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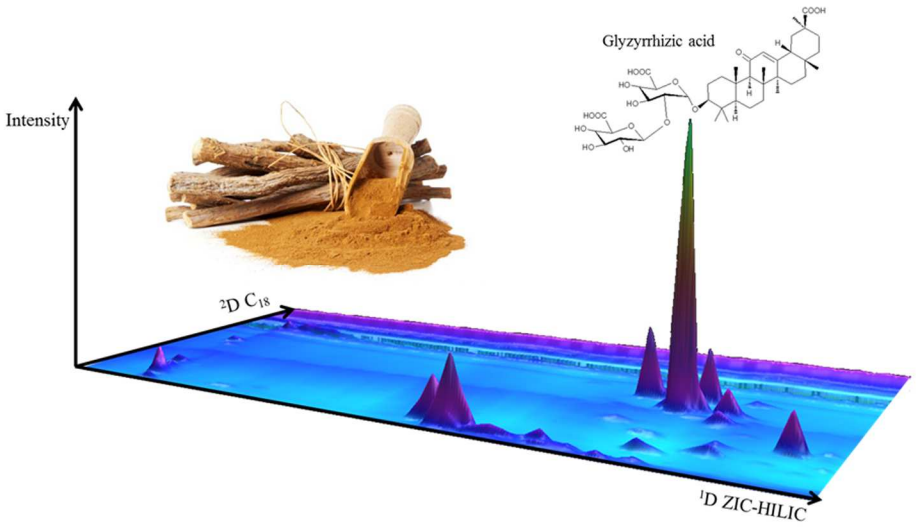

METABOLITE PROFILING OF LICORICE (*Glycyrrhiza glabra*) FROM DIFFERENT LOCATIONS USING COMPREHENSIVE TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY COUPLED TO DIODE ARRAY AND TANDEM MASS SPECTROMETRY DETECTION

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ABSTRACT

Profiling of the main metabolites from several licorice (*Glycyrrhiza glabra*) samples collected at different locations is carried out in this work by using comprehensive two-dimensional liquid chromatography (LC × LC) coupled to diode array (DAD) and mass spectrometry (MS) detectors. The optimized method was based on the application of a HILIC-based separation in the first dimension combined with fast RP-based second dimension separation. This set-up was shown to possess powerful separation capabilities allowing separating as much as 89 different metabolites in a single sample. Identification and grouping of metabolites according to their chemical class were achieved using the DAD, MS and MS/MS data. Triterpene saponins were the most abundant metabolites followed by glycosylated flavanones and chalcones, whereas glyzyrrhizic acid, as expected, was confirmed as the main component in all the studied samples. LC × LC-DAD-MS/MS was able to resolve these complex licorice samples providing with specific metabolite profiles to the different licorice samples depending on their geographical origin. Namely, from 19 to 50 specific compounds were exclusively determined in the 2D-chromatograms from the different licorice samples depending on their geographical origin, which can be used as a typical pattern that could potentially be related to their geographical location and authentication.

Keywords: LC × LC, licorice, metabolite, triterpene saponins, phenolic compounds, authentication.

1. INTRODUCTION

Licorice (*Glycyrrhiza glabra*) is an herbaceous perennial plant, belonging to the Leguminosae family and is one of the oldest and most popular herbal medicines in the world. A wide array of biological activities have been ascribed to this plant, including antiulceric, anti-inflammatory [1], antispasmodic, expectorant, antiallergic, antidepressive [2], antiviral [3], antifungal [4] and antioxidant [5] activities. Besides, licorice has been used in the food industry as a sweetener and a flavor enhancer while it is considered as a safe food ingredient (GRAS) by the US Food and Drug Administration [6]. The mentioned potential health beneficial effects have been associated to the secondary metabolites present in licorice which essentially consist on triterpene saponins and phenolic compounds, including flavanones, chalcones, flavones, isoflavones and isoprenylated flavonoids [7]. Triterpene saponins present in *G. glabra* belong to the oleane-type and are the major active substances. In particular, glycyrrhizic acid is regarded the most important constituent of licorice, pointed out to be the main responsible of the beneficial effects attributed to this plant [8].

There exist around 30 different *Glycyrrhiza* species over the world. Their differentiation based only on root morphology is very complicated. As a consequence, it is important to search metabolic markers that may allow the correct identification of licorice species [9]. The content on secondary metabolites in licorice may vary significantly depending on the geographical area of origin, the stage of maturity of the plant, the environmental conditions and the harvesting/production procedures [7]. In this regard, the content of secondary metabolites could be employed for the geographical identification of licorice, as the product characteristics will be greatly influenced by its particular chemical composition. In relation to these differences, licorice from the region of Calabria (Italy) has been described as one of those with highest quality [10] thanks to the amount of

bioactive compounds present on it as well as its typical organoleptic characteristics including volatiles composition. This region presents a Mediterranean climate, characterized by dry summers and mild winters, whereas springs and autumns last only few months, that could provide the special characteristics to the Calabrian licorice [10]. The quality of the Calabrian licorice has been recognized with the Protected Designation of Origin ‘‘Licorice from Calabria’’ in 2011 by the European Union (Reg. (CE) N. 1072/2011 EU) [11].

The metabolic profile of licorice has been widely studied through different analytical techniques such as HPLC-DAD [12,13], HPLC-DAD/MS [10,12-14], UHPLC-DAD [6], UHPLC-MS [6,15-17] or NMR [9,17]. However, aiming the complete profiling of the bioactive phenolics and saponins from licorice is rather difficult since numerous different structures and their isomers are present as well as many compounds with similar retention behavior coexisting in its composition. In this regard, comprehensive two-dimensional liquid chromatography (LC × LC) may be a useful analytical technique to provide a broad separation of the whole composition of the chemically complex licorice extracts. LC × LC allows the separation of a sample through two separation processes simultaneously connected on-line by means of a modulator or interface. This technique provides the advantage of greatly enhancing the peak capacity attainable compared to monodimensional LC due to the use of columns with low correlated separation mechanisms in the first (^{1}D) and the second (^{2}D) dimension, thus, maintaining a high orthogonality degree [18]. Although LC × LC is not still a commonly-employed analytical technique, there are many applications in which this tool has been employed for the successful attainment of the metabolic profile of complex samples [19-21]. However, LC × LC has only been scarcely explored for the separation of licorice compounds. Concretely, a RPLC × RPLC method was developed

for the separation of an ethyl lactate fraction of a licorice extract in order to identify flavonoid aglycones present [22]. The developed RPLC × RPLC approach provided a good separation of some components present in a licorice extract fraction, with higher resolving power than one dimensional methods. However, the use of non-correlated separation mechanisms in the two dimensions may significantly increase the attainable peak capacity as well as the orthogonality of the two-dimensional system. Possible combinations of non-correlated mechanisms include NPLC × RPLC [23-25], SEC × RPLC [26], and IEC × RPLC [27-29] approaches. However, these couplings can cause immiscibility and incompatibility problems with the mobile phases employed in both dimensions. The coupling between hydrophilic interaction liquid chromatography (HILIC) and RP separations is useful to partially solve these problems, since the solvents employed in the mobile phases are miscible. In this regard, different HILIC × RP methods have recently been developed for the separation and identification of a variety of compounds in several food-related samples. In particular, HILIC × RPLC methods have been widely demonstrated to be extremely useful for the separation of highly complex and closely related mixtures of phenolic compounds, such as procyanidins [30-33], anthocyanins [34] or phlorotannins [35,36]. The use of DAD and MS detectors connected in series together with the information related to the relative position of each peak in the 2D space allow increasing the identification capabilities of this technique. In this regard, this alternative can be also proposed as a potentially successful approach for the separation and identification of the entire phenolic compounds and saponins profile of licorice samples. The acquisition of these profiles might be later on used to differentiate among samples of different geographical origin. Thus, in the present work, a new HILIC × RPLC method employing a microbore ZIC-HILIC column in the ^{1}D and a C_{18} partially porous column in the ^{2}D has been developed

for the characterization of the polyphenol and saponin profile of licorice extracts from different geographical locations with the aim of searching for metabolites that could be pointed out as potential markers for the identification and assignment of the geographical origin as well as for the authentication of each sample.

2. MATERIALS AND METHODS

2.1. Samples and chemicals

Licorice samples (*Glycyrrhiza glabra*) from China, Iran and Azerbaijan were produced in 2009, whereas licorice from Crotone and Villapiana (Calabria Region, Italy) were collected in 2007 and 2013, respectively.

HPLC grade ethanol and acetonitrile were purchased from VWR Chemicals (Barcelona, Spain) whereas acetic and formic acids were acquired from Sigma-Aldrich (Madrid, Spain) and ammonium acetate was from Panreac (Barcelona, Spain).

2.2. Sample preparation

The extraction of the metabolites from licorice was carried out following the preparation of the extracts described by Montoro et al. [7]. Briefly, the root material was ground and extracted with a solid-liquid extraction assisted by ultrasonic agitation. The solvent employed was ethanol/water (1:1, *v/v*) with a sample to solvent ratio of 1:5 (*w/v*), and the extraction was carried out during 1 h. Then the mixture was maintained in darkness overnight at room temperature. The extract was vacuum filtered and finally diluted 1:10 with ethanol/water (1:1, *v/v*). Prior to the chromatographic analysis, an aliquot of each extract was evaporated to dryness and redissolved in ethanol/ACN (1:1, *v/v*).

2.3. LC × LC-DAD-MS/MS

144 *2.3.1. Instrumentation*

145 The LC × LC-DAD instrumentation consisted on a first dimension (^{1}D) composed by an
146 Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA)
147 equipped with an autosampler. In order to obtain more reproducible low flow rates and
148 gradients, a Protecol flow-splitter (SGE Analytical Science, Milton Keynes, UK) was
149 placed between the ^{1}D pump and the autosampler. Additionally, a LC pump (Agilent
150 1290 Infinity) performed the second dimension (^{2}D). Both dimensions were connected
151 by an electronically-controlled two-position ten-port switching valve acting as
152 modulator equipped with two identical 30 µl injection loops. Modulation time of the
153 switching valve was 1.3 min. A diode array detector was coupled after the second
154 dimension in order to register every ^{2}D analysis. Besides, an Agilent 6320 Ion Trap
155 mass spectrometer equipped with an electrospray interface working in negative
156 ionization mode was coupled in series using the following conditions: dry temperature,
157 350 °C; dry gas flow rate, 12 L min^{-1}; nebulization pressure, 40 psi; mass range, m/z
158 90–2,200 Da. The LC data were elaborated and visualized using LC Image software
159 (version 1.0, Zoex Corp., Houston, TX).

160

161 *2.3.2. LC x LC separation conditions*

162 The ^{1}D separation was carried out employing a SeQuant ZIC-HILIC (150 x 1 mm, 3.5
163 µm d.p., Merck, Darmstadt, Germany) column. The analysis was run using (A)
164 acetonitrile and (B) 10 mM ammonium acetate at pH 5.0 as mobile phases, eluted
165 according to the following gradient: 0 min, 3% B; 5 min, 3% B; 10 min, 5% B; 15 min,
166 10% B; 30 min, 20% B; 40 min, 20% B; 50 min, 30% B; 60 min, 30% B; 65 min, 40%
167 B; 80 min, 40% B. The injection volume was 2.5 µL and the flow rate was set at 15 µL
168 min^{-1}.

On ^{2}D, an Ascentis Express C_{18} (50 x 4.6 mm, 2.7 µm d.p., Supelco, Bellefonte, CA) partially porous column was employed. Mobile phases consisted of (A) water (0.1 % formic acid) and (B) acetonitrile, eluted at 3 mL min^{-1}. During the LC × LC analysis two ^{2}D gradients were employed in order to obtain the best ^{2}D separations in agreement with the compounds eluting from the ^{1}D. Therefore, from 0 min to 23.4 min the ^{2}D gradient elution was: 0 min, 0% B, 0.1 min, 5% B; 0.5 min, 35% B; 0.9 min, 70% B; 1 min, 90% B; 1.01 min, 0% B; 1.3 min, 0% B. On the other hand, from 23.4 to 80 min the employed gradient was programmed as follows: 0 min, 0% B; 0.1 min, 5% B; 0.3 min, 35% B; 0.5 min, 40% B; 0.9 min, 50% B; 1 min, 90% B; 1.01 min, 0% B; 1.3 min, 0% B. UV-Vis spectra were collected in the range of 190-550 nm using a sampling rate of 20 Hz, while 254, 280 and 330 nm signals were also independently recorded. The eluent from the ^{2}D column was splitted before entering the MS instrument, so that the flow rate introduced in the MS detector was 0.6 mL min^{-1}.

3. RESULTS AND DISCUSSION

The metabolic profile of licorice presents compounds with different chemical nature, being the main ones triterpene saponins and phenolic compounds including flavanones, chalcones, flavones, isoflavones and isoprenylated flavonoids [7]. Within this complex mixture, isomers of some compounds may coexist in its composition as well as other compounds with closely related chemical structures. Therefore, they present the same or close molecular weights as well as related chromatographic behavior. For this reason, the exhaustive separation of the whole array of these kinds of compounds present in licorice is difficult to be carried out by conventional analytical techniques such as HPLC or UHPLC. In fact, many of these compounds coelute when separated under a single retention mechanism. Hence, the employment of analytical tools able to separate

194 the sample by two separation process like LC x LC may be effective for obtaining the
195 complete separation of the metabolic profile of licorice.
196
197 **3.1. Optimization of the 2D profiling of licorice.**
198 The combination of HILIC in the ^{1}D and RPLC in the ^{2}D has been shown to provide a
199 high orthogonality degree and therefore good results in terms of peak capacity [30-36].
200 However, due to the complexity of the samples that are usually analyzed by LC × LC
201 together with the challenging handling of this technique, the development of each new
202 LC × LC application has to be carefully optimized. In this regard, in the present
203 development each dimension was separately studied and optimized.
204 To obtain appropriate conditions, slow separations are needed in ^{1}D, whereas ^{2}D
205 separations should be as fast as possible; this way, individual ^{2}D analysis time will
206 directly influence the modulation time, and thus, sampling from the ^{1}D eluate. On the
207 other hand, the use of very low flow rates in ^{1}D will allow maintaining the transfer
208 volume to a minimum and also increasing the sampling from each ^{1}D separated peak
209 that could be cut more times into the ^{2}D. Due to these requirements, the use of
210 microbore columns in ^{1}D is advisable as they present several advantages in terms of
211 separation and resolution attainable at very low flow rates. Consequently, the use of
212 these conditions will permit the collection and injection of fractions continuously to the
213 ^{2}D, providing enough time between fractions to completely finish each ^{2}D analysis.
214 Once the basic morphology of the ^{1}D column was selected, three different HILIC-
215 compatible stationary phases were tested, namely silica, diol and ZIC-HILIC columns.
216 For each type of column several gradients and aqueous mobile phases were tested
217 including 10 mM ammonium acetate at pH 5, pH 5.5 and pH 7.5 or mixtures of
218 acetonitrile, methanol, water and acetic acid to find the best conditions independently.

Figure 1 shows a comparison of the separation attainable using each column at the specific optimum conditions. It has to be remarked that the silica column was used for comparison in spite of not having the required dimensions to act a ^{1}D. Still, this test allowed us to discard this stationary phase for further use. After the study and optimization of the experimental conditions that affect the separation, the ZIC-HILIC column was selected employing acetonitrile and 10 mM ammonium acetate at pH 5.0 as the optimum mobile phases for the ^{1}D separation. As it can be observed in Figure 1, the ZIC-HILIC column provided better resolution than the other columns with reasonable analysis times at 15 µL min^{-1}, which is an appropriate flow rate in order to keep to a minimum the total volume of each transfer to the ^{2}D.

Next, the ^{2}D separation was optimized individually by injecting the whole sample. This provides a good measure of the separation capabilities of the system even if in real conditions no such complex fraction will be transferred into this ^{2}D. Two short RP columns (50 x 4.6 mm), containing C_{18} and PFP (pentafluorophenyl) partially porous particles (2.7 µm), were tested. Typically, the separation carried out in the ^{2}D has to be fast in order to finish each ^{2}D analysis before the next ^{1}D fraction is transferred. In this regard, the employment of short partially porous columns in this kind of separation is advantageous since these columns generate lower backpressures and provide better efficiency in short analysis times. For the optimization of the ^{2}D separation, different mobile phases including acetonitrile, methanol and different combinations of acetonitrile/methanol as well as water and acidified water, and gradients were studied.

After careful comparison of the best attainable separations, C_{18} stationary phase was selected as provided a higher resolution between the compounds contained in such a complex sample. The optimized conditions involved the use of water (0.1% formic acid) and acetonitrile as mobile phases.

Starting from the best possible separation conditions already determined for the separation of licorice compounds in each dimension, the optimization of the LC × LC method as a whole was carried out. After the first analyses it became evident that the huge diversity of compounds contained in the sample meant that the obtained metabolite profiles were clearly grouped in two different areas in the 2D plane (Figure 2A). Those compounds eluting first from the ^{1}D (low polarity compounds, prenylated flavonoids) were less separated in the ^{2}D under the chosen conditions that those eluting later from the ^{1}D (high polarity compounds, triterpene saponins) that were less retained in the ^{2}D (Figure 2A). Consequently, with the aim to provide a profile as separated as possible, it was decided to reoptimize the ^{2}D method by including two different gradients along the LC × LC analysis. Figures 2B and C show a comparison of the two-dimensional separation of licorice compounds eluting first from the ^{1}D (tr~8-22 min). While in Figure 2B the same gradient is maintained along the 2D analysis (the same used for the second part of the analysis), in Figure 2C a newly optimized ^{2}D gradient was used in order to maximize the separation of the compounds eluting in this area (see details in Section 2.3.). As can be seen, significantly better separation could be obtained using the new gradient. Thus, under the finally optimized LC × LC conditions, two different ^{2}D gradients were adopted in order to achieve the best possible separation of the licorice metabolites in agreement with their chemical nature, allowing a theoretical peak capacity value of 1306, calculated according to Li et al. [37], which considers the ^{2}D time cycle as well as the influence of undersampling of the ^{1}D eluate. Moreover, as it can be observed from the 2D plots illustrated in Figure 2, the retention in both dimensions was clearly non-correlated, assuring a good degree of orthogonality.

3.2. Phenolic compounds and saponins profiling of licorice by HILIC × RP-DAD-MS/MS.

Once the ZIC-HILIC × C_{18} method was completely optimized, an MS instrument equipped with an ESI interface working in negative ionization mode was coupled in series to the DAD for the characterization and identification of each separated compound. The employed MS analyzer was an ion trap that offered valuable information about the chemical structure of the separated compounds thanks to its capacity to work in MS/MS mode. However, the use of a high resolution MS detector with high scanning speed could significantly improve the obtained results in terms of reducing the number of non-identified compounds and increasing the certainty of the identifications. The five licorice samples studied, belonging to diverse locations, namely, Calabria-Italy (Villapiana and Crotone), Iran, China and Azerbaijan, were injected and analyzed in detail. Table 1 shows the compounds tentatively identified in more than one sample, whereas Table 2 shows those compounds that were exclusively found in just one location (potential markers of geographical origin). Figure 3 shows the two-dimensional plots obtained for each sample in which the assigned peaks are marked.

The main licorice metabolites are triterpene saponins and phenolic compounds, including flavanones, chalcones, flavones, isoflavones and isoprenylated flavonoids. Some of these flavonoids may be found as aglycones or glycosylated. Each group of compounds described in licorice can be identified attending to their mass fragmentation pathway [42]. This information was collected and studied in order to tentatively assign the separated peaks in the different samples.

In any case, as can be seen in Figure 3 the profile of the 5 licorice samples is rather similar being the main detected compounds (those with higher intensities) present in all locations.

3.2.1. Flavonoid aglycones and prenylated flavonoids.

At the beginning of the analysis, a group of prenylated flavonoids was found together with other flavonoid aglycones, clearly located in the upper side of the 2D plot, as can be observed in Figure 2. Among prenylated flavonoids, only kanzonol Y (peak 1, *m/z* 409.6) was described in more than a sample. Others were just found to be potential markers of location, such as glabridin (peak E4) from Villapiana, hispaglabridin (peak C1) and glyasperin E (peak C2) from Crotone or kanzonol H (peak A4) from China.

The most relevant flavonoid aglycone found was liquiritigenin (peak 4), a flavanone with $[M-H]^-$ at m/z 255.0, which provided fragment ions at m/z 135 and 119 through the fragmentation via retro Diels-Alder (RDA) reaction. Figure 4A shows the MS/MS fragmentation pattern of this compound.

3.2.2.Glycosylated flavanones and chalcones.

Glycosylated flavanones and their related glycosylated chalcones were found to be the major phenolic compounds in the metabolites profile of all the studied samples. Depending on the position of the OH- groups of the molecule, the fragmentation pathway of these compounds was different. Among them, different peaks presented MS/MS fragmentation patterns that gave rise to a characteristic fragment with *m/z* 255, corresponding to the aglycone liquiritigenin. Thus, the presence of the fragment at *m/z* 255 meant that these compounds suffered the loss of the saccharide groups. In particular peaks 8, 9 and 11 with $[M-H]^-$ at *m/z* 725.3 were assigned to licorice glycoside A or

licorice glycoside C1 or C2. The chemical structure of these compounds presents two glycoside groups (an apiose and a glucose units), and a p-coumaroyl group with a methylated group that decreases their polarity; therefore, their retention on the HILIC ^{1}D was low. Figure 4B shows the chemical structure of these components as well as their corresponding MS/MS spectrum. As can be observed, their fragmentation produced ions at *m/z* 549, 531, 417, 255; these fragments could correspond to the loss of 176 Da ([M-H - $C_{10}H_8O_3$]$^-$), 194 Da ([M-H- $C_{10}H_8O_3$ - H_2O]$^-$), 308 Da ([M-H- $C_{10}H_8O_3$ - Api]$^-$) and 470 Da ([M - H - $C_{10}O_3H_8$ - Api - Glu]$^-$), respectively.

The same behavior was observed for licorice glycoside B or D1/D2 (peak 14) formed by a liquiritigenin moiety linked to a hexose, a pentose and a p-coumaroyl group. This compound presented a [M-H]$^-$ at *m/z* 695.3 and a fragmentation pattern characterized by ions at *m/z* 549 (loss of p-coumaroyl group), 531 (loss of p-coumaroyl group and a water molecule), 417 (loss of p-coumaryl and pentose molecules) and 255 (removal of the three linked groups: p-coumaroyl, pentose and hexose). Figure 4C illustrates these data. On the other hand, liquiritin apioside and isoliquiritin apioside (peaks 16 and 18), with [M-H]$^-$ at *m/z* 549.6, have a chemical structure composed by a liquiritigenin and a hexose and a pentose units. As it is shown in Figure 4D, their MS/MS pattern was characterized by the occurrence of an ion at *m/z* 417 attributed to the loss of apioside (neutral loss of 132 Da), *m/z* 297 to the combination of the loss of the apioside unit and the cleavage of the hexose (-132 Da and -120 Da, respectively) and *m/z* 255 due to the loss of the two glycosidic units [49].

Other compounds belonging to these groups were also tentatively identified or putatively assigned to this group thanks to their MS characteristics, as can be observed in Tables 1 and 2, as well as to their typical UV maxima (270 nm for chalcones and 360 nm for flavanones).

341

342 *3.2.3.Triterpene saponins.*

343 Triterpene saponins are regarded as the main bioactive metabolites present in licorice,
344 eluting together in the last part of the two-dimensional analyses. The triterpene saponins
345 of licorice belong to oleanane-type triterpene saponins and their chemical structure
346 consist on a 30-carbon aglycone (sapogenin) with multi-sugar attached units. Under
347 MS/MS experiments carried out in negative ionization mode, oleanane-type triterpene
348 saponins mainly give rise to a $[M - aglycone - H]^-$ fragment ion. Glycyrrhizic acid (peak
349 54) presented a $[M - H]^-$ at *m/z* 821.5 and its fragmentation pattern showed ions at *m/z*
350 803 ($[M - H_2O - H]^-$), 645 ([M - Glucuronic acid-H]$^-$), 351 ([2 Glucuronic acid - H]$^-$).
351 As mentioned in the introduction section, many compounds and their isomers and
352 compounds with the same molecular weight coexist in the licorice extract, hence,
353 several separated peaks presented $[M-H]^-$ at m/z 821. Glycyrrhizic acid is the main
354 licorice metabolite and thus, peak 54 was assigned to this component as it was the most
355 intense peak in all studied samples. Besides this component,18-α-glycyrrhizic acid,
356 macedonoside C, yunganoside L2, uralsaponin A, licorice saponin H2 and licorice
357 saponin K2 presented the same molecular ion and fragmentation pattern, and thus, the
358 peaks that presented this pattern (peaks 40, 55, 58, 59, 65 and 72) could not be
359 unequivocally assigned.

360 Other important saponins were identified in the samples according to their $[M-H]^-$
361 values and MS/MS spectra. Table 1 summarizes this information. Among them, 22-
362 acetoxyl-glycyrrhizin (peak 53), licorice saponin A3 (peak 66) and licorice saponin G2
363 (peak 71) were found in all the studied samples. Additionally, other compounds that
364 could not be identified were putatively assigned as triterpene saponins based on the
365 typical MS/MS behavior.

366

367 **3.3. Geographical differentiation of licorice by HILIC × RP-DAD-MS/MS.**

368 The huge potential that comprehensive two-dimensional LC may provide for the
369 separation of very complex samples, like those studied in the present work, is
370 complemented by the possibility of attaining 2D-plots that may be employed to
371 discriminate among samples. In an effort to avoid frauds when dealing with protected
372 designation of origin foods, metabolite profiling can be used to effectively differentiate
373 samples with different geographical origin. In this work, a first approach to this strategy
374 is employed using the optimized HILIC × RP-DAD-MS/MS method in order to
375 demonstrate the potential of this technique to point out possible markers of geographical
376 origin in different licorice samples. Besides the qualitative information collected
377 described in section 3.2, the generation of 2D-plots may be used to produce
378 characteristic patterns of each type of sample. Figure 5 shows reconstructed 2D-plots
379 corresponding to the individual markers found in each sample. Thus, only those
380 compounds that were found in just one sample are included. As can be observed in that
381 Figure, each particular pattern was formed by a variety of peaks at different points along
382 the complete 2D-plot. The use of those points could effectively offer an advantage for
383 the identification of unknown or suspected samples. Consequently, these patterns might
384 be used to visually assign a sample to a particular location, although the analysis of a
385 significantly higher number of samples is required to statistically validate the found
386 candidate markers. Chinese licorice was the one that presented a higher amount of
387 typical compounds (50), whereas the Calabrian samples (C and E) presented just 19 and
388 22 exclusive peaks, respectively, being clearly more similar.

389 Once this method has been optimized and its usefulness to analyze licorice samples
390 demonstrated, its extension to a higher number of samples of each location to

391 statistically confirm the validity of the found markers to discriminate according their
392 location will be assessed in a forthcoming paper.
393
394 **4. CONCLUSIONS**
395 The development of a new LC × LC-DAD-MS/MS method to obtain a complete
396 phenolic compounds and saponins profile of licorice samples has been carried out in
397 this work. The optimized method was able to separate a large number of compounds (up
398 to 89 in the Iranian sample), which were grouped in the obtained 2D-plots according to
399 their chemical class. Triterpene saponins were the most abundant metabolites followed
400 by glycosylated flavanones and chalcones. Glyzyrrhizic acid was confirmed as the main
401 component in all the studied samples. Moreover, the developed method not only
402 permitted the assignment of compounds to a particular chemical family according to
403 their position in the 2D-plot, but it was also employed to produce a typical pattern of
404 each sample that could be later on be used to differentiate among geographical
405 locations, once statistically validated. A good number of unique components (from 19
406 to 50) were found in all the samples. Thus, the usefulness of this method to generate
407 patterns that could be potentially employed to confirm the authenticity and geographical
408 origin of unknown or suspected licorice samples is demonstrated.
409
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621

622 **FIGURE LEGENDS**

623 **Figure 1.** First dimension chromatograms (280 nm) obtained under optimum conditions
624 for each type of column studied. Separations using: A) Silica column (250 x 2.5 mm, 5
625 µm d.p., Synchronis HILIC, Thermo Scientific) eluted using acetonitrile (with 0.1%
626 HCOOH), and water (containing 0.1% HCOOH), at 0.5 mL min^{-1}; B) Diol column (150
627 x 1.0 mm, 5 µm d.p., Lichrospher diol-5, HiChrom) eluted using acetonitrile and 10
628 mM ammonium acetate at pH 5.5 at 15 µL min^{-1}, and; C) ZIC-HILIC column (150 x 1.0
629 mm, 3.5 µm d.p., SeQuant ZIC-HILIC, Merck) at the optimum conditions detailed in
630 Section 2.3.2. For other separation conditions, see text.

631 **Figure 2.** Two-dimensional licorice metabolites profiles (280 nm) obtained under the
632 studied conditions using the same ^{2}D gradient along the whole analysis (A).
633 Comparison of the separation obtained in the first area using the same ^{2}D gradient (B)
634 and the newly optimized ^{2}D gradient. For separation conditions, see text.

635 **Figure 3.** Two-dimensional HILIC × RP licorice metabolites profiles (280 nm) obtained
636 for licorice samples collected from China (A), Iran (B), Crotone (Italy, C), Azerbaijan
637 (D) and Villapiana (Italy, E). For peak identification, see Tables 1 and 2. Separation
638 details in section 2.3.

639 **Figure 4.** Chemical structure and MS/MS fragmentation patterns for A) liquiritigenin,
640 B) licorice glycoside A, C) licorice glycoside B, and D) liquiritin apioside, detected in
641 the licorice samples.

642 **Figure 5.** Reconstructed two-dimensional HILIC × RP traces of metabolites exclusively
643 present in the indicated licorice sample. A) China, B) Iran, C) Crotone (Italy), D)
644 Azerbaijan and E) Villapiana (Italy).

645

Table 1. Tentatively identified common metabolites present in more than one licorice sample together with their MS and MS/MS information. Source: A) China, B) Iran, C) Crotone (Italy), D) Azerbaijan and E) Villapiana (Italy).

Peak	^{2}D t_R (s)	Total t_R (min)	$[M-H]^-$	Main MS/MS fragments detected	Identification	Structure class	Source	Ref
1	67.9	8.93	409.3	405, 391, 365, 235, 217	Kanzonol Y	Prenylated favonoid	B, C, E	[38]
2	53.3	9.99	576.8	539, 518, 419, 331, 261, 187	NI		B, D	
3	70.9	10.28	409.4	391, 235	NI		C, D, E	
4	40.7	11.08	255.0	134, 119	Liquiritigenin or isomer	Flavanone	B, D. E	[39-42]
5	35.3	12.29	727.7	613, 549, 532, 255	NI	Flavanone	A, B, C, D	
6	37.5	12.33	727.4	549, 531, 389, 255	NI	Flavanone	B, C, D	
7	31.0	14.82	417.2	255, 135	(Iso)liquiritin / Neo(iso)liquiritin	Flavanone	B, E	[43-45]
8	34.4	42.17	725.3	549, 531, 399, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	B, E	[9,22,42,44]
9	33.3	42.81	725.2	695, 512, 575, 549, 532, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	B, E	[9,22,42,44]
10	31.1	43.42	419.4	391, 279, 256, 201, 135	NI		B, E	
11	37.3	44.82	725.3	549, 531, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	B, E	[9,22,42,44]
12	31.0	46.02	633.8	587, 549, 511, 426, 339, 295, 229	Sarcaglaboside D	Sesquiterpene Glycoside	A, D, E	[45]
13	32.6	46.04	549.7	494, 481, 419, 256		Flavanone	A, B, C, D, E	
14	34.1	46.07	695.3	649, 549, 531, 417, 255	Licorice glycoside B/D1/D2	Flavanone	C, E	[9,27,42]
15	29.6	47.28	759.4	549, 467, 209	NI		A, B, E	
16	38.8	47.45	549.2	429, 255	(Iso)liquiritin apioside	Flavanone	A, D	[7,22,44,45]
17	30.5	48.61	549.9	494, 481, 419, 256	NI	Flavanone	B, D, E	
18	32.8	48.65	549.2	429, 417, 297, 255	(Iso)liquiritin apioside	Flavanone	A, B, C, D, E	[7,22,44,44]
19	37.1	48.72	549.8	494, 480, 418, 255	NI	Flavanone	C, D	
20	29.3	51.19	577.3	559, 526, 503, 488, 441, 406	(Iso)violantin	Flavone	B, C, D, E	[9,22]
21	30.3	51.21	665.9	619, 551, 530, 505, 447, 383, 239	NI		A, E	
22	27.1	51.80	721.3	677, 619, 577, 559, 487, 457, 383	NI	Flavone	A, C	
23	60.7	52.36	777.6	715, 627, 538, 470, 427	Apioglycyrrhizin	Triterpene saponin	A, B, C, D, E	[7]
24	29.4	52.49	577.2	559, 526, 503, 488, 441, 406	(Iso)violantin	Flavone	B, C, D	[9,22]
25	27.8	53.76	711.1	674, 649, 591, 549, 531, 443, 423, 298, 255	Glucoliquiritin apioside or isomer	Flavanone	C, E	[39,46]
26	29.4	53.79	837.8	828, 791, 672, 588, 472	Cicloheptaleucyl	Cyclopeptide	A, B, C, D, E	[42]
27	49.2	54.12	983.7	965, 921, 879, 838, 733, 715, 687, 645, 439	NI		A, E	
28	28.5	54.43	563.1	545, 503, 473, 443, 383, 353	Shaftoside/Apigenin 6-C-Glucoside-C-Arabinoside	Flavone	C, D	[22,39,47]
29	52.2	54.82	807.3	745, 627, 609, 583, 537, 469, 351	Licorice saponin B2 or isomer	Triterpene saponin	A, B, C, D, E	[42]
30	63.0	55.00	793.5	775, 732, 645, 522, 351	NI	Triterpene saponin	D, E	
31	28.6	55.08	711.2	674, 649, 591, 549, 531, 443, 423, 298, 255	Glucoliquiritin apioside or isomer	Flavanone	A, B, C, D, E	[39,46]
32	30.9	55.12	649.2	631, 613, 604, 565, 523, 444, 392, 259	NI		A, B, D, E	

33	41.1	55.29	939.6	921, 879, 879, 777, 645, 523, 437	NI	Triterpene saponin	B, C, D	
34	49.0	55.42	865.3	847, 821, 803, 727, 689, 608, 351	22-acetyl licorice saponin B2 or isomer	Triterpene saponin	A, B, D	[47]
35	50.1	55.44	865.3	805, 690, 607, 351	22-acetyl licorice saponin B2 or isomer	Triterpene saponin	A, E	[47]
36	27.0	56.35	711.4	674, 649, 591, 549, 531, 443, 423, 298, 255	Glucoliquiritin apioside or isomer	Flavanone	C, D, E	[39,46]
37	29.6	56.39	707.3	647, 617, 563, 545, 473, 443, 353, 255, 205	3-Hydroxyl-3-methylglutaroyl-(iso)schaftoside	Flavone	C, E	[42]
38	65.1	56.99	807.0	791, 745, 632, 351, 334, 289, 261	Licorice saponin B2 or isomer	Triterpene saponin	A, B, C, D, E	[7,9,42,44]
39	44.5	59.24	865.0	848, 821, 803, 689, 351	22-acetyl licorice saponin B2 or isomer	Triterpene saponin	B, C, E	[47]
40	52.5	59.38	821.0	801, 757, 644, 351, 333	18-α-glycyrrhizin / macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	B, C	[7,17,42,45]
41	57.6	59.46	807.0	789, 746, 632, 351, 334, 290	Licorice saponin B2 or isomer	Triterpene saponin	A, B, C, D, E	[7,9,42,44]
42	65.6	59.59	939.8	921, 878, 523, 776, 622, 497, 435	NI	Triterpene saponin	A, B, E	
43	28.4	59.63	675.9	549, 531, 255	NI		B, E	
44	37.0	59.77	881.6	864, 819, 705, 351	22-Acetoxyl licorice saponin J2	Triterpene saponin	A, D	[46]
45	41.0	59.83	969.9	953, 925, 909,835, 824, 793, 351	Albiziasaponin B or isomer	Triterpene saponin	B, D	[42]
46	49.4	59.97	1011.6	993, 949, 867, 831, 689, 643, 629, 497, 321	Licorice saponin D3	Triterpene saponin	B, C, E	[7]
47	57.4	60.11	955.8	938, 847, 633, 611, 497, 435, 339	Yunganoside A1/C1/B1	Triterpene saponin	B, C	[9]
48	59.8	60.15	737.3	719, 647, 617, 593, 503, 473, 393, 353	6-(3-Hydroxy-3-methylglutaroyl)-vicenin-2 or isomer	Flavone	C, D, E	
49	57.0	60.21	807.5	789, 745, 631, 611, 351, 289	Licorice saponin B2 or isomer	Triterpene saponin	C, E	[7,9,42,44]
50	60.6	60.27	953.8	937, 892, 849, 790, 633, 497, 339, 321	NI	Triterpene saponin	A, B, C, D	
51	27.9	60.27	593.3	575, 503, 474, 441, 406, 354	Apigenin 6,8-di-C-glucoside (Vicenin-2)	Flavone	B, C, D, E	[47]
52	35.3	60.39	823.5	805, 779, 761, 647, 539, 351, 333, 289	Licorice saponin J2 / Uralsaponin C	Triterpene saponin	A, B, C, D, E	[33,44-46]
53	41.4	60.49	879.8	861, 800, 703, 685, 643, 584, 351, 333, 315	22-Acetoxyl-glycyrrhizin	Triterpene saponin	A, B, C, D, E	[45,47]
54	52.6	60.68	821.5	803, 759, 645, 351	Glycyrrhizic acid	Triterpene saponin	A, B, C, D, E	[17,42,45]
55	60.0	60.80	821.4	803, 759, 645, 351	18-α-glycyrrhizin/ Yunnanglysaponin B/ macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	A, B, C, D, E	[17,42,45]
56	27.7	62.86	737.3	675, 635, 619, 593, 575, 503, 473, 353	6-(3-Hydroxy-3-methylglutaroyl)-vicenin-2 or isomer	Flavone	B, C, E	
57	28.7	62.88	737.5	675, 635, 619, 593, 575, 503, 473, 353	6-(3-Hydroxy-3-methylglutaroyl)-vicenin-2 or isomer	Flavone	A, B, C, D, E,	
58	49.6	63.23	821.3	803, 759, 645, 351	18-α-glycyrrhizin / macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	B, C, D, E	[17,42,45]
59	52.6	63.28	821.4	803, 759, 645, 351	18-α-glycyrrhizin / macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	B, C	[17,42,45]
60	31.8	64.23	969.6	951, 904, 837, 793, 711, 351	Albiziasaponin B or isomer	Triterpene saponin	A, C, D	[42]
61	33.8	64.26	969.1	951, 793, 497, 436, 351	Albiziasaponin B or isomer	Triterpene saponin	D, E	[42]
62	37.0	63.72	1025.7	1007, 956, 908, 645, 497, 321	22-Acetoxyl-rhaoglycyrrhizin	Triterpene saponin	A, C, E	[45,48]

63	47.8	65.80	837.6	819, 776, 704, 661, 485, 351, 333, 289	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	A, B	[7,9,17, 22,42,44-47]
64	48.2	73.03	837.5	819, 776, 704, 661, 485, 351, 333, 289	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	A, B, C, D, E	[7,9,17, 22,42,44-47]
65	52.3	65.87	821.5	803, 759, 645, 351	18αglycyrrhizin / macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	A, B, C, D, E	[17,42,45]
66	37.0	66.92	983.5	923, 863, 821, 803, 760, 645, 351, 289	Licorice saponin A3 or isomer	Triterpene saponin	A, B, C, D, E	[45]
67	39.7	66.93	983.5	923, 863, 821, 803, 760, 645, 351, 289	Licorice saponin A3 or isomer	Triterpene saponin	B, C, D, E	[45]
68	48.6	67.11	969.7	951, 904, 837, 793, 711, 351	Albiziasaponin B or isomer	Triterpene saponin	B, C, D, E	[42]
69	52.8	67.18	967.7	949, 906, 833, 645, 497, 321	Yunganoside J1/L1	Triterpene saponin	B, C	[42]
70	44.8	68.35	837.6	819, 781, 661, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	C, D, E	[7,9,17, 22,42,44-47]
71	50.3	68.44	837.7	819, 775, 661, 644, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	A, B, C, D, E	[7,9,17, 22,42,44-47]
72	59.7	68.60	821.5	803, 759, 645, 351	18αglycyrrhizin / macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	B, C, D, E	[17,42,45]
73	31.1	70.72	1115.9	1097, 793, 497, 435	NI		A, D	
74	34.6	70.78	999.6	881, 837, 819, 661, 351	22-Hydroxyl-licorice saponin A3	Triterpene saponin	A, B, C, E	[42,45,48]
75	36.9	70.82	989.6	966, 924, 821, 803, 645, 501, 351, 289	Licorice saponin A3 or isomer	Triterpene saponin	A, B, C, D, E	[45]
76	39.9	70.87	969.8	951, 907, 793, 351, 289	Albiziasaponin B or isomer	Triterpene saponin	C, E	[42]
77	41.8	72.19	837.6	821, 776, 661, 485, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	B, C, D, E	[7,9,17, 22,42,44-47]
78	36.0	73.40	837.7	821, 776, 661, 485, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	B, C, D, E	[7,9,17, 22,42,44-47]
79	38.1	71.74	853.6	837, 835, 677, 502, 351	22-Hydroxy licorice saponin G2	Triterpene saponin	B, C, D, E	[42,48]
80	42.9	74.82	983.7	965, 880, 821, 661, 497, 339, 321	Licorice saponin A3 or isomer	Triterpene saponin	B, C, D, E	[45]

649

650

651 **Table 2.** Tentatively identified metabolites exclusively found present in the indicated
652 licorice source.

China ID	^{2}D t_R (s)	Total t_R (min)	$[M-H]^-$	Main MS/MS fragments detected	Identification	Structure class	Ref.
A1	51.2	8.65	331.2	311, 293, 229, 211, 171, 139, 99	NI		
A2	53.4	8.69	269.8		Emodin		[49]
A3	72.1	10.30	407.4	379, 284, 235, 177, 135	NI		
A4	74.2	10.34	423.9	391, 347, 322, 229, 207, 193, 177	Kanzonol H or isomer	Prenylated flavonoid	[50,51]
A5	31.2	12.22	417.2	297, 255, 174, 135	(Iso)liquiritin / Neo(iso)liquiritin	Flavanone	[43-45]
A6	34.3	12.27	433.3	385, 301, 271, 176, 151	5-Hydroxyliquiritin	Flavanone	[52]
A7	38.9	12.35	695.3	549, 531, 399, 255	Licorice glycoside B/D1/D2	Flavanone	[9,27,42]
A8	40.7	12.38	417.4	297, 255, 135	(Iso)liquiritin / Neo(iso)liquiritin	Flavanone	[43-45]
A9	43.1	12.42	475.3*	417, 345, 311, 267, 252	Ononin	Prenylated flavonoid	[53]
A10	54.2	12.60	255.0	134, 119	Liquiritigenin or isomer	Flavanone	[39-42]
A11	59.1	12.69	369.3	352, 339, 323, 309, 297, 284	NI		
A12	61.0	12.72	423.4	405, 387, 355, 264, 213, 148	NI		
A13	31.4	13.52	419.0	298, 256, 153, 134, 119	NI		
A14	33.3	13.56	551.1	515, 445, 429, 419, 297, 255, 221	NI	Flavanone	
A15	32.0	14.83	586.7	549, 539, 504, 399	NI		
A16	31.0	47.32	481.4	438, 432, 417, 381, 321, 255	NI	Flavanone	
A17	35.3	47.39	551.6	515, 431, 419, 389, 297, 257	NI	Flavanone /Chalcone	
A18	37.3	47.42	551.0	429, 417, 297, 255	NI	Flavanone /Chalcone	
A19	38. 5	47.44	478.5	423, 378, 319, 271, 167	NI		
A20	32.7	49.95	777.1	716, 627, 537	Apioglycyrrhizin	Triterpene saponin	[42]
A21	27.5	51.16	579.4	547, 417, 324, 255	Liquiritigenin -7, 4' diglucoside / Glucoliquiritin	Flavanone	[53]
A22	28.9	51.18	528.7	509, 483, 410, 273, 247	NI		
A23	46.9	51.48	836.0	775, 685, 626	Cicloheptaleucyl	Cyclopeptide	[42]
A24	27.7	53.76	590.8	549, 531, 471, 459, 297, 255	Liquiritigenin 4'-[3-acetylapiosyl-(1-2)] glucoside	Triterpene saponin	
A25	31.8	53.83	881.8	864, 819, 754, 705, 644, 584, 351	22-Acetoxyl glycyrrhizic acid / 22β-acetoxyl licorice saponin J2	Triterpene saponin	[43]
A26	46.9	54.08	866.3	805, 626	NI		
A27	50.2	54.14	966.3	947, 896, 863, 757, 717, 671, 629, 579, 537	NI		
A28	60.6	54.31	809.3	745, 627, 537	NI		
A29	37.3	55.22	810.3	629, 540	NI		
A30	40.7	55.28	923.9	905, 877, 861, 777, 716, 627, 609, 537	NI		
A31	41.9	55.30	1014.1	995, 951, 909, 867, 805, 782, 763, 745, 687, 645, 601, 487, 371	NI		
A32	46.9	55.38	997.9	935, 893, 787, 747, 652, 629, 579, 539	NI		
A33	52.1	55.47	868.0	830, 806, 690, 599, 487, 351	NI	Triterpene saponin	
A34	41.0	56.58	982.7	963, 921, 903, 859, 815, 685, 669, 643, 625, 595, 581, 535	NI		

A35	50.5	56.74	864.8	847, 803, 687, 351	22-Acetoxyl licorice saponin C2	Triterpene saponin	[46]
A36	28.7	57.68	709.6	647, 617, 563, 473, 443, 383	NI		
A37	37.5	57.83	1012.2	993, 951, 889, 845, 744, 699, 685, 667, 643, 625, 595, 535, 423	NI		
A38	28.7	58.33	955.8	937, 893, 793, 747, 643, 539	Hederagenin-3-Orhamnosyl glucoryl arabinosyl glucuronide or isomer	Triterpene saponin	[55,55]
A39	37.7	58.48	837.3	817, 773, 701, 659, 351, 289	Licorice saponin G2 or isomer / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P/ Macedenosin B / Macedenosin A	Triterpene saponin	[7,9,17,22, 42,44-47]
A40	26.4	58.94	676.7	632, 550, 475, 402, 297, 256	NI		
A41	44.7	59.25	823.1	654, 351	Licorice saponin J2 / Uralsaponin C or isomer	Triterpene saponin	[39,44,46]
A42	28.7	60.28	823.2	806, 761, 647, 351	Licorice saponin J2 / Uralsaponin C or isomer	Triterpene saponin	[36,44-46]
A43	40.9	60.48	881.5	862, 819, 575, 705, 643, 466, 351	22-Acetoxyl glycyrrhizic acid / 22β-acetoxyl licorice saponin J2	Triterpene saponin	[46]
A44	35.1	62.99	967.7	933, 907, 794, 351	NI	Triterpene saponin	
A45	37.7	64.33	895.6	877, 833, 721, 351	22-Acetoxyl licorice saponin G2	Triterpene saponin	[46]
A46	33.4	65.56	841.6	821, 663, 351	NI	Triterpene saponin	
A47	41.1	65.69	882.1	863, 803, 351	NI	Triterpene saponin	
A48	33.5	72.06	1129.7	1060, 967, 951, 931, 807, 627, 497, 321	NI	Triterpene saponin	
A49	33.5	73.36	837.2	820, 661, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A	Triterpene saponin	[7,9,17,22, 42,44-47]
A50	30.35	74.61	1132.6	1114, 1072, 1052, 497	NI	Triterpene saponin	

Iran

ID	^{2}D t_R (s)	Total t_R (min)	[M-H]$^-$	Main MS/MS fragments detected	Identification	Structure class	Ref.
B1	40.7	9.78	577.2	559, 539, 472, 386, 329	NI		
B2	61.3	10.12	557.1	539, 521, 499, 381, 323, 261, 173	NI		
B3	67.9	10.23	423.0	403, 352, 309, 229, 193	Kanzonol H or isomer	Prenylated flavonoid	[53]
B4	70.6	10.28	468.4	441, 423, 405, 335, 248, 178	NI		
B5	32.6	12.24	477.7*	443, 433, 317, 271, 252, 176	Naringenin-7-Oglucoside or isomer	Flavanone	[52]
B6	68.6	12.84	742.7	725, 672, 633, 539, 417, 309	NI		
B7	31.2	13.52	496.6	481, 460, 418, 297, 230, 162	NI		
B8	34.4	13.57	725.3	632, 612, 549, 533, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	[9,22,42,44]
B9	37.6	13.63	695.3	549, 531, 399, 255	Licorice glycoside B/D1/D2	Flavanone	[9,27,42]
B10	68.6	14.14	525.7	456, 334	NI		
B11	30.4	20.01	491.4	446, 283, 267, 211	NI		
B12	35.1	27.89	578.2	534, 387, 326, 283, 268, 194	NI	Isoflavone	
B13	35.2	29.19	695.3	576, 549, 531, 255	Licorice glycoside B/D1/D2	Flavanone	[9,27,42]
B14	33.6	42.16	563.6	483, 427, 310, 267, 253, 183	NI		

B15	34.0	44.77	697.5	662, 551, 533, 255	NI	Flavanone /Chalcone	
B16	39.5	44.86	903.5	885, 873, 725, 531, 255	NI	Flavanone	
B17	29.3	45.99	518.9	446, 385, 307, 205, 153	NI		
B18	31.0	46.02	633.9	587, 549, 417, 339	NI		
B19	33.4	46.06	921.7	903, 873, 725, 549	NI		
B20	37.1	46.12	727.4	685, 550, 532, 309, 255	NI	Flavanone	
B21	38.8	48.75	565.3	471, 433, 271, 161	Naringenin 7-O-(2-β-D-apiofuranosyl)-β-D-glucopyranoside	Flavanone	[42]
B22	52.2	52.87	808.3	745, 627, 539, 469	NI	Triterpene saponin	
B23	40.1	55.27	1013.7	996, 952, 928, 909, 805, 763, 745, 687, 645, 601, 469	NI		
B24	46.9	56.68	824.0	804, 779, 762, 643, 600, 554, 485	NI	Triterpene saponin	
B25	27.0	57.65	563.1	545, 503, 485, 473, 443, 383, 353	Shaftoside	Flavone	[56]
B26	50.7	58.05	924.0	905, 862, 777, 716, 627, 537	NI	Triterpene saponin	
B27	67.3	58.32	805.5	787, 746, 631, 351, 289	Licorice saponin C2	Triterpene saponin	[9]
B28	51.3	59.36	1222.1	1204, 1088, 1045, 965, 869, 789, 352	NI	Triterpene saponin	
B29	45.4	60.56	1027.8	1009, 984, 922, 706, 559, 497, 339	NI	Triterpene saponin	
B30	34.0	64.27	825.6	808, 779, 765, 649, 599, 554, 351, 333, 259	NI	Triterpene saponin	
B31	32.1	65.53	969.7	953, 925, 909,835, 824, 793, 351	Albiziasaponin B or isomer	Triterpene saponin	[42]
B32	45.0	68.35	837.6	819, 661, 485, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B / Macedenosin A or isomers	Triterpene saponin	[7,9,17,22, 42,44-47]
B33	52.9	68.48	839.1	821, 777, 715, 663, 645, 488, 351, 334, 289, 261, 235	Yunganoside G2 or isomer	Triterpene saponin	[9]
B34	50.2	69.74	969.7	951, 924, 887, 833, 647, 497, 405, 339, 321	Albiziasaponin B or isomer	Triterpene saponin	[42]
B35	56.9	71.15	839.1	837, 821, 663, 351	Yunganoside G2 or isomer	Triterpene saponin	[9]
B36	50.8	73.65	985.8	967, 923, 851, 663, 497, 435, 321	Yunganoside K1 or isomer	Triterpene saponin	[17]

Crotone (Italy)

ID	^{2}D t_R (s)	Total t_R (min)	$[M-H]^-$	Main MS/MS fragments detected	Identification	Structure class	Ref.
C1	53.5	8.69	391.0		Hispaglabridin	Prenylated flavonoid	[43]
C2	62.5	8.84	385.6	367, 340, 311, 162	Glyasperin E	Prenylated flavonoid	[57]
C3	31.2	12.22	419.2	255, 135	NI		
C4	34.1	12.31	420.2	401, 297, 257, 119	NI		
C5	71.5	12.89	667.4	644, 553, 471, 290, 210	NI		
C6	31.3	13.52	659.7	642, 548, 481, 335	NI		
C7	30.1	20.00	549.2	488, 445, 429, 325, 255	NI	Flavanone	
C8	31.2	47.32	549.7	494, 430, 342, 293, 256	NI	Flavanone	
C9	29.4	48.59	634.0	598, 549, 492	NI		
C10	33.1	51.25	724.3*	677, 633, 577, 550, 283, 225	NI		
C11	30.8	54.41	721.9	703, 647, 617, 577, 559, 457, 383, 353	Apigenin 6-C-α-L-rhamnopyranoside-8-C-[6'''-(3-methylglutaroyl)-β-D-glucopyranoside]	Flavone	[58]
C12	53.3	54.79	793.7	775, 750, 731, 644, 485, 384, 351	NI	Triterpene saponin	

C13	43.9	55.33	807.3	789, 765, 633, 524, 423, 351	Licorice saponin B2 or isomer	Triterpene saponin	[42]
C14	55.7	55.53	863.5	845, 687, 393, 351, 263	22-Acetoxy-glycyrrhaldehyde	Triterpene saponin	[49]
C15	44.8	59.25	807.3	791, 747, 631, 527, 351	Licorice saponin B2 or isomer	Triterpene saponin	[42]
C16	41.3	59.84	953.6	936, 862, 803, 497, 321	Yunganoside D1 / H1 / I1	Triterpene saponin	[17]
C17	64.7	60.23	821.4	803, 759, 645, 351	18αglycyrrhizin/ Yunnanglysaponin B/ Macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	[17,42,45]
C18	42.8	64.41	895.6	877, 660, 351	22β-Acetoxyl licorice saponin G2	Triterpene saponin	[46]
C19	40.7	72.68	969.7	952, 922, 793, 351	Albiziasaponin B or isomer	Triterpene saponin	[42]

Azerbaijan

ID	^2D t_R (s)	Total t_R (min)	$[M-H]^-$	Main MS/MS fragments detected	Identification	Structure class	Ref.
D1	62.5	10.14	559.0	541, 421, 375, 313, 223	NI		
D2	66.3	10.21	556.9	539, 487, 391, 336, 253	NI		
D3	68.2	10.24	411.5	392, 236, 217, 177	NI		
D4	41.0	11.08	661.3	642, 549, 481, 335	NI		
D5	32.8	12.25	725.2	549, 531, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	[9,22,42, 44]
D6	39.5	12.36	737.3	691, 653, 613, 543, 381, 267	NI		
D7	70.5	12.88	757.1	741, 711, 521, 349, 297	NI		
D8	31.2	13.52	480.9	444, 376, 283, 137	NI		
D9	34.4	13.57	727.7	669, 551, 533, 399, 311, 254	NI		
D10	36.6	13.61	697.5	632, 550, 532, 430, 281	NI		
D11	33.8	16.16	648.1	561, 322, 267	NI		
D12	33.8	17.46	598.3	561, 867	NI		
D13	35.1	33.09	561.2	482, 309, 267, 252	Glycyroside or isomer	Isoflavone	[42,51]
D14	33.5	48.66	561.1	484, 401, 309, 267	Glycyroside or isomer	Isoflavone	[42,51]
D15	31.5	48.63	711.1	693, 681, 601, 417, 385	NI		
D16	33.3	48.66	670.8	626, 549, 531, 255	NI	Flavanone /Chalcone	
D17	38.7	54.60	921.4	903, 874, 835, 790, 725, 550, 531	NI		
D18	53.6	55.49	955.6	937, 747, 630	Hederagenin-3-O-rhamnosyl glucoryl arabinosyl glucuronide or isomer	Triterpene saponin	[54,55]
D19	45.1	55.35	867.6	848, 691, 351	NI	Triterpene saponin	
D20	51.1	59.35	867.3	847, 803, 689, 593, 351	NI	Triterpene saponin	
D21	65.9	60.25	807.4	790, 744, 676, 630, 454, 351	NI	Triterpene saponin	
D22	30.2	64.20	713.5	674, 593, 550, 255	NI	Flavanone /Chalcone	
D23	41.6	64.39	985.5	863, 823, 805, 647, 497, 351	Yunganoside K1 or isomer	Triterpene saponin	[17]
D24	43.6	64.43	1027.8	990, 983, 959, 942, 646, 497	NI	Triterpene saponin	
D25	48.4	64.51	837.5	819, 793, 775, 661, 351	Licorice saponin G2 / 24-hydroxyl-glycyrrhizin/ YunganosideK2 / Macedonoside P / Macedenosin B / Macedenosin A	Triterpene saponin	[7,9,17,22, 42,44-47]
D26	49.9	64.53	837.6	819, 793, 775, 661, 351	Licorice saponin G2 / 24-hydroxyl-glycyrrhizin/ YunganosideK2 / Macedonoside P /	Triterpene saponin	[7,9,17,22, 42,44-47]

D27	58.7	64.68	821.4	805, 760, 647, 627, 351	Macedenosin B / Macedenosin A 18αglycyrrhizin/ Yunnanglysaponin B/ macedonoside C / Yunganoside L2 / Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	[17,42,45]
D28	37.2	66.92	983.5	965, 923, 863, 821, 804, 760, 645, 351	Licorice saponin A3	Triterpene saponin	[45]
D29	39.8	68.26	971.2	953, 909, 791, 585, 497, 435, 351	NI	Triterpene saponin	
D30	31.8	70.73	969.7	951, 907, 807, 793, 643, 553, 497, 351	Albiziasaponin B or isomer	Triterpene saponin	[42]
D31	31.1	72.02	1115.9	1098, 981, 951, 858, 793, 497, 435		Triterpene saponin	
D32	30.7	74.61	985.5	983, 967, 924, 810, 496, 405, 351	Yunganoside K1 or isomer	Triterpene saponin	[17]
D33	48.2	76.20	969.7	946, 864, 821, 803, 645, 527, 351, 289	NI	Triterpene saponin	
D34	35.9	77.30	987.2	967, 923, 810, 351	NI	Triterpene saponin	

Villapiana (Italy)

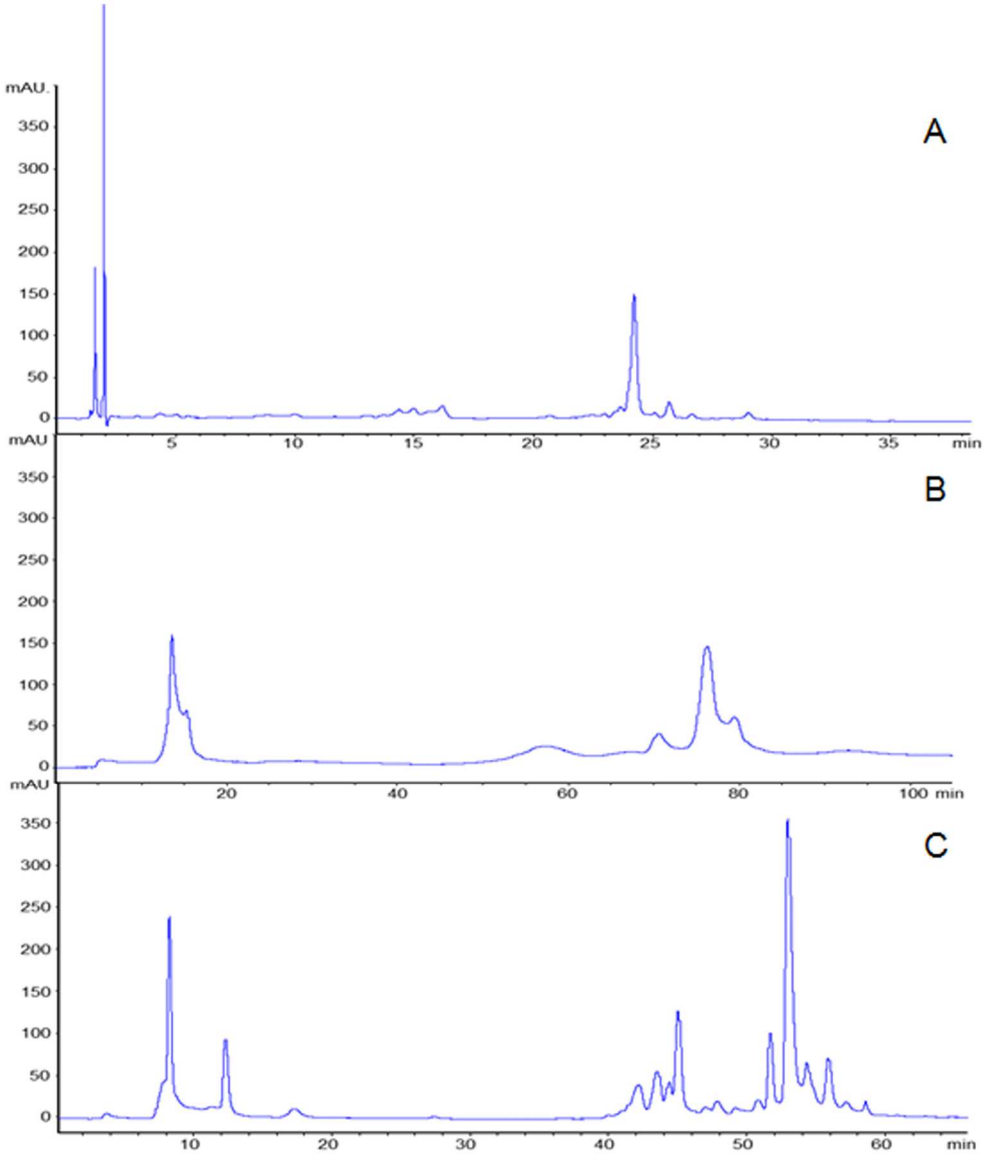
ID	^{2}D t_R (s)	Total t_R (min)	[M-H]$^-$	Main MS/MS fragments detected	Identification	Structure class	Ref.
E1	53.8	8.70	345.4	235, 166	NI		
E2	57.4	8.76	472.2	386, 296, 178			
E3	62.2	8.84	359.0	311, 269, 177			
E4	71.8	9.00	323.2	253, 213, 201, 135, 121	Glabridin	Prenylated flavonoid	[9,43]
E5	35.4	9.69	477.1	432, 353, 268, 253, 221	Naringenin-7-Oglucoside or isomer	Flavanone	[42]
E6	32.5	9.64	592.4	549, 531, 255	NI		
E7	34.7	9.68	725.2	611, 549, 532, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	[9,22,42, 44]
E8	37.3	9.72	695.3	549, 531, 255	Licorice glycoside B/D1/D2 or isomer	Flavanone	[9,27,42]
E9	30.1	42.10	725.4	678, 605, 577, 562, 549, 531, 310, 255	Licorice glycoside A/C1/C2 or isomer	Flavanone	[9,22,42, 44]
E10	29.3	43.39	591.5	549, 531, 473, 399, 255	NI	Flavanone	
E11	34.6	51.28	549.2	429, 417, 297, 255	(Iso)liquiritin apioside	Flavanone	[7,22,44, 45]
E12	34.6	55.18	723.7*	677, 659, 577, 457, 383	Ciclohexaleucyl	Cyclopeptide	[42]
E13	36.7	55.21	925.7	908, 894, 879, 717, 603, 539, 509, 469	NI		
E14	32.9	60.35	939.6	922, 877, 777, 732, 644, 524, 457	dHex-Hex-HexA-Soyasapogenol E	Triterpene saponin	[54]
E15	44.4	60.54	707.2	674, 648, 617, 563, 545, 443, 383, 353, 255	3-Hydroxyl-3-methylglutaroyl-(iso)schaftoside	Triterpene saponin	[42]
E16	34.6	62.98	865.3	847, 804, 689, 582, 351	22-acetyl licorice saponin B2	Triterpene saponin	[47]
E17	40.8	63.08	823.5	805, 761, 648, 351	Licorice saponin J2 / Uralsaponin C or isomer	Triterpene saponin	[33,44-47]
E18	57.7	63.36	1011.7	994, 923, 689, 497, 339	Licorice saponin D3	Triterpene saponin	[7]
E19	43.2	65.72	821.3	804, 760, 646, 351	18αglycyrrhizin/ Yunnanglysaponin B/ Macedonoside C/ Yunganoside L2/ Uralsaponin A/ Licorice saponin H2/ Licorice saponin K2	Triterpene saponin	[17,42,45]
E20	41.3	73.49	939.2	922, 878, 732, 643, 554, 485, 356	NI	Triterpene saponin	
E21	40.8	74.78	837.5	821, 775, 661, 351	Licorice saponin G2 or isomers / 24- hydroxyl-glycyrrhizin/ YunganosideK2/ Macedonoside P / Macedenosin B /	Triterpene saponin	[7,9,17,22, 42,44- 47]

Macedenosin A or isomers

653 * ions detected as $[M-H + HCOOH]^-$

654

655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250
200
150
100
50
0
B
20
40
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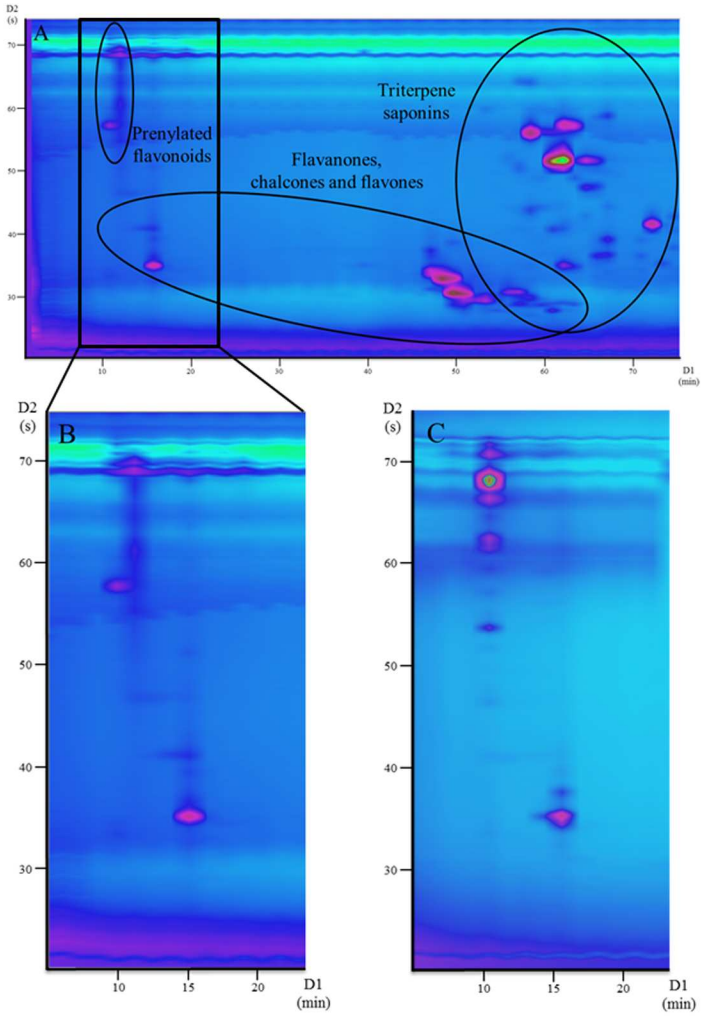
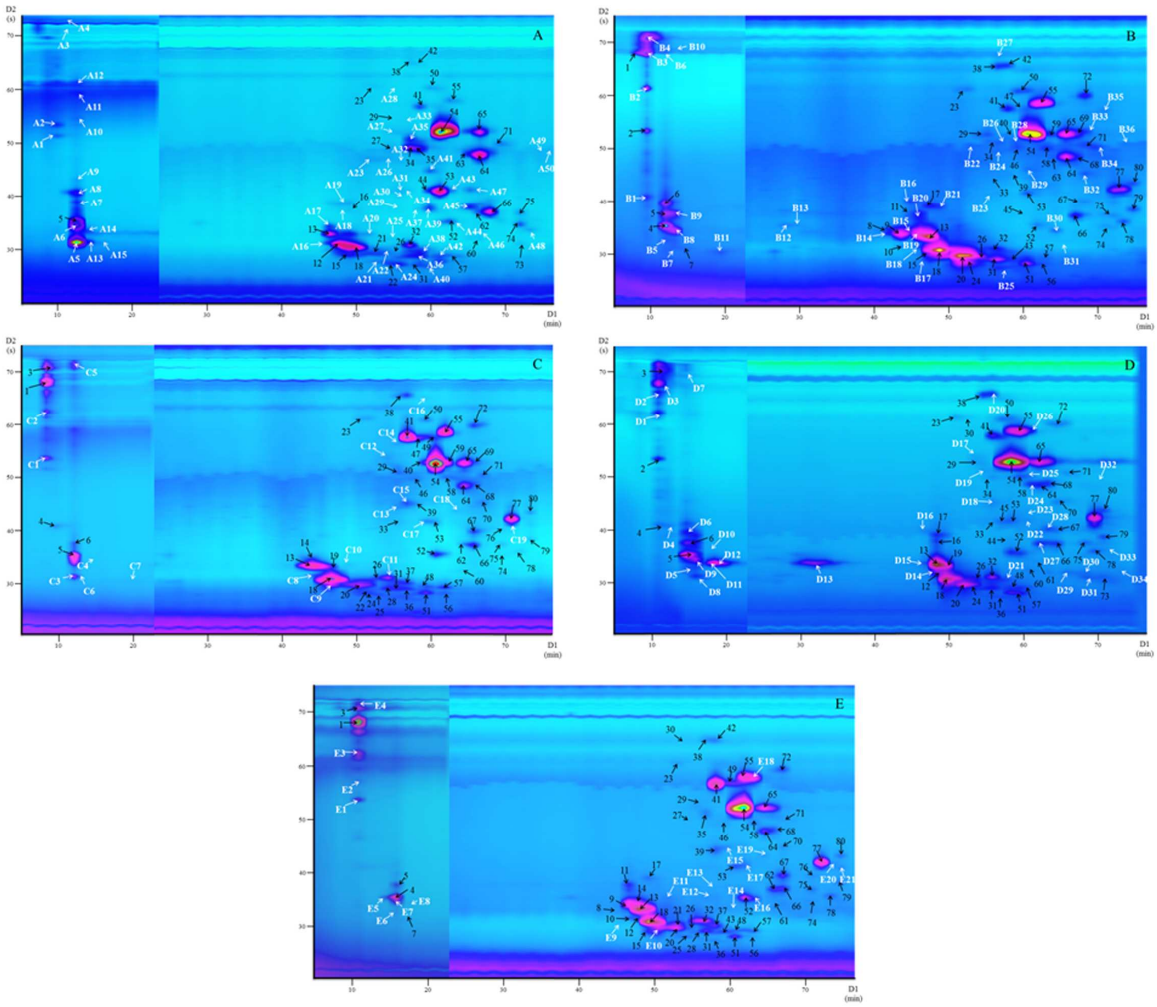
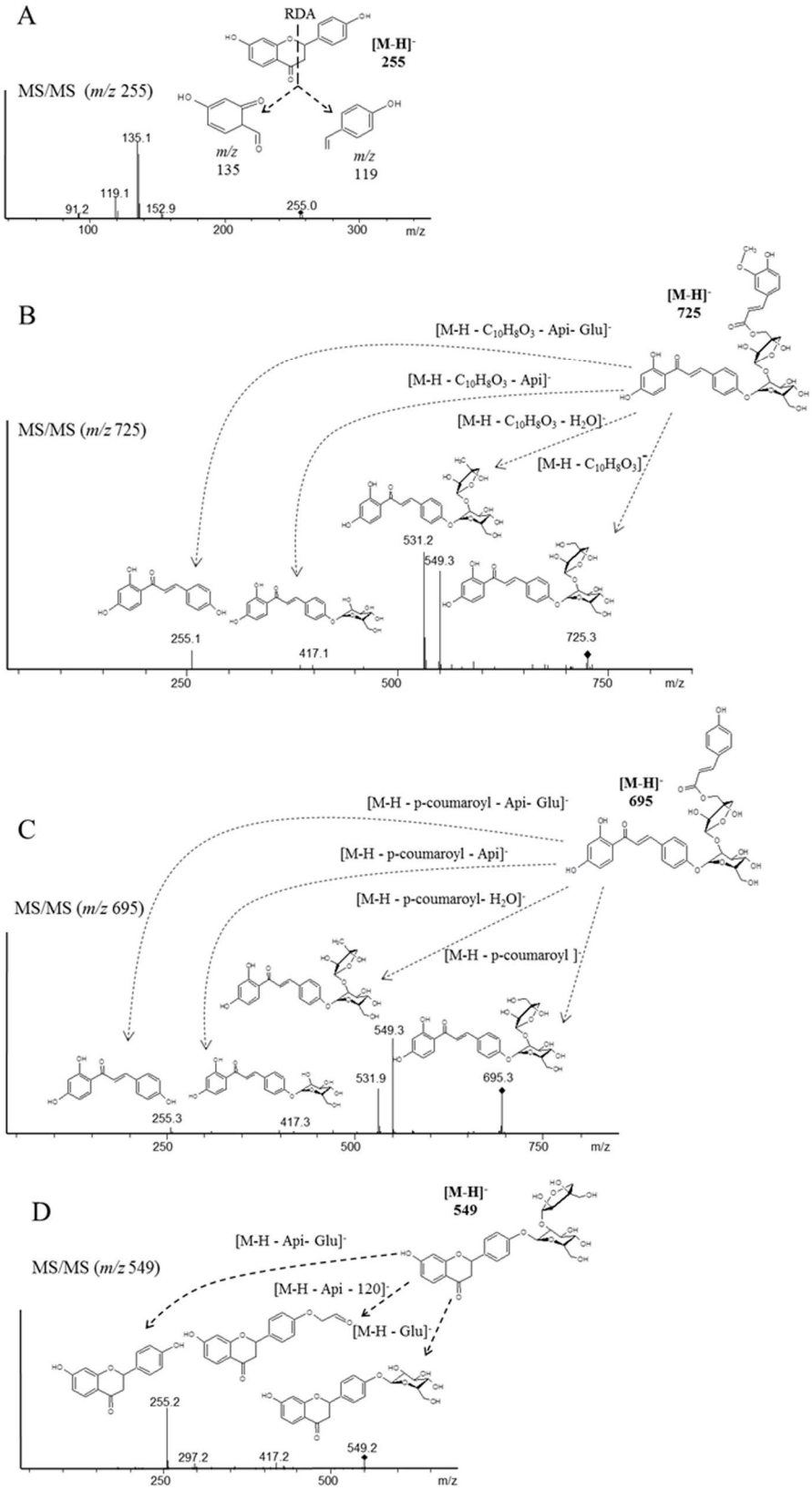
min



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m/z
135
m/z
119
100
200
300
m/z
B
[M-H]-
725
[M-H - C10H8O3 - Api- Glu]-
[M-H - C10H8O3 - Api]-
[M-H - C10H8O3 - H2O]-
[M-H - C10H8O3]-
MS/MS (m/z 725)
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255.1
417.1
725.3
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500
750
m/z
C
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m/z
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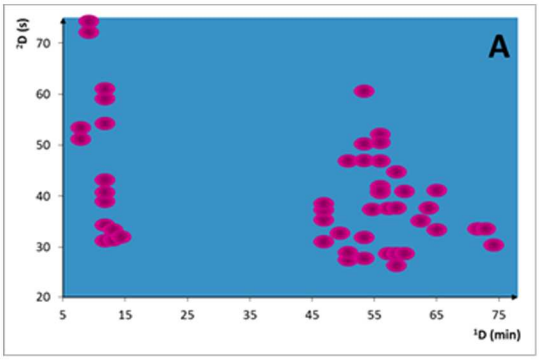
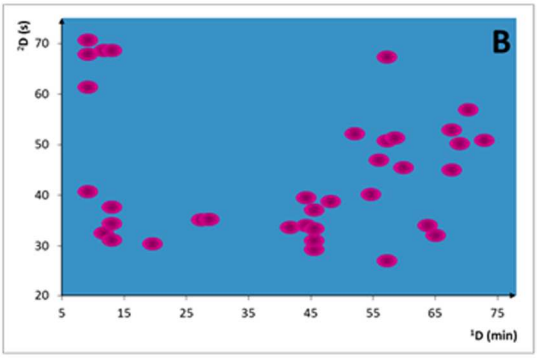
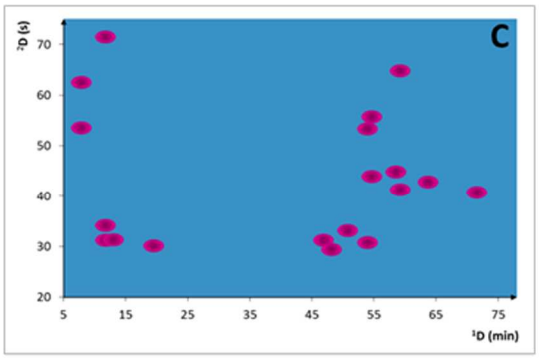
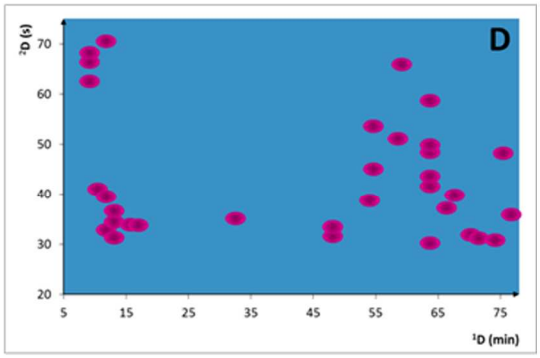
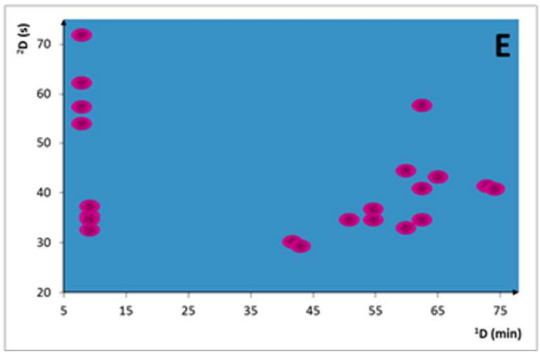
m/z











HIGHLIGHTS.

- Non-correlated comprehensive LC is applied to licorice roots metabolic profiling for the first time

- Complex 2D-plots for different licorice samples are attained

- Typical metabolite patterns potentially helpful to assess origin are obtained

- Different gradients in D2 are employed to improve separation

- Up to 89 compounds are separated and detected in the metabolite profile