin)	-	1.15	-
61	Terpenoid (saponin)	1.30	1.38	1.22
63	Terpenoid	1.13	1.06	1.20
64	Terpenoid (saponin)	-	1.31	-
66	Terpenoid (saponin)	1.38	1.88	-
68	Terpenoid (saponin)	1.46	1.98	-
69	Terpenoid (saponin)	1.03	-	1.37
70	Terpenoid	1.20	-	1.56
72	Terpenoid (saponin)	1.09	-	1.45
73	Terpenoid (saponin)	1.24	1.43	1.03
74	Terpenoid (saponin)	1.17	-	1.39
75	Terpenoid (saponin)	-	-	1.11
76	Terpenoid (saponin)	1.28	-	1.70
77	Terpenoid (saponin)	1.26	1.11	1.39
78	Terpenoid	1.15	-	1.45
79	Terpenoid (saponin)	-	-	1.12
80	Terpenoid	1.08	-	1.44
81	Terpenoid (saponin)	1.54	-	1.95
84	Terpenoid (saponin)	1.23	-	1.45

87	Terpenoid (saponin)	1.09	1.48	-
89	Terpenoid (saponin)	1.61	2.01	1.15
90	Terpenoid (saponin)	-	-	1.02
91	Terpenoid (saponin)	-	-	1.27
94	Terpenoid (saponin)	1.25	1.65	-
95	Terpenoid (saponin)	1.20	-	1.57
96	Terpenoid	-	-	1.21
97	Terpenoid	1.06	-	1.36
98	Terpenoid (saponin)	1.56	2.13	-
99	Terpenoid	-	1.15	-
100	Terpenoid (saponin)	1.53	2.08	-
101	Terpenoid (saponin)	1.35	-	1.70
102	Terpenoid (saponin)	-	1.14	-
103	Terpenoid	1.37	1.81	-
104	Terpenoid (saponin)	1.48	1.34	1.60
106	Phenolic acid	-	1.12	-
107	Organic acid	-	1.02	-
110	Organic acid	1.09	-	1.23
111	Organic acid	-	1.18	-

As can be seen in the Venn diagram of Figure 7 (and in Supplementary Table 1), from the total of 73 bioactive compounds critical for differentiation, 12 were exclusively identified in G and 12 in P quinoa samples, suggesting their importance as makers for discriminating between the classes. Regarding bioactive compounds found in more than one class, 10 were found in W, G,

and P quinoa samples, 15 in W and G quinoa samples, and 24 in W and P quinoa samples. Despite the advantageous information obtained after applying PLS-DA and VIPs, a more complete study using the areas of the identified compounds, as well as statistical methods to find significant differences between, will be applied. In this way, we will be able, not only to stablish which compounds are critical for differentiation, but also to find if they are up-regulated, down-regulated, or unaltered between classes.

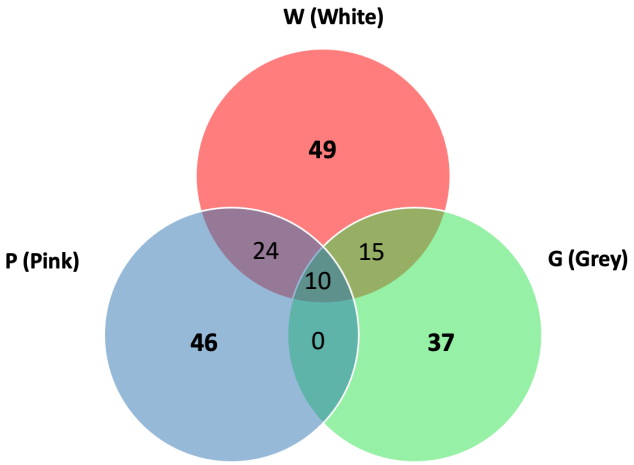

Figure 7. Venn diagram showing the relationship between the number of identified low molecular mass compounds critical for discriminating W, G, an P quinoa samples. (Only the identification in one of the two accessions of the same color was enough to be considered in the representation).

10. CONCLUSIONS

The LC-MS metabolomics approach applied in this work, after the creation of a comprehensive database and the application of an appropriate workflow, allowed the identification of 111 low molecular mass bioactive compounds in six colored quinoa seed accessions from different regions of Peru (two W, two G, and two P samples). It was observed that, among all the compound classes (terpenoids (including saponins), flavonoids, organic acids, phenolic acids, and monosaccharides/disaccharides), saponins represented the highest percentage of identified compounds in all the quinoa seed accessions. However, as the number of bioactive compounds was similar in all the studied samples, it was necessary to consider differences at the concentration level for a more confident discrimination. For this purpose, a matrix containing the areas of the identified compounds in all the samples was built and subjected to multivariate data analysis, i.e., PCA and PLS-DA.

Unsupervised PCA allowed organizing the samples in three main classes: W, G, and P. After that, supervised PLS-DA in combination with VIPs allowed quantifying the influence of the different low molecular mass compounds on the separation between the classes. A total of 73 out of the 111 identified bioactive compounds were found to be important for discriminating between W, G, and P samples. Among them, 12 compounds were exclusively identified in W, and 12 in P quinoa samples. Regarding bioactive compounds found in more than one class, 10 were found in W, G, and P quinoa samples, 15 in W and G quinoa samples, and 24 in G and P quinoa samples. In the future, a more complete study using the areas of the identified compounds, as well as statistical methods to find significant differences between, will be applied. In addition, MS/MS data will also be interpreted, in order to improve the reliability of the identity assignments, as well as unequivocally confirm the structure of the proposed compounds.

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APPENDICES

APPENDIX 1: SUPPLEMENTARY TABLE 1

Supplementary table 1. Peak number (from 1 to 111), t_r (with the percentage of relative standard deviation, %RSD), theoretical m/z of the $[M-H]^-$ and/or $[M-2H]^{2-}$ molecular ions, mass error (in ppm), compound name, compound class, and presence or not (+ and -, respectively) of the identified bioactive compounds in the six quinoa seed accessions.

[a]Peak num.	[b]t_r (min)	RSD t_r (%)	m/z $_{theo}$ $[M-H]^-$ $[M-2H]^{2-}$	[c,d]Error ppm	Identification	Compound class	W1	W2	G1	G2	P1	P2
1	1.02	0.9	181.0718	1.6	D-Mannitol	Monosaccharide	-	+	+	+	+	+
2	1.06	0.0	165.0405	0.9	D-Xylonic acid	Organic acid	-	+	-	-	-	-
3	1.07	0.9	341.1089	1.5	Maltose	Disaccharide	+	+	+	+	+	+
4	1.09	0.0	99.0088	5.1	Succinic anhydride	Organic acid	-	+	-	-	-	-
5	1.09	2.1	149.0455	1.9	Arabinose	Monosaccharide	-	+	+	+	+	+
6	1.15	0.8	133.0142	2.8	3-Dehydro-L-Threonic Acid	Organic acid	-	+	+	+	-	-
7	1.17	0.6	191.0197	3.5	Isocitric Acid	Organic acid	-	-	+	+	-	-
8	1.36	0.0	341.1089	0.7	Maltose	Disaccharide	-	+	-	-	-	-
9	1.46	0.9	191.0197	2.6	Citric Acid	Organic acid	-	+	+	+	-	-
10	1.52	7.0	128.0353	2.9	1-Pyrroline-4-hydroxy-2-carboxylic acid	Organic acid	+	+	+	+	+	+
11	1.57	0.0	117.0193	2.1	Methylmalonic acid	Organic acid	-	-	-	-	+	-
12	1.65	1.0	341.1089	1.9	Maltose	Disaccharide	+	-	+	+	-	+
13	1.87	0.0	341.1089	0.1	Maltose	Disaccharide	-	-	-	-	+	-
14	3.93	0.0	109.0295	0.9	Hydroquinone	Phenolic acid	-	-	-	-	+	-
15	3.97	1.9	153.0193	1.6	2,6-Dihydroxybenzoic acid	Phenolic acid	-	-	+	-	+	-
16	6.47	0.9	137.0244	2.7	P-Salicylic acid	Phenolic acid	-	-	+	+	+	-
17	7.17	0.0	355.1035	2.5	6-O-Feruloyl-D-Glucose	Phenolic acid	-	-	+	-	-	-
18	7.97	0.0	121.0295	0.4	Benzoic acid	Phenolic acid	-	-	-	-	+	-
19	8.24	0.3	755.204	1.4	Kaempferol-3-O-neohesperidoside-7-O-glucoside	Flavonoid	+	+	+	+	+	+

20	8.36	0.7	741.1884	2.5	Luteolin-7-O-Sophoroside-5-O-arabinoside	Flavonoid	+	+	+	+	+	+
21	8.37	0.0	159.0452	3.1	6-MethylCoumarin	Organic acid	-	-	-	+	-	-
22	8.49	0.3	739.2091	1.6	Apigenin-7-O-glucoside-4'-O-rutinoside	Flavonoid	+	+	+	+	+	+
23	8.57	0.3	769.2197	1.6	Isorhamnetin-3-O-rutinoside-7-O-rhamnoside	Flavonoid	-	-	+	+	+	-
24	8.58	0.1	163.0401	4.1	Caffeic aldehyde	Phenolic acid	-	-	+	+	-	-
25	8.59	0.9	609.1461	1.4	Kaempferol-3-O-sophoroside	Flavonoid	+	-	+	-	+	+
26	8.64	0.2	595.1305	0.5	Quercetin-3-O-sambubioside	Flavonoid	+	-	-	-	+	+
27	8.66	0.2	631.0941	1.2	Myricetin 7-(6' -galloside)	Flavonoid	-	-	+	-	+	-
28	8.66	0.3	725.1935	1.5	Kaempferol-3-O-(2-O-Xylosyl-6-O-Rhamnosyl) Glucoside	Flavonoid	+	+	+	+	+	+
29	8.71	0.0	609.1461	0.8	Kaempferol-3-O-sophoroside	Flavonoid	+	-	-	-	-	-
30	8.80	0.0	567.3175	1.0	Chenodeoxycholic Acid 24-Acyl-|A-D-Glucuronide	Organic acid	+	+	-	-	-	+
31	8.85	0.3	607.1305	2.3	Luteolin-7-O-glucuronide-5-O-rhamnoside	Flavonoid	-	-	+	+	+	-
32	8.89	1.0	593.1512	1.1	Luteolin-7-O-neohesperidoside (Lonicerin)	Flavonoid	-	+	-	-	-	+
33	8.89	0.3	758.1911	1.6	Delphinidin 3-lathyroside 5-glucoside	Flavonoid	-	-	+	+	+	-
34	8.94	0.0	579.1355	0.8	Quercetin-3-O-rhamnosyl (1→2) arabinoside	Flavonoid	+	-	-	-	-	+
35	8.97	0.3	477.0675	1.8	Quercetin-3-O-glucuronide	Flavonoid	-	+	+	+	+	+
36	8.97	0.3	163.0401	0.9	Caffeic aldehyde	Phenolic acid	+	+	-	+	+	+
37	9.07	0.0	133.0142	0.5	3-Dehydro-L-Threonic Acid	Organic acid	-	-	-	-	+	-
38	9.17	0.0	623.1618	2.0	Chrysoeriol-5,7-di-O-glucoside	Flavonoid	+	-	-	-	-	-
39	9.39	0.0	285.0405	0.9	Kaempferol	Flavonoid	-	+	-	-	-	-
40	9.41	0.3	461.0725	1.4	Kaempferol-3-O-glucuronoside	Flavonoid	+	+	+	+	+	+
41	9.58	0.1	943.4908	2.4	3,23,30-trihydroxyolean-12-en-28-oic acid 3-O-β-d-Glucopyranosyl-(1,3)-α-l-	Terpenoid (saponin)	-	-	-	+	-	+

					arabinopyranosyl-28-O-β-d-glucopyranoside								
42	9.79	0.3	187.0976	2.2	Azelaic acid	Organic acid	+	-	+	+	+	+
43	9.79	0.0	985.465	1.7	Phytolaccagenic acid 3-O- [α-l-arabinopyranosyl-(1,3)-β-d-glucuronopyranosy]-28 -O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	-	-	+	-
44	9.84	0.0	581.2709	1.6	Phytolaccagenic acid 3-*O*-β-d-Glucopyranosyl-(1,4)-β-d-glucopyranosyl-(1,4) -β-d-glucopyranosyl 28-*O*-β-d-glucopyranoside	Terpenoid (saponin)	-	+	-	-	-	+
45	9.85	0.0	971.4857	0.9	Phytolaccagenic acid 3-O-[β-d-glucopyranosyl-(1,3)-α-l-arabinopyranosyl]-28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	-	+	-	-
46	9.96	0.1	943.4908	2.0	3,23,30-trihydroxyolean-12-en-28-oic acid 3-O-β-d-Glucopyranosyl-(1,3)-α-l-arabinopyranosyl-28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	+	+	-	-	-
47	9.97	0.1	827.4435	2.2	1beta,2alpha,3beta,19alpha-Tetrahydroxy-12-ursen-28-oic acid 28-O-[b-D-Glucopyranosyl-(1→2)-b-D-glucopyranosyl] ester	Terpenoid	-	+	+	-	-	-
48	10.07	0.0	955.4544	1.6	Medicagenic acid-3-O-glucuronide-28-O-rhamnosyl (1,2)-arabinoside	Terpenoid	-	-	-	-	+	+
49	10.14	0.1	1001.4963	2.1	Phytolaccagenic acid 3-O-β-d-Glucopyranosyl-(1,3)-β-d-galactopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	-	+
50	10.25	0.1	987.4806	2.1	Medicagenic acid-3-O-glucosyl-(1,6)-glucosyl-(1,3)-glucoside	Terpenoid	+	+	+	+	-	+
51	10.27	0.0	1133.5385	2.4	Phytolaccagenic acid 3-O-β-d-glucopyranosyl-(1,2)-β-d-glucopyranosyl-(1,3) -α-l-arabinopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	+	-	-
52	10.37	1.5	566.2656	1.2	Phytolaccagenic acid 3-O-[β-d-glucopyranosyl-(1,3)-β-d-xylopyranosyl-(1,2)-β-d-	Terpenoid (saponin)	-	+	-	-	+	-

					glucopyranosyl]-28-O-β-d-glucopyranoside								
53	10.37	0.0	827.4435	3.8	1beta,2alpha,3beta,19alpha-Tetrahydroxy-12-ursen-28-oic acid 28-O-[b-D-Glucopyranosyl-(1→2)-b-D-glucopyranosyl] ester	Terpenoid	-	-	+	-	-	-
54	10.55	2.3	985.465	1.2	Phytolaccagenic acid 3-O- [α -l-arabinopyranosyl-(1,3)-β-d-glucuronopyranosyl]-28 -O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	+	+
55	10.58	0.2	809.4329	2.0	Phytolaccagenic acid 3-O-α -l-arabinopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	-	-
56	10.69	0.0	811.4485	10.8	Kudzusaponin SA1	Terpenoid	+	-	-	-	-	-
57	10.69	2.0	971.4857	1.6	Phytolaccagenic acid 3-O-[β-d-glucopyranosyl-(1,3)-α-l-arabinopyranosyl]-28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	-	+	-
58	10.77	1.1	957.5065	2.0	Hederagenin 3-O-β-d-Glucopyranosyl-(1,3)-α-l-galactopyranosyl 28-O- β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	-	-	+
59	10.93	0.0	939.4595	1.8	Melilotussaponin O2	Terpenoid	+	-	-	-	-	-
60	10.95	0.4	301.0354	2.1	Morin	Flavonoid	-	-	-	+	+	-
61	11.02	0.3	1089.5487	2.2	3,23-bis(*O*-β-d-Glucopyranosylox) olean-12-en28-oic acid28-O-α-l-arabinopyanosyl-(1,3) -β-d-glucopyranoside	Terpenoid (saponin)	-	+	+	+	-	-
62	11.02	0.0	809.4329	0.7	Phytolaccagenic acid 3-O-α -l-arabinopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	-	-	-	-	-
63	11.03	0.0	807.4172	3.0	Licoricesaponin B2	Terpenoid	+	-	-	-	-	-
64	11.04	0.6	581.2709	1.7	Phytolaccagenic acid 3-O-β-d-Glucopyranosyl-(1,4)-β-d-glucopyranosyl-(1,4) -β-d-glucopyranosyl 28-*O*-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	+	+
65	11.05	0.0	807.4172	1.5	Licoricesaponin B2	Terpenoid	-	+	-	-	-	-
66	11.09	0.9	809.4329	2.6	Phytolaccagenic acid 3-O-α -l-arabinopyranosyl 28-	Terpenoid (saponin)	+	+	+	+	+	-

					O-β-d-glucopyranoside							
67	11.12	0.8	1117.5436	1.5	Serjanic acid-glucopyranosyl-(1,2)-β-D-glucopyranosyl-(1,3)-α-L-arabinopyranosyl-28-O-β-D - 3-O-β-d-glucopyranosyl-(1,2)-β-d-glucopyranosyl-(1,3) glucopyranoside -α-l-arabinopyranosyl-28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	-	+
68	11.12	0.0	941.4752	1.1	Hederagenin 3-O-β-d-Xylopyranosyl-(1,3)-β-d-glucuronopyranosyl-28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	-	-	+	+
69	11.13	1.4	927.4959	1.5	Hederagenin-3-O-glucosyl (1-2) glucosyl(1-4)arabinoside	Terpenoid (saponin)	-	-	+	+	+	-
70	11.13	0.0	987.4806	1.9	Medicagenic acid-3-O-glucosyl-(1,6)-glucosyl-(1,3)-glucoside	Terpenoid	-	-	-	-	+	-
71	11.18	0.0	581.2709	2.3	Phytolaccagenic acid 3-O-β-d-Glucopyranosyl-(1,4)-β-d-glucopyranosyl-(1,4) -β-d-glucopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	+	+	-	-	+
72	11.20	0.1	1089.5487	2.4	3,23-bis(O-β-d-Glucopyranosyloxy)olean-12-en28-oicacid28-O-α-l-arabinopyanosyl-(1,3) -β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	+	-	+
73	11.21	0.1	809.4329	2.1	Phytolaccagenic acid 3-O-α -l-arabinopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	+	-	-
74	11.32	0.1	765.4431	1.9	Hederagenin 3-O-β-d-glucopyranosyl-(1,3)- α-l-arabinopyranoside	Terpenoid (saponin)	+	+	+	+	-	+
75	11.34	0.0	1001.4963	1.9	Phytolaccagenic acid 3-O-β-d-Glucopyranosyl-(1,3)-β-d-galactopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	-	-	-
76	11.38	1.0	955.4908	1.2	3-O-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl serjanic acid 28-O-β-D-glucopyranosyl ester	Terpenoid (saponin)	-	-	-	+	+	+
77	11.39	0.1	837.4278	1.4	Serjanic acid 3-O-β-d-glucuronopyranosyl-	Terpenoid (saponin)	+	+	+	-	-	-

					28-O-β-d-glucopyranoside								
78	11.41	0.2	939.4595	0.9	Melilotussaponin O2	Terpenoid	+	-	-	-	+	-
79	11.41	0.0	985.465	0.8	Phytolaccagenic acid 3-O- [α -l-arabinopyranosyl-(1,3)-β-d-glucuronopyranosyl]-28 -O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	-	-	+	-
80	11.42	0.1	851.4435	2.0	3-((3'-Malonyl)Xyl)-28-Glu-2-hydroxy oleanolic acid	Terpenoid	+	+	+	+	-	+
81	11.47	0.1	566.2656	2.0	Phytolaccagenic acid 3-*O*-β-d-glucopyranosyl-(1,2)-β-d-glucopyranosyl-(1,3) -α-l-arabinopyranosyl 28-*O*-β-d-glucopyranoside	Terpenoid (saponin)	+	-	-	-	+	+
82	11.57	2.1	955.4544	2.5	Medicagenic acid-3-O-glucuronide-28-O-rhamnosyl (1,2)-arabinoside	Terpenoid	+	-	+	+	+	-
83	11.57	0.1	807.4172	1.7	Licoricesaponin B2	Terpenoid	-	+	-	-	-	+
84	11.59	0.1	1001.4963	1.7	Phytolaccagenic acid 3-O-β-d-Glucopyranosyl-(1,3)-β-d-galactopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	-	+
85	11.68	1.2	971.4857	1.9	Phytolaccagenic acid 3-*O*-[β-d-glucopyranosyl-(1,3)-α-l-arabinopyranosyl]-28-O-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	+	+
86	11.80	0.0	285.0405	1.2	Kaempferol	Flavonoid	-	-	-	-	-	+
87	11.86	0.1	809.4329	2.2	Phytolaccagenic acid 3-O-α -l-arabinopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	+	-	+
88	11.89	0.0	1119.5593	2.3	Hederagenin 3-O-β-d-Glucopyranosyl-(1,4)-β-d-glucopyranosyl-(1,4)-β-d-glucopyranosyl-28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	-	-	-
89	11.89	0.0	1073.5538	2.5	Oleanolic acid 3-O-β-d-glucopyranosyl-(1,2)-β-d-glucopyranosyl-(1,3)-α-l-arabinopyranosyl 28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	+	+	+	-	-
90	11.89	0.5	925.4802	0.9	Oleanolic acid 3-O-α-l-arabinopyranosyl-(1,3)-β-d-glucuronopyranosyl-	Terpenoid (saponin)	-	-	+	-	+	-

					28-O-β-D-glucopyranoside							
91	11.91	0.1	971.4857	2.3	Phytolaccagenic acid 3-O-[β-d-glucopyranosyl-(1,3)-α-l-arabinopyranosyl]-28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	+	+	-	-	-
92	11.91	0.0	811.4485	2.3	Kudzusaponin SA1	Terpenoid	+	-	-	+	-	+
93	12.08	0.0	478.2496	2.0	Hederagenin 3-O-β-d-Glucopyranosyl-(1,3)-α-l-galactopyranosyl 28-O- β-d-glucopyranoside	Terpenoid (saponin)	+	-	-	-	-	-
94	12.09	0.1	455.2468	2.7	Oleanolic acid 3-O-[β-d- glucopyranosyl-(1,3)-α-l-arabinopyranosyl]-28-O-β-D-glucopyranoside	Terpenoid (saponin)	-	-	+	+	-	-
95	12.24	0.4	793.438	1.7	Serjanic acid 3-*O*-α-l-arabinopyranosyl-28-*O*-β-d-glucopyranoside	Terpenoid (saponin)	+	+	+	+	-	-
96	12.38	1.6	851.4435	1.7	3-((3'-Malonyl)Xyl)-28-Glu-2-hydroxy oleanolic acid	Terpenoid	+	+	+	-	-	-
97	12.62	0.7	853.4591	1.7	Hovenidulcioside B1	Terpenoid	+	+	+	+	-	+
98	12.63	0.2	955.4908	1.8	3-O-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl serjanic acid 28-O-β-D-glucopyranosyl ester	Terpenoid (saponin)	-	-	+	+	+	+
99	12.72	0.1	939.4595	1.4	Melilotussaponin O2	Terpenoid	-	+	-	-	-	+
100	12.73	0.1	985.465	2.6	Phytolaccagenic acid 3-O- [α -l-arabinopyranosyl-(1,3)-β-d-glucuronopyranosyl]-28 -O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	+	-	+
101	13.01	0.0	765.4431	2.5	Hederagenin 3-O-β-d-glucopyranosyl-(1,3)- α-l-arabinopyranoside	Terpenoid (saponin)	+	+	+	+	-	-
102	13.01	0.0	793.438	2.3	Serjanic acid 3-O-α-l-arabinopyranosyl-28-O-β-d-glucopyranoside	Terpenoid (saponin)	-	-	+	-	-	-
103	13.15	1.2	795.4536	1.9	3-Gal(1-2)GluA Soyasapogenol B	Terpenoid	+	-	+	+	-	-
104	13.23	1.4	925.4802	1.7	Oleanolic acid 3-O-α-l-arabinopyranosyl-(1,3)-β-d-glucuronopyranosyl-28-O-β-D-glucopyranoside	Terpenoid (saponin)	+	+	+	-	-	-
105	14.22	0.3	763.4274	2.0	Oleanolic acid-3-O-xylosyl(1→3) glucuronide	Terpenoid (saponin)	+	-	+	-	-	-

106	14.27	0.1	791.3859	1.9	5,6-dihydropenicillic acid	Phenolic acid	+	+	+	+	-	+
107	14.46	0.1	313.2384	1.5	Sebacate	Organic acid	+	+	+	+	+	+
108	14.61	0.0	313.2384	3.4	Sebacate	Organic acid	-	-	+	-	-	-
109	16.24	0.1	295.2279	1.4	(±)12(13)-EpOME	Organic acid	+	+	+	+	+	+
110	16.94	1.2	297.2435	1.6	(E)-6-hydroxyoctadec-4-enoic acid	Organic acid	-	+	+	+	+	+
111	17.58	0.0	295.2279	3.6	(±)12(13)-EpOME	Organic acid	-	-	-	+	-	-

[a]Peak numbers are ordered by t_r.
[b]t_r is presented as an average of all the samples where the compounds were identified.
[c]Error in ppm is calculated as $(m/z_{exp} - m/z_{theo}) / m/z_{exp} * 10^6$ (in absolute value).
[d]Error is presented as an average of all samples where the compounds were identified.