A metabolomic approach to quality determination and authentication of raw plant material in the fragrance field. Iris rhizomes: A case study

A metabolomic approach to quality determination and authentication of raw plant material in the fragrance field. Orris roots: a case study

Jerome Masson[1,2], Erica Liberto[2], Hugues Brevard[1], Carlo Bicchi[2], Patrizia Rubiolo[2*]

[1] Robertet SA, Research Division, 37 Avenue Sidi Brahim, F-06130 Grasse, France
[2] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, I-10125 Torino, Italy

Corresponding author:

Prof. Dr. Patrizia Rubiolo

Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, I-10125 Torino, Italy; Tel: +39 011 6707662, fax: +39 011 6707687;

e-mail: patrizia.rubiolo@unito.it

Abstract

This study aimed to discriminate twenty-two samples of commercial *Iris* rhizomes (orris root) by species and origin (*Iris germanica* (Morocco), *I. albicans* (Morocco), *I. pallida* (Morocco), *I. pallida* (China), *I. pallida* (Italy)) by applying a strategy derived from those adopted in metabolomics. The specimens' fingerprints from conventional analysis methods (LC-UV and/or LC-MS) were unable to provide clear discrimination. A strategy combining UHPLC/TOF-HRMS, in positive and negative modes, with multivariate statistical methods was therefore applied. Exact Mass/Retention Time (EMRT) pairs obtained by UHPLC-TOF/HRMS were successfully submitted to statistical processing by Principal Component Analysis (PCA), Partial Least Square Discriminant Analysis (PLS-DA), and then Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA), to extract the discriminating EMRT pairs through their trend views. 146 EMRT pairs were selected on the basis of their trend views, because they significantly varied, and 104 of them were included to discriminate between species and origins. 32 of them were tentatively identified as discriminating markers (flavonoids, isoflavonoids, triterpenoids, benzophenone derivatives and related glycosides …) from the reference database created on the basis of *Iris* genus components reported in the literature: eight of them specific for *I. albicans*, four for *I. germanica*, five for *I. pallida* (Italy), five for *I. pallida* (China), and ten for *I. pallida* (Morocco). The reliability of this strategy was confirmed by identifying species and origin of two unknown samples submitted to the same analytical procedure.

Keywords:

Iris species and origins; Metabolomic strategy, Phytochemical analysis; UHPLC/TOF-MS; statistical data treatment, discriminant markers

1. Introduction

Raw plant materials used in the flavor and fragrance field often differ in terms of species and origins and, as a consequence, they present variable chemical compositions, resulting in different characteristic organoleptic properties. The most recent developments in analytical technologies have greatly contributed to an improved knowledge of the chemical composition of vegetable matrices, thus affording more precise definition of their identity and origin.

Metabolomics is one of the most recent "omics" sciences. It was introduced at the end of the 20th century, and first defined by Oliver et al. in 1998 [1] as "*the systematic study of the unique chemical metabolite fingerprints (the metabolome) resulting from specific cellular processes*". This discipline entails defining a matrix fingerprint or profile, mainly consisting of small molecules, that is representative of the biological system investigated. Several practical approaches have been developed to meet the requirements of metabolomics, some being of great interest for quality evaluation and authentication of raw plant materials. The most promising approaches in this connection are *fingerprinting* and *profiling,* which differ in terms of results achieved and methodology adopted. *Fingerprinting* is an *untargeted method,* in which samples are compared on the basis of their profiles, to detect quali- and quantitative differences between them, or to classify samples within a data set. *Fingerprinting* comprises general and rapid high-throughput screening, with the aim of discriminating and classifying samples. As a consequence, it can also be achieved through a non-separative approach (HS-MS, direct infusion-MS, NMR, FT-IR (MIR, NIR, etc.) combined with appropriate statistical data processing. *Profiling* is a *targeted method,* in which samples are compared on the basis of the distribution of known target analytes (markers). *Profiling* produces a detailed analytical profile of the sample, by combining separation with a spectroscopic technique (*e.g.* GC/MS, LC/MS, CE/MS etc.) with the aim of identifying and quantifying a selected number of diagnostic components [2].

Recent advances in analytical technologies, in particular the on-line combination of UHPLC with TOF-MS, make it possible to acquire rapidly a full set of exact masses present in different matrices, although the resulting huge amount of data may be complex to manage. The use of this approach, in combination with advanced statistical methods [3] deriving from metabolomics, may be a further decisive option in the area of phytochemical analysis. It can

73 successfully be used to define specific markers, and to establish identity profiles for
74 unequivocal identification and authentication of raw plant materials.
75 *Iris* rhyzome essential oil, commonly known as "Orris butter", is one of the most important,
76 and most expensive, raw materials used in the fragrance field [4]. It is valued for its unique
77 violet-like scent, and is obtained by hydrodistillation of *Iris* rhizomes. Its distinctive odor is
78 due to irones (**Figure 1**), first described by Tiemann and Krüger in 1893 [5]; their structure
79 was defined by Naves [6] and Ruzicka [7], and their stereochemistry elucidated by
80 Rautenstrauch and Ohloff [8, 9].
81 Several studies on species belonging to the *Iris* genus have dealt with their biological activity
82 [10-13], with the formation of irones and identification of their precursors [14-25], and have
83 lead to the isolation of different classes of secondary metabolites, namely triterpenoids,
84 flavonoids and phenols [25-27]. In 1983, Krick [14] showed that iripallidal and iriflorental are
85 the irone precursors (Fig. 1). Subsequently, Marner's group [15, 16] isolated and described
86 several mono-cyclic [17-19] and bicyclic iridal structures [20], for a total of more than forty
87 triterpenic compounds, and also clarified their biosynthetic pathway [21-24]. In 1993, Bicchi
88 and Rubiolo [25] showed that iridals were also present as esters of myristic acid.
89 The phenolic fraction of *Iris* rhizomes has also been investigated in depth. In 1983, Ali [26]
90 determined the structure of several flavonoids and isoflavonoids (**Figure 1**) in *I. germanica*
91 rhizomes, including irigenin, irisolidon, and iridin, all known for their biological activities.
92 Bicchi and Rubiolo [25] identified irisflorentin, iristectogenin B, and iriflophenone in *I.*
93 *pallida.* More recently, Roger et al. [27] isolated, identified, and quantified 11 flavonoids in *I.*
94 *pallida* and *I. germanica* rhizomes, and characterized a benzophenone derivative typical of *I.*
95 *pallida* (2,6,4'-trihydroxy-4-methoxybenzophenone).
96 Over the last 30 years, the geographical and botanical origins of "orris butter" have been the
97 objective of several studies, mostly based on the isomeric abundances of irones and iridals
98 [28, 29], and more recently also on the flavonoid fraction, whose composition was used to
99 discriminate *I. germanica* from *I. pallida* from Morocco [27].
100 This study aimed to apply a metabolomic approach, based on both UHPLC/TOF-MS analyses
101 and statistical elaboration, to authenticate species and origins of a set of samples of *Iris*
102 rhizomes, thanks to the definition of specific discriminating markers. The reliability of the
103 strategy applied was confirmed by controlling the authenticity of unknown samples.
104

2. Experimental

2.1 Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), ortophosphoric acid, formic acid, and ammonium formate (UHPLC/MS grade) were purchased from Biosolve (Dieuze, France). Distilled water was purified 'in-house' using an ELGA MilliQ system VeoliaWater STI (Le Plessis-Robinson, France). CH_2Cl_2 was purchased from Carlo Erba (Val de Reuil, France). Leucine-Enkephalin was from Waters (Milford, USA)

2.2 Plant material

Five groups of certified samples of *Iris* rhizomes rhizomes available from Robertet (Grasse, France) for a total of 24 samples were used for this study. They comprised *Iris germanica* (3, origin: Morocco) (*g*,MA), *I. albicans* (4, Morocco) (*a*,MA), *I. pallida* (3, Morocco) (*p*,MA), *I. pallida* (7, China) (*p*,CN), *I. pallida* (5, Italy) (*p*,IT) and 2 unknown samples.

2.3 Sample preparation

Dried plant material was crushed and extracted in a Soxtec system (Foss, France), with the aim of developing an exhaustive and, at the same time, simple and automated sample preparation technique compatible with demands for applicability to a routine quality control process. Different experiments were carried out to optimize the extraction parameters (extraction and rinsing times, extraction solvents, etc.). Five different solvent mixtures (methanol 100%; methanol/methylene chloride 70/30, 50/50; methanol/chloroform 70/30, 30/70) were tested. The extraction yield was evaluated through the relative areas of five components, representing different chemical classes already identified in the *Iris* genus (irigenin; iripallidal, iriflorental, iridin, iriflophenone) analyzed by UHPLC/TOF-MS at two different collision capillary voltages (1kV and 3kV). On the basis of the results of these preliminary tests, the following method was applied thereafter: plant material (25g) was submitted to an exhaustive extraction with MeOH:CH_2Cl_2 (70:30) (100mL) for two hours at boiling temperature, and to rinsing for another two and a half hours. The resulting extracts were then analyzed by HPLC/DAD-UV and UHPLC/TOF-MS in duplicate.

2.4 HPLC/DAD-UV analysis

HPLC analyses were run on an Agilent 1200 system. A Nucleodur HTec 5µm 4.6 x 250mm C18 column (Macherey-Nagel, France) at 45°C was used for chromatographic separations. Solvent A was water with orthophosphoric acid (0.5%), solvent B was methanol (MeOH), and solvent C was acetonitrile (ACN). The gradient profile was: 2.5% of solvent B and solvent C for 5 min in isocratic mode; then from 2.5% to 47.5% of solvent B and Solvent C over 40 min, hold for 5 min; the system was then re-equilibrated for 10 min at the initial conditions. The flow rate was 1 ml/min, and the injection volume 10µL. Analyses were run at a wavelength of 230nm, with a bandwidth of 4nm.

2.5 UHPLC/TOF-MS analysis

UHPLC analyses were carried out on a Waters ACQUITY® UPLC - Class system. Chromatographic separation was achieved on an ACQUITY® UPLC HSS, 1.8µm, 2.1 x 100mm C18 stationary phase (Waters, Milford, USA), which was maintained at 45°C; mobile phase: eluent A: 5mM ammonium formate solution adjusted to pH=3.8 with formic acid; eluent B: methanol/acetonitrile (50:50) with ammonium formate (5mM) and 0.1% formic acid. Mobile phase gradient: from 5% B (1 min) to 95% of B in 10 min (3 min); re-equilibration at initial condition: 2 min; flow rate: 0.45 ml/min; injection volume: 1µL.

UHPLC/TOF-MS analyses were run with a Waters XEVO® G2 TOF system. ESI source parameters: capillary voltage: 1kV, cone voltage: 30V, extraction cone: 4V, source temperature: 120°C, desolvation temperature: 400°C, gas flow (nitrogen): 10L/h, desolvation gas flow: 1200L/h. The analyses were performed in positive and negative modes. The mass axis was calibrated with sodium formate in the range 50 to 1200 Da; mass lockspray: Leucine Enkephalin was used to correct masses; two masses were checked in both modes (positive mode: 278.1141; 556.2771 and negative mode: 236.1035; 554.2615).

Each analysis was also carried out at a high capillary voltage (3kV) in both positive and negative modes. The acquisitions were performed in MS^E mode, using argon as collision gas.

2.6 Data processing

The resulting data set was processed through the package Markerlynx; (Waters, Milfors USA) based on the determination of Exact Mass Retention Time pairs (EMRT).

168 The EMRT pairs were first processed by Principal Component Analysis (PCA), followed by
169 Partial Least Square-Discriminant Analysis (PLS-DA) to improve group separation and classify
170 components. Then, specific data treatment, known as Orthogonal Partial Least Square-
171 Discriminant Analysis (OPLS-DA), was used to extract the EMRT pairs responsible for the
172 group separation; OPLS-DA entails a two-by-two comparison for either species or origin
173 discrimination.
174
175 *2.7 Building up the reference database*
176 A specific database containing 250 compounds identified in the investigated *Iris* species was
177 built up in the Masslynx Software package (Waters, Milfors USA) with the data retrieved
178 from the free data base KNApSAcK [30], and from the original articles in which each
179 compound had first been described. The resulting database included exact mass, molecular
180 formula, RN from CAS, and structure, for each component included, and was then used to
181 identify discriminant markers on the basis of their molecular formula or exact mass.
182 The following procedure was adopted for tentative identification:
183 a) the EMRT pair of each eluting component obtained from UHPLC-TOF-MS was matched
184 with the *Iris* database, by its exact mass or elemental composition; the former was preferred
185 because it is less variable. Mass accuracy was used as diagnostic parameter for
186 identification. An output corresponding to components having similar exact mass (or
187 elemental composition) in both ionization modes (positive or negative) was obtained.
188 b) The resulting tentative identification was verified by comparing the exact masses of the
189 experimental fragments of each EMRT pair, obtained at high collision energy, to those of the
190 theoretical fragmentation, obtained via Mass Fragment software (Waters, Milfors USA). The
191 probability of a tentative identification being correct therefore increases markedly if, and
192 only if, experimental and theoretical fragmentations agree.
193 The reported identifications from analyses in the MS^E mode, in combination with the above
194 mentioned database, may be considered as tentative/putative, in agreement with the
195 recommendations of both the Chemical Analysis Working Group (CAWG) of the
196 Metabolomics Standards Initiative (MSI) [31], [32], and Schymanski et al. for the
197 environmental field [33].
198 In any case, the definitive identification must always be confirmed with the injection of
199 authentic standards, although, in most cases, they are not available commercially.

3. Results and discussion

The strategy adopted in this study is closely linked to the potential offered by new and effective technologies, which can support comprehensive metabolite studies in discriminating complex matrices of vegetable origin. Thus an untargeted approach was first applied to the investigated samples, to obtain sample fingerprints for comparison, to select components without considering peak identification. A targeted approach was then applied, to validate discriminant markers from each species and origin, for differentiation of the samples. This section comprises a critical step-by-step discussion of the results obtained from the application of this strategy to the authentication of *Iris* rhizomes, in terms of species and origin.

3.1 Untargeted approach

3.1.1 HPLC-DAD-UV analysis versus UHPLC-TOF-MS analysis

Figure 2 reports the HPLC-DAD-UV chromatographic profiles of all samples. It is clear that, under the analytical conditions adopted, the differences are very small. The most significant difference concerns two components (peaks at retention time (Rt) = 23.35 min and at Rt = 30.16 min respectively) that are present in all extracts although in different abundances. Conventional HPLC-DAD-UV at 230 nm analyses took about 50 minutes; analyses were also carried out at different wavelengths (250, 300, and 350 nm) but the resulting chromatographic patterns were not very significant (data not shown).

The same samples were then submitted to UHPLC/TOF-MS to obtain more informative results in a shorter time. **Figures 3A and 3B** give the UHPLC/TOF-MS patterns of the extracts of the investigated species and origins, in both positive and negative ESI ionization modes. Thanks to the high efficiency of the analytical system (i.e. UHPLC, sub 2µm columns and a TOF-MS detector) analysis time was reduced to 14 min.

Although, due to the TOF-MS detection, the chromatographic patterns of the investigated *Iris* samples varied, again, only minor differences in composition were seen, either in positive or in negative ionization modes. The main variations between samples in positive mode (**Figure 3A**) concerned the different abundance of some components (markers). Moreover, all *I. pallida* extracts showed very similar chromatographic profiles, making it impossible to discriminate them in terms of origin. The fingerprints of the extract of all samples were very similar, with the exception of *I. albicans,* which presented a characteristic component eluting

232 at Rt = 8.97 min with an exact mass m/z of 388.1148 Da. Similar results were obtained in
233 negative mode (**Figure 3B**), in which the differences mainly concerned the abundance of
234 some components within the set of samples. For instance, *I. pallida* samples from all origins
235 showed a peak at Rt = 6.20 min with an exact mass m/z = 260.0685 Da; its abundance was
236 higher than those detected in the other two species. *I. albicans* and *I. germanica* samples
237 showed a common abundant peak at Rt = 8.81 min with an exact mass of m/z = 314.0788
238 Da. However, the differences detected in this first set of experiments, in either ionization
239 mode, were not sufficient to make an unequivocal and correct assignment of species and
240 origin.
241
242 *3.1.2 Metabolite fingerprinting*
243 The main aim of this study was to differentiate orris root samples in terms of origin and
244 species, on the basis of the composition of their extracts. Meaningful information from the
245 very large number of data produced by modern analytical platforms (UHPLC-TOF-MS) can
246 only be obtained by adopting suitable strategies supported by dedicated data processing.
247 The software used in this study automatically converted each data point into an Exact Mass
248 Retention Time (EMRT) pair of data; about 39000 variables were extracted from the analysis
249 in positive ionization mode, and tabulated into a 2D matrix, and 5000 variables were
250 obtained from the negative mode, and processed independently.
251 The data were first processed by Principal Component Analysis (PCA), *i.e.* an unsupervised
252 clustering method able to reduce the dimensionality of the multivariate data while keeping
253 the intrinsic variability of the data set [3]. PCA processing was preceded by UV-scaling pre-
254 processing (Unit Variance scaling); this is one of the scaling systems most widely adopted
255 with PCA but, in this case, it suffered from considerable statistical noise (72% in positive
256 mode), (data not reported). This noise may decrease the meaningfulness of the resulting
257 information to below 50%. Other scaling procedures were therefore applied (center scaling
258 and Pareto scaling) to minimize the noise level, reducing it from 72% to 39%. **Figure 4** shows
259 the PCA discrimination of UHPLC/positive-TOF-MS data on the investigated *Iris* rhizome
260 species and origins, using Pareto-scaling. The PCA score plot showed a satisfactory
261 separation between samples of *I. pallida* from different origins and other species, achieving
262 an explained variance of 34 % (PC1 *vs.* PC2), despite the dispersion of the samples within the
263 same group (with the exception of the samples of *I. germanica* from Morocco (g, MA)).

Moreover, *I. pallida* from Morocco (p, MA) and Italy (p, IT) were not fully discriminated. These results increase the difficulty of establishing which EMRT pairs could clearly differentiate samples or groups.

Partial Least Square-Discriminant Analysis (PLS-DA) was then applied, to increase the separation between the different groups, and to decrease the dispersion of samples within groups. This model uses a fixed variable, in this case the species and/or origin of a group. PLS-DA data treatment was also combined with different scaling approaches (UV-scaling, center scaling, and Pareto scaling). Pareto scaling was chosen because it offered the lowest level of statistical noise in both ionization modes (**Figures 5A and 5B**) resulting in an explained variance of 35% in positive mode and 41% in negative mode; this difference is due to the much greater number of variables in positive mode (39000 *vs.* 5000). This statistical tool provided good separation of all groups on the first component, especially with the negative ionization data sets; in particular, *I. pallida* samples were clearly separated from *I. albicans* and *I. germanica*, and within *I. pallida* samples, the origins Italy, Morocco and China were discriminated in negative ionization mode, showing the ability of the model to describe the distribution of samples also in function of their geographical origin.

OPLS-DA statistical data treatment was then applied to clarify which components (discriminating markers) made the biggest contribution to differentiating species and origins and, as a consequence, to reducing the huge number of variables. The main benefit of introducing an OSC (orthogonal signal correction) filter is the possibility of applying OPLS-DA statistical treatment, to better separate predictive from non-predictive (orthogonal) variations. This treatment enables the specificity of each group to be determined by pair comparison. The models were internally fully cross-validated (by the leave-one-out method) and sensitivity and specificity were calculated, being respectively 95% and 100% in negative mode, and 100% and 100% in positive mode. The sensitivity of a model indicates the percentage of acceptance relative to its own objects, while its specificity indicates the percentage of the objects of another class that are rejected by the model. This over-fitting was unsurprising, because of a) the high level of statistical noise, in particular in positive mode, and b) the statistically limited number of samples analyzed, in comparison to the number of variables considered. Through a scatter plot (S-Plot) based on OPLS-DA, the discriminant markers that differentiate two groups of samples can be determined. In the S-Plot, each point represents a unique EMRT pair. The x-axis shows the contributions of each

EMRT to discrimination between the two sample groups: the farther is the data point from zero, the greater the contribution it gives to sample variance. The y-axis shows sample correlations within a single sample group. As a consequence, the EMRT pairs at both ends of the S-shaped curve represent the EMRT pairs (potential discriminating markers) contributing the most to discrimination, with the highest level of confidence, from each sample group.

Twelve different comparisons by OPLS-DA were made, in positive and negative modes: three in each mode between the different geographical origins within the same species (*I. pallida*), and the other three pair-wise among the three different species from the same geographical origin. **Figure 6** gives an example of an OPLS-DA diagram and its corresponding S-Plot diagram, obtained by comparing *I. pallida* Italy *vs. I. pallida* China samples, in positive mode (39000 variables). The EMRT pairs at both extremities of the diagrams were selected, and imported into Markerlynx software for further detailed investigations. In this example, (i.e. *p*,IT vs. *p*,CN), 83 potential discriminant markers were extracted.

The twelve OPLS-DA treatments gave approximately 850 potential markers, extracted in both positive and negative ionization modes. This first set of data could be reduced by eliminating the non-significant EMRT pairs (e.g. those close to the noise level). From these 850 EMRT pairs, the true discriminating markers were defined after visualizing the whole set of data, through the so-called trend views, i.e. the results of the comparison of the respective abundance (normalized area) of an EMRT pair in all investigated samples. Through visualization of the trend views of each EMRT, potential markers can be selected arbitrarily, by comparing their abundance in the different samples. In a first step, a total of 200 components were selected as potential discriminating markers, from the trend views of each EMRT for all investigated samples. As an example, **Figure 7** shows the trend view in positive mode of the EMRT at 6.82 min with an exact mass at 375.1075 DA. This compound can be taken as discriminant marker, because of its abundance in *I. pallida* samples from China and its near absence in all other specimens.

Conversely, irigenin, an isoflavonoid characteristic of the *Iris* genus, cannot be taken as a discriminating marker, because its abundance does not vary significantly in the set of samples investigated.

3.2 Targeted approach

The 200 EMRT can be considered potential diagnostic statistical variables for each species and origin. The possibility to associate one or more ions obtained in positive and negative ionization modes univocally to a single substance and, when possible, to identify those ions, was investigated by comparing the measured exact masses to those in the home-made ~~laboratory made?~~ orris root database. The exact masses for each component were correlated to the protonated and deprotonated ions $[M+H]^+$, $[M-H]^-$ or all other ionized adducts *e.g.* $[M+NH_4]^+$or $[M-H+HCOOH]^-$. 146 compounds were characterized, (table 1 Supplementary Material, 1SM) on the basis of their trend views, and used to reprocess the data by the above multivariate analyses. PLS-DA data re-processing with the discriminant markers gave better separations between *Iris* species and origins than those obtained with the whole set of variables. The explained variances increased significantly (by 45% for the positive mode and 58% for the negative mode) while the noise level decreased to approximately 3% (data not shown).

UHPLC-TOF-MS analyses were also run in MS^E mode to obtain complementary spectral information. With MS^E acquisition, high resolution spectra at low (parent ions) and high (daughter ions) collision energies can be collected simultaneously. Daughter ions were obtained through the deconvolution of each potential marker, and were tentatively associated to the theoretical fragmentations obtained from the reference compounds in the data bank, thus increasing the reliability of the tentative identification. A number of discriminating markers were then characterized and, where possible, tentatively identified by matching experimental and reference spectra.

104 components were found to be specific of a species or an origin, and 42 non-specific. In particular, 15 discriminating components were found to be characteristic of *I. albicans* from Morocco, 12 of *I. germanica* from Morocco, 19 of *I. pallida* originating from Italy, 28 from China, and 30 from Morocco. Common compounds but with significantly different abundances can also be taken as diagnostic for discrimination between species or origins, *e.g.* compound 7 is present in *I. pallida* of all origins, while the triterpenoids 132 ~~132~~ and 137 ~~137~~ are present in all species originating from Morocco.

~~52~~ 53 compounds on 104 were tentatively identified as discriminant markers and, on the basis of their trend view profiles, 32 of them were taken as specifically discriminative both for species and for *I. pallida* origins, meaning that their abundance in one or more species or

origins is decidedly higher than in the others: they may be present in other species or origins, but in significantly smaller amounts. Table 1 reports the compounds tentatively identified in the methanol/dichloromethane extracts by UHPLC-ESI-TOF-MS in both positive and negative ionization modes that are discriminant for each *Iris* species and origins. The tentatively identified compounds are grouped by species and origins; the peak number was assigned according to the overall elution order in the UHPLC-MS pattern resulting from the reference database (table 1SM).Table 1 also includes retention time (min), exact mass (Da), ionization mode, elemental composition, mass accuracy (ppm), tentative identification of the component with the considered elemental formula, references, and fragments in positive and negative modes, and where available, daughter ions. Most of the tentatively identified components have already been reported in *Iris* genus extracts. These components therefore discriminate the investigated *Iris* species and origins on the basis of the adopted statistical strategy, and may be different from those identified through conventional phytochemical studies and usually used for *Iris* species characterization. Two main classes of secondary metabolites have been detected as discriminating for species and origins within the samples investigated, i.e. phenolic componds and terpenoids. In particular they respectively include four subclasses of phenolic compounds (flavonoids, isoflavonoids, xanthone and benzophenone derivatives and related glycosides) and a subgroup of triterpenoids, the iridals (i.e. the irone precursors).

The following paragraphs list the tentatively identified discriminant markers that can be used to distinguish between different *Iris* species (*I. pallida, I. germanica and I. albicans)* and/or different origins of *I. pallida* (Italy, China and Morocco).

Eight components specifically discriminant for *I. albicans* were tentatively identified, in particular an irisxanthone (18), iriflogenin (61), an irigenin isomer (67), 5-hydroxy-3',4',5'-trimethoxy-6,7-ethylenedioxyisoflavone (71), irisjaponin B isomer 2 (78) and isomer 3 (82), an irisolidone isomer (86) and belamcandin (90).

Four discriminating components for *I. germanica* from Morocco were (tentatively) identified as discriminant markers through their trend view profiles, in particular 4'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone 4'-O-glucoside (43), 4'-hydroxy-5,3'-dimethoxy-6,7-methylene- dioxyisoflavone 4'-O-glucoside (45), a trihydroxy-7-methoxy flavone isomer (66) and an iriflogenin analogue (80).

Five components were identified and found to be specific of *I. pallida* from Italy. Two of them belong to the class of triterpenoids, i.e iriflorental (121) and iripallidal (122), and three are benzophenone derivatives and isoflavonoids, i.e. 2,6,4'-trihydroxy-4-methoxybenzophenone (42) and a 2,6,4'-trihydroxy-4-methoxybenzophenone isomer (51), and a homotectorigenin-7-glucoside isomer (32).The high quality of *I. pallida* from Italy is undoubtedly linked to the greater abundance of iriflorental (121) and iripallidal (122), from which irones are formed during the maturation steps.

Five specific discriminating markers were also found for *I. pallida* from China, in particular mangiferin (13), an irisxanthone isomer (23), irisjaponin B isomer 1 (58), and two triterpenoids, i.e. trieneiridal (107) and trieneiridal isomer (115).

Ten discriminant components for *I. pallida* from Morocco were found, in particular two iridals, spiroiridal-3 and spiroiridal-3 isomer (98, 101) and two trihydroxy-trimethoxy isoflavone 7-O-(6''-glucosylglucoside) isomers (21, 25), iso-swertiajaponin (26), three iridin isomers (28, 33, 46), a homotectorigenin-7-glucoside isomer (29), and an irigenin isomer (47).

3.3 Confirmation of the reliability of the metabolomic approach

Two unknown samples were submitted to the same analytical procedure as the whole set of previous samples. A predictive statistical data treatment was carried out in order to classify these unknown samples within the different groups. The PLS-DA prediction obtained in positive mode classified the species, but not their origins unequivocally, while that in negative mode clearly associated the two samples to Italian origin. **Figures 8A and 8B** report the PLS-DA diagrams of the total set of samples (including unknowns) obtained using the discriminating markers listed in Table 1 ~~100~~, using EMRT pairs in both positive and negative ionization modes.

This predictive approach was confirmed, on the one hand, by comparing the trend views of the 19 discriminating markers of *I. pallida* from Italy (in particular of those tentatively identified *i.e.* 32, 42, 51,121,122) in the unknown samples and, on the other, by comparing those identified as characteristic of *I. pallida* from Morocco (*i.e.* 21, 25, 26, 28, 29, 33, 46, 47, 98, 101, 105). The profiles of the marker trend views of the unknown samples were found to be very similar to those of reference samples of *I. pallida* from Italy, and completely different

422 from those from Morocco, thus unequivocally classifying origin and species of the unknown
423 samples.
424
425 **4. Conclusions**
426 Modern HPLC and MS instrumentation (UHPLC/TOF-HRMS) in combination with advanced
427 statistical tools open up the field of phytochemical analysis to the use of strategies adopted
428 in metabolomics. The above approaches made it possible to authenticate a natural raw
429 material that is important for the fragrance field, authentication that is difficult with
430 conventional methods: i.e. *Iris* rhizomes and their extracts. The strategy used here (EMRT
431 pairs combined with an advanced statistical strategy) enabled three *Iris* species *I. albicans*, *I.*
432 *germanica*, and *I. pallida*, the latter from three different origins (Italy, China and Morocco),
433 to be discriminated. This discrimination was achieved by detecting 104 discriminating
434 markers; 53 of them were tentatively identified and 32 of them were found to be specific for
435 species and origins. These results can successfully be used to define trend views of specific
436 discriminating markers, to automatically authenticate species and origins, in view of the
437 method's application to routine analysis. Further studies are under way concerning this
438 latter aspect.
439
440 **Acknowledgment**
441 The authors thank Dr. Anne Chartogne from Waters Company for her support and her
442 expertise, and Stéphanie Millo-Lamote from Robertet SA. for her technical support
443 JM, CB, EL, PR are indebted with the project "*Studio di metaboliti secondari biologicamente*
444 *attivi da matrici di origine vegetale*" financially supported by the Ricerca Locale (Ex60%2012)
445 of University of Turin, Turin (Italy).
446

447 **References**

448

449 [1] S.G. Oliver, M.K. Winson, D.B. Kell, F. Baganz, Systematic functional analysis of the yeast
450 genome, Trends Biotechnol. 16 (1998) 373-378.

451 [2] J.M. Halket, D. Waterman, A.M. Przyborowska, R.K.P. Patel, P.D. Fraser, P.M. Bramley,
452 Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and
453 LC/MS/MS, J. Experim. Botany 56 (2005) 219-243.

454 [3] L. Eriksson, E. Johansson, N. Kettaneh-Wold, J. Trygg, C. Wikstrom, S. Wold, Multi- and
455 Megavariate Data Analysis. Part I. Basic principles and applications (2nd revised and enlarged
456 edition),- Umetrics Academy, Sweden, 2006.

457 [4] J.C. Ellena, M.C. Grasse, L. Peyron, Un jour, une plante : l'Iris dans tous ses états. MIP (Editor),
458 Grasse, 2005.

459 [5] F. Tiemann, P. Kruger, Zum nachweis von ionon und iron, Ber. Dtsch. Chem. Ges. 28 (1895)
460 1754-1757.

461 [6] Y.R. Naves, A.V. Grampoloff, P. Bachmann, Etudes sur les matières végétales volatiles. Etudes
462 dans les méthyl-3-linalols, des méthyl-3-citrals et des méthyl-6-ionones, Helv. Chim. Acta 30
463 (1947) 1599.

464 [7] L. Ruzicka, C.F. Seidel, H. Schinz, M. Pfeiffer, Die konstitution des irons, Helv. Chim. Acta 30
465 (1947) 1807-1810.

466 [8] V. Rautenstrauch, G. Ohloff, Die stereochemie der irone, Helv. Chim. Acta 54 (1971) 1776-
467 1778.

468 [9] V. Rautenstrauch, B. Willhalm, W. Thommen, G. Ohloff, On the stereochemistry of the irones,
469 Helv. Chim. Acta 67 (1984) 325-331.

470 [10] E. Wollenweber, J.F. Stevens, K. Klimo, J. Knauft, N. Frank, C. Gerhauser, Cancer
471 chemopreventive in vitro activities of isoflavones isolated from *Iris germanica*, Planta Med.
472 69 (2003) 15-20.

473 [11] D. Rigano, C. Formisano, A. Grassia, G. Grassia, A. Perrone, S. Piacente, M.L. Vuotto, F.
474 Senatore, Antioxidant flavonoids and isoflavonoids from rhizomes of *Iris pseudopumila*,
475 Planta Med. 73 (2007) 93-96.

476 [12] Atta-ur-Rahman, S. Nasim, I. Baig, S. Jalil, I. Orhan, B. Sener, M.I. Choudhary, Anti-
477 inflammatory isoflavonoids from the rhizomes of *Iris germanica*, J. Ethnopharmacol. 86
478 (2003) 177-180.

479 [13] J.J. Paufique, Principe actif riche en isoflavones, son procédé d'extraction et ses applications
480 cosmétiques, FR Patent 2 815 539, 2000

481 [14] W. Krick, F.J. Marner, L. Jaenicke, Isolation and structure determination of the precursors of
482 α- and γ-irone and homologous compounds from *Iris pallida* and *Iris florentina*, Z.
483 Naturforsch. 38C (1983) 179-184.

[15] F.J. Marner, L. Jaenicke, Die absolute konfiguration der iridale und cycloiridale unterschiedlicher herkunft, Helv. Chim. Acta 72 (1989) 287-294.

[16] F.J. Marner, B. Hanisch, Hoogianal, a β-irone precursor from *Iris hoogiana* Dykes (Iridaceae), Helv. Chim. Acta 84 (2001) 933-938.

[17] F.J. Marner, W. Krick, B. Gellrich, L.Jaenicke, W.Winter, Irigermanal and iridogermanal: two new triterpenoids from rhizomes of *Iris germanica* L, J. Org. Chem. 47 (1982) 2531-2536.

[18] F.J. Marner, I. Longerich, Isolation and structure determination of new iridals from *Iris sibirica* and *Iris versicolor,* Liebigs Ann. Chem. (1992) 269-272.

[19] F.J. Marner, K. Simic, B. Scholz, B. Kuster, Isolation and structure determination of a new iridal from *Iris sibirica*, J. Nat. Prod. 58 (1995) 299-301.

[20] F.J. Marner, A. Littek, R. Arold, K. Seferiadis, L. Jaenicke, Isolation and structure determination of new spiro-bicyclic triterpenoids from *Iris pseudacorus,* Liebigs Ann. Chem. (1990) 563-567.

[21] F.J. Marner, D. Gladtke, L. Jaenicke, Studies on the biosynthesis of iridals and cycloiridals, Helv. Chim. Acta 71 (1988) 1331-1338.

[22] F.J. Marner, B. Kerp, Composition of iridals, unusual triterpenoids from sword-lilies, and the seasonal dependence of their content in various parts of different Iris species, Z. Naturforsch. 47c (1992) 21-25.

[23] F.J. Marner, I. Ritzdorf, G. Johnen, Metabolism of iridals in plant cell cultures of *Iris pallida*, Phytochemistry 33 (1993) 573-579.

[24] F.J. Marner, T. Kasel, Biomimetic synthesis of the iridal skeleton, J. Nat. Prod. 58 (1995) 319-323.

[25] C. Bicchi, P. Rubiolo, Analysis of constituents of Iris rhizomes. Part I. High performance liquid chromatographic-particle beam-mass spectral analysis of iridals from *Iris pallida* rhizomes, Phytochem.~~ical~~ analysis 4 (1993) 171-177.

[26] A.A. Ali, N.A. El-Emary, M.A. El-Moghazi, F.M. Darwish, A.W. Frahm, Three isoflavonoids from *Iris germanica*, Phytochemistry 22 (1983) 2061-2063.

[27] B. Roger, V. Jeannot, X. Fernandez, S. Cerantola, J. Chahboun, Characterisation and quantification of flavonoids in *Iris germanica* L. and *Iris pallida* Lam. resinoids from Morocco, Phytochem.~~ical~~ analysis 23 (2012) 450-455.

[28] A.Guenet, Comparaison des beurres d'iris de diverses origines botaniques et géographiques, 12èmes Journées Internationales Huiles Essentielles (Digne, 1993) (1993) 492-492.

[29] M. Remy, J.C. Bayle, M. Derbesy, R. Uzio, H. Casabianca, J.B. Graff, Les beurres d'iris. Dosage des irones par chromatographie gazeuse sur phase chirale, vérification des origines, Journées Internationales Huiles Essentielles 12èmes (Digne, 1993) (1993) 506-506.

[30] Y. Nakamura, H. Asahi, Md. Altaf-Ul-Amin, K. Kurokawe, S. Kanaya, KNApSAcK, http://kanaya.aist-nara.ac.jp/KNApSAcK/ (2013).

521 [31] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W.M. Fan, O. Fiehn,
522 R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane,
523 J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reily, J.J. Thaden, M.R. Viant, Proposed minimum
524 reporting standards for chemical analysis Chemical Analysis Working Group (CAWG)
525 Metabolomics Standards Initiative (MSI), Metabolomics 3 (2007) 211-221.

526 [32] D.J. Creek, W.B. Dunn, O. Fiehn, J.L. Griffin, R.D. Hall, Z. Lei, R. Mistrik, S. Neumann, E.L.
527 Schymanski, L.W. Sumner, Metabolite identification: are you sure? And how do your peers
528 gauge your confidence, Metabolomics 10 (2014) 350-353.

529 [33] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P.Singer, J.Hollender, Identifying
530 small molecules via high resolution mass spectrometry: Communicating confidence, Environ.
531 Sci. Technol. (2014) 2097-2098.

532 [34] M. Arisawa, N. Morita, Y.Kondo, T. Takemoto, Studies on constituents of genus *Iris*. VII. The
533 constituents of *Iris unguicularis* Poir, Chem. Pharm. Bull. 21 (1973) 2323-2328.

534 [35] M.Fujita, T.Inoue, Studies on the constituents of *Iris florentina* L. II. *C*-Glucosides of
535 xanthones and flavones from the leaves, Chem. Pharm. Bull. 30 (1982) 2342-2348.

536 [36] J.B. Harborne, H. Baxter, G.P. Moss, Xanthones in J.B.Harborne, H.Baxter, G.P.Moss (Eds),
537 Phytochemical Dictionary. A Handbook of Bioactive Compounds from Plants (2nd ed.), Taylor
538 & Francis, Padstow, 1999, p. 582-590.

539 [37] H. Minami, A. Okubo, M. Kodama, Y. Fukuyama, Highly oxygenated isoflavones from *Iris*
540 *japonica,* Phytochemistry 41 (1996) 1219-1222.

541 [38] A. Littek, F.J. Marner, Structure determination of new iridals from *Iris pallida* and *Iris*
542 *foetidissima,* Helv. Chim. Acta 74 (1991) 2035-2037.

543 [39] Atta-ur-Rahman, S. Nasim, I. Baig, I. Orhan, B. Sener, F. Ayanoglu, M.I C houdhary,
544 Isoflavonoid glycosides from the rhizomes of *Iris germanica,* Helv. Chim. Acta 86 (2003) 3354-
545 3362.

546 [40] J.W. Wallace, G. Morris, C-glycosylflavones in *Gnetum gnemon,* Phytochemistry 17 (1978)
547 1809-1810.

548 [41] J.B. Harborne, H. Baxter, C-Glycosylflavones in J.B.Harborne, H.Baxter (Editors), The
549 Handbook of Natural Flavonoids (Vol. 1), Wiley, Chichester, 1999, p. 549-644..

550 [42] P. Kassak, Secondary metabolites of the choosen genus *Iris* species, Acta Univ. Agricult.
551 Silvicult. Mendalianae Brunensis 60 (2012) 269-280.

552 [43] J.B. Harborne, H. Baxter, G.P. Moss, Isoflvonoids and neoflavonoids in J.B.Harborne,
553 H.Baxter, G.P.Moss (Editors), Phytochemical Dictionary - A Handbook of Bioactive
554 Compounds from Plants (2nd ed.). Taylor & Francis, Padstow, 1999, p. 458-477.

555 [44] H. Ito, S. Onoue, T. Yoshida, Isoflavonoids from *Belamcanda chinensis,* Chem. Pharm. Bull. 49
556 (2001) 1229-1231.

557 [45] Atta-ur-Rahman, S. Nasim, I. Baig, I.A. Jahan, B. Sener, I. Orhan, M.I. Choudhary, Isoflavonoid
558 glycosides from the rhizomes of *Iris germanica*, Chem. Pharm. Bull. 50 (2002) 1100-1102.

559 [46] J.B. Harborne, H. Baxter, Isoflvonoids and neoflavonoids in J.B.Harborne, H.Baxter (Editors),
560 The Handbook of Natural Flavonoids (Vol. 2). Wiley, Chichester, 1999, p. 517-708.

561 [47] A. Kawase, N. Ohta, K. Yagishita, On the chemical structure of a new isoflavone glucoside,
562 homotectoridin, isolated together with tectoridin from the rhizomes of *Iris germanica*
563 Linnaeus, Agric. Biol. Chem. 37 (1973) 145-147.

564 [48] M. Arisawa, N. Morita, Studies on constituents of *Iris* genus plants. IV. The constituents of *Iris*
565 *florentina* L. (2), Chem. Pharm. Bull. 24 (1976) 815-817.

566 [49] M. Moriyasu, Y. Igi, M. Ichimaru, K. Iwasa, J. Kobayakawa, F. Sato-Nishimori, Y. Matsukawa,
567 C. Nagaze, New isoflavones from *Belamcandae* rhizome, J. Nat. Med. 61 (2007) 329-333.

568 [50] M.I. Choudhary, M. Nur-e-Alam, I. Baig, F. Akhtar, A. Khan, P. Ndognii, T. Badarchiin, G.
569 Purevsuren, N. Nahar, Atta-ur-Rahman, Four new flavones and a new isoflavone from *Iris*
570 *bungei*, J. Nat. Prod. 64 (2001) 857-860.

571 [51] K.L. Dhar, A.K. Kalla, Isoflavones of *Iris kumaonensis* and *Iris germanica,* Phytochemistry 11
572 (1972) 3097-3098.

573 [52] U. Mahmood, V.K. Kaul, L. Jirovetz, Alkylated benzoquinones from *Iris kumaonensis,*
574 Phytochemistry 61 (2002) 923-926.

575 [53] Y. Miyake, H. Ito, T. Yoshida, Identification of iridals as piscicidal components of iradaceous
576 plants and their conformations associated with CD spectra, Canadian J. Chem. 75 (1997) 734-
577 741.

578 [54] F. Abe, R.F. Chen, T. Yamauchi, Iridals from *Belamcanda chinensis* and *Iris japonica,*
579 Phytochemistry 30 (1991) 3379-3382.

580 [55] L. Jaenicke, F.J. Marner, On irones, dihydroirones and their precursor, Topics in Flavour
581 Research (Proc. International conference Freising-Weihenstephan), (1985), p. 253-253.

582 [56] W. Krick, F.J. Marner, L. Jaenicke, On the stereochemistry of natural irones, dihydroirones
583 and their precursors, Helv. Chim. Acta 67 (1984) 318-324.

584 [57] O. Potterat, C. Herzog, M. Raith, S.N. Ebrahimi, M. Hamburger, Irigermanone, a noriridal with
585 unprecedented methylketone function, from *Iris germanica,* Helv. Chim. Acta 97 (2014) 32-
586 38.

587 [58] W.S. Woo, E.H. Woo, An isoflavone noririsflorentin from *Belamcanda chinensis*,
588 Phytochemistry 33 (1993) 939-940.

589 [59] J.B. Harborne, H.Baxter, Flavonones and dihydroflavonols in J.B.Harborne, H.Baxter (Editors),
590 The Handbook of Natural Flavonoids (Vol. 2). Wiley, Chichester, 1999, p. 218-354.
591
592

593 Captions to figures

594 Figure 1 Main skeletons of secondary metabolites identified in the *Iris* genus.

595 Figure 2 HPLC/DAD-UV chromatographic profile of orris extracts (for analysis conditions see
596 text).

597 Figure 3 UHPLC/TOF-MS chromatographic profiles of the investigated orris root extracts: a)
598 in positive mode, b) in negative mode. For analysis conditions see text.

599 Figure 4 PCA score plot with pareto scaling of the investigated orris root samples analyzed by
600 UHPLC-TOF-MS in positive mode.

601 Figure 5: PLS-DA score plot with pareto scaling of the investigated orris root samples
602 analyzed by UHPLC-TOF-MS a) in positive mode, b) in negative mode.

603 Figure 6 OLPS-DA and S-plot diagram from the comparison between *Iris pallida* Italy *vs. Iris*
604 *pallida* China samples.

605 Figure 7 Trend view of a selected discriminating marker (EMRT 6.82, 375.1075) in the
606 investigated samples.

607 Figure 8: PLS-DA prediction of unknown orris root samples with pareto scaling: a) with
608 dataset obtained by UHPLC-TOF-MS in positive mode, b) with dataset obtained by UHPLC-
609 TOF-MS in negative mode.

610
611

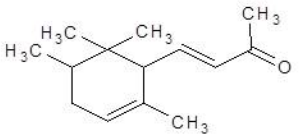
Table 1 List of the tentatively identified discriminant markers in agreement with species and origin, and UHPLC retention times. Legend: *I. pallida* China (*p*, CN), *I.pallida* Morocco (*p*, MA), *I.pallida* Italy (*p,* IT), *I.albicans* Morocco (*a,* MA), *I.germanica* Morocco (*g,* MA).

										Fragments in pos. mode			Fragments in neg. mode		
	N°	**Rt (min)**	**Exact mass (Da)**	**Ioniz. mode**	**Elemental composit.**	**Mass accuracy (ppm)**	**Tentative identification**	**References**	**Species and/or origin**	**Exact mass (Da)**	**Element. compos.**	**Mass accuracy (ppm)**	**Exact mass (Da)**	**Element. compos.**	**Mass accuracy (ppm)**
1	**13**	4.38	422.0838	±	$C_{19}H_{18}O_{11}$	2.63	**Mangiferin**	[34], [35]	***p*, CN**	327.0578 303.0516 273.0416	$C_{17}H_{11}O_7$ $C_{15}H_{11}O_7$ $C_{14}H_9O_6$	22.39 3.703 6.18	331.0446 301.0338	$C_6H_{11}O_8$ $C_{15}H_9O_7$	2.39 3.41
2	**23**	5.30	436.1004	-	$C_{20}H_{20}O_{11}$	0.37	**Irisxanthone isomer**	[36]	***p*, CN**				315.0505 272.0344	$C_{16}H_{11}O_7$ $C_{14}H_8O_6$	0.07 8.49
3	**58**	6.82	374.0997	+	$C_{19}H_{18}O_8$	1.25	**irisjaponin B isomer 1**	*[37]*	***p*, CN**	360.0849 342.0740	$C_{19}H_{16}O_8$ $C_{18}H_{14}O_7$	1.06 0.14			
4	**107**	10.75	456.3595	+	$C_{30}H_{48}O_3$	1.85	**Trieneiridal isomer 1**	[24], [38]	***p*, CN**	439.3567 421.3433	$C_{30}H_{47}O_2$ $C_{30}H_{40}O$	2.06 8.80			
5	**115**	11.49	456.3599	+	$C_{30}H_{48}O_3$	0.98	**Trieneiridal isomer 2**	[24], [38]	***p*, CN**	439.3584 421.3474	$C_{30}H_{47}O_2$ $C_{30}H_{45}O$	1.81 0.85			
6	**21**	5.12	684.1897	±	$C_{30}H_{36}O_{18}$	0.68	**5,7,3'-Trihydroxy-6,4',5'-trimethoxy isoflavone 7-O-(6''-glucosyl glucoside) isomer 1**	[39]	***p*, MA**	523.1453 361.0920 331.0825	$C_{24}H_{27}O_{13}$ $C_{18}H_{17}O_8$ $C_{17}H_{15}O_7$	0.26 0.95 2.18	521.1276 359.0747 329.0656	$C_{24}H_{25}O_{13}$ $C_{18}H_{15}O_8$ $C_{17}H_{13}O_7$	3.67 5.55 1.60
7	**25**	5.41	684.1897	±	$C_{30}H_{36}O_{18}$	0.68	**5,7,3'-Trihydroxy-6,4',5'-trimethoxy isoflavone 7-O-(6''-glucosyl glucoside) isomer 2**	[39]	***p, MA***	523.1453 361.0914	$C_{24}H_{27}O_{13}$ $C_{18}H_{17}O_8$	0.26 2.61	359.0751	$C_{18}H_{15}O_8$	4.44
8	**26**	5.46	462.1162	±	$C_{22}H_{22}O_{11}$	0.03	**Iso-swertiajaponin**	[40], [41]	***p*, MA**	301.0711 286.0474	$C_{16}H_{13}O_6$ $C_{15}H_{10}O_6$	0.38 1.18	299.0559 284.0325	$C_{16}H_{11}O_6$ $C_{15}H_8O_6$	1.13 1.45

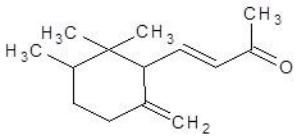
										Fragments in pos. mode			Fragments in neg. mode		
	N°	Rt (min)	Exact mass (Da)	Ioniz. mode	Elemental composit.	Mass accuracy (ppm)	Tentative identification	References	Species and/or origin	Exact mass (Da)	Element. compos.	Mass accuracy (ppm)	Exact mass (Da)	Element. compos.	Mass accuracy (ppm)
9	**28**	5.56	522.1367	+	$C_{24}H_{26}O_{13}$	1.23	**Iridin isomer_1**	*[25], [26], [42], [43], [44], [45]*	***p*, MA**	361.0921	$C_{18}H_{17}O_8$	0.67			
10	**29**	5.60	492.1262	±	$C_{23}H_{24}O_{12}$	1.17	**Homotectorigenin-7-O-glucoside isomer**	*[46], [47]*	***p*, MA**	331.0806 316.0572	$C_{17}H_{15}O_7$ $C_{16}H_{12}O_7$	3.56 3.49	329.0668 314.0424	$C_{17}H_{13}O_7$ $C_{16}H_{10}O_7$	2.04 0.81
11	**33**	5.89	522.1367	-	$C_{24}H_{26}O_{13}$	1.23	**Iridin isomer 2**	*[25], [26], [42], [43], [44], [45]*	***p*, MA**				506.1063 359.0762 344.0526	$C_{23}H_{22}O_{13}$ $C_{18}H_{15}O_8$ $C_{17}H_{12}O_8$	0.51 1.37 1.79
12	**46**	6.40	522.1368	-	$C_{24}H_{26}O_{13}$	1.04	**Iridin**	*[25], [26], [42], [43], [44], [45]*	***p*, MA**				506.1054 359.0762 344.0529	$C_{23}H_{22}O_{13}$ $C_{18}H_{15}O_8$ $C_{17}H_{12}O_8$	1.27 1.37 0.92
13	**47**	6.41	360.0842	+	$C_{18}H_{16}O_8$	0.88	**Irigenin isomer**	*[25], [26], [30], [42], [48], [49], [50], [45], [51], [52]*	***p*, MA**	346.0695	C17H14O8	1.83			
14	**98**	9.98	470.3390	+	$C_{30}H_{46}O_4$	1.30	**Spiroiridal-3**	*[22], [23], [25], [38], [53], [54]*	***p*, MA**	453.3354 219.1380	$C_{30}H_{45}O_3$ $C_{14}H_{19}O_2$	3.24 2.30			
15	**101**	10.22	470.3388	+	$C_{30}H_{46}O_4$	1.72	**Spiroiridal-3 isomer**	*[22], [23], [25], [38], [53], [54]*	***p*, MA**	453.3353 219.1357	$C_{30}H_{45}O_3$ $C_{14}H_{19}O_2$	3.46 12.80			
16	**32**	5.84	492.1261	±	$C_{23}H_{24}O_{12}$	1.37	**Homotectorigenin-7-O-glucoside isomer**	*[46], [47]*	**p ,IT**	331.0818 316.0587	$C_{17}H_{15}O_7$ $C_{16}H_{12}O_7$	0.24 1.26	329.0652 314.0411	$C_{17}H_{13}O_7$ $C_{16}H_{10}O_7$	2.81 4.94
17	**42**	6.21	260.0684	±	$C_{14}H_{12}O_5$	0.28	**2,6,4'-trihydrox-4-methoxybenzophenone**	[27]	***p*, IT**	167.0355 121.0301 93.0345	$C_8H_7O_4$ $C_7H_5O_2$ C_6H_5O	6.38 9.46 4.95	165.0188 121.0309 93.0344	$C_8H_5O_4$ $C_7H_5O_2$ C_6H_5O	0.11 6.07 3.87
18	**51**	6.45	260.0682	-	$C_{14}H_{12}O_5$	1.05	**2,6,4'-trihydrox-4-methoxybenzo-phenone isomer**	[27]	***p*, IT**				243.0303 215.0727 151.0034	$C_{13}H_7O_5$ $C_{18}H_5O_2$ $C_7H_3O_4$	3.92 5.32 1.76
19	**121**	11.88	486.3704	±	$C_{31}H_{50}O_4$	1.05	**Iriflorentale**	*[14], [23], [25], [55], [56], [57]*	***p*, IT**	469.3665 451.3565 217.1967 191.1811	$C_{31}H_{49}O_3$ $C_{31}H_{47}O_2$ $C_{16}H_{35}$ $C_{14}H_{33}$	3.56 2.45 6.78 5.88	467.3533 437.3425 237.1497	$C_{31}H_{47}O_3$ $C_{30}H_{45}O_2$ $C_{14}H_{21}O_3$	1.67 1.25 2.66

										Fragments in pos. mode			Fragments in neg. mode		
	N°	Rt (min)	Exact mass (Da)	Ioniz. mode	Elemental composit.	Mass accuracy (ppm)	Tentative identification	References	Species and/or origin	Exact mass (Da)	Element. compos.	Mass accuracy (ppm)	Exact mass (Da)	Element. compos.	Mass accuracy (ppm)
20	**122**	12.06	486.3701	+	$C_{31}H_{50}O_4$	1.67	**Iripallidal**	*[14], [23], [25], [55], [56], [57]*	***p*, IT**	469.3655 451.3556 255.2309 217.1967 191.1814	$C_{31}H_{49}O_3$ $C_{31}H_{47}O_2$ $C_{16}H_{31}O_2$ $C_{16}H_{35}$ $C_{14}H_{33}$	5.69 4.44 5.89 9.09 7.48			
21	**18**	5.06	436.1001	-	$C_{20}H_{20}O_{11}$	1.06	**Irisxanthone**	[36]	***a*, MA**				345.0606 315.0500 272.0319	$C_{17}H_{13}O_8$ $C_{16}H_{11}O_7$ $C_{14}H_8O_6$	1.28 1.52 0.69
22	**61**	6.97	328.0580	-	$C_{17}H_{12}O_7$	0.92	**Iriflogenin**	*[42]*, [27]	***a*, MA**				312.0272 255.0288 240.0428	$C_{16}H_8O_7$ $C_{14}H_7O_5$ $C_{14}H_8O_4$	0.63 2.15 2.25
23	**67**	7.40	360.0840	±	$C_{18}H_{16}O_8$	1.44	**Irigenin isomer**	*[25], [26], [30], [42], [48], [49], [50], [45], [51], [52]*	***a*, MA**	346.0690 328.0580 313.0346	$C_{17}H_{14}O_8$ $C_{17}H_{12}O_7$ $C_{16}H_9O_7$	0.38 0.92 0.73	344.0533 329.0302	$C_{17}H_{12}O_8$ $C_{16}H_9O_8$	0.24 1.39
24	**71**	7.70	372.0838	+	$C_{19}H_{16}O_8$	1.93	**5-hydroxy-3',4',5'-trimethoxy-6,7-methylenedioxy isoflavone**	[58]	***a*, MA**	358.0693	$C_{18}H_{14}O_8$	1.21			
25	**78**	8.21	374.0997	+	$C_{19}H_{18}O_8$	1.25	**Irisjaponin B isomer 2**	*[37]*	***a*, MA**	360.0820 345.0623 342.0740	$C_{18}H_{16}O_8$ $C_{17}H_{13}O_8$ $C_{18}H_{14}O_7$	6.99 3.65 0.14			
26	**82**	8.36	374.0997	+	$C_{19}H_{18}O_8$	1.25	**Irisjaponin B isomer 3**	*[37]*	***a*, MA**	360.0823 345.0618 342.0741	$C_{18}H_{16}O_8$ $C_{17}H_{13}O_8$ $C_{18}H_{14}O_7$	6.16 2.20 0.43			
27	**86**	8.62	314.0787	-	$C_{17}H_{14}O_6$	1.08	**Irisolidone isomer**	*[26], [27], [42], [43], [46], [37]*	***a*, MA**				298.0474 283.0242 255.0287	$C_{16}H_{10}O_6$ $C_{15}H_7O_6$ $C_{14}H_7O_5$	1.13 0.22 2.54
28	**90**	8.86	358.1045	+	$C_{19}H_{18}O_7$	2.10	**Belamcandin**	*[46]*	***a*, MA**	344.0893 327.0868 315.0871 300.0637	$C_{18}H_{16}O_7$ $C_{18}H_{15}O_6$ $C_{17}H_{15}O_6$ $C_{16}H_{12}O_6$	0.88 0.19 0.75 1.04			

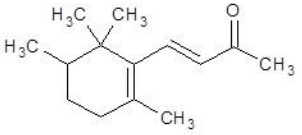
										Fragments in pos. mode			Fragments in neg. mode		
	N°	Rt (min)	Exact mass (Da)	Ioniz. mode	Elemental composit.	Mass accuracy (ppm)	Tentative identification	References	Species and/or origin	Exact mass (Da)	Element. compos.	Mass accuracy (ppm)	Exact mass (Da)	Element. compos.	Mass accuracy (ppm)
29	**43**	6.28	474.1160	±	$C_{23}H_{22}O_{11}$	0.45	**4'-Hydroxy-5-methoxy-6,7-methylenedioxy isoflavone 4'-O-glucoside**	*[42], [45]*	***g*, MA**	313.0714	$C_{17}H_{13}O_6$	0.6	311.0518	$C_{17}H_{11}O_6$	0.20
30	**45**	6.39	504.1263	+	$C_{24}H_{24}O_{12}$	0.95	**4'-Hydroxy-5,3'-dimethoxy-6,7-methylenedioxy isoflavone 4'-O-glucoside**	*[42], [45]*	***g*, MA**	343.0819 328.0580	$C_{18}H_{15}O_7$ $C_{17}H_{12}O_7$	0.36 0.92			
31	**66**	7.37	302.0790	-	$C_{16}H_{14}O_6$	0.13	**5,8,2'-Trihydroxy-7-methoxyflavone**	[59]	***g*, MA**						
32	**80**	8.30	328.0579	+	$C_{17}H_{12}O_7$	1.23	**Iriflogenin isomer**	*[42],* [27]	***g*, MA**	314.0423 297.0398 180.0054	$C_{16}H_{10}O_7$ $C_{16}H_9O_7$ $C_8H_4O_5$	1.12 0.38 2.63			



α-Irone

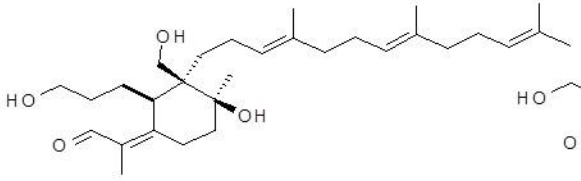


γ-Irone

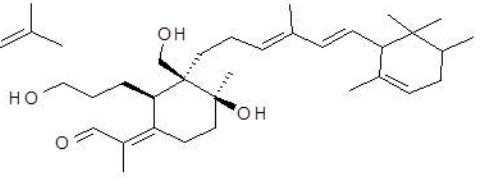


β–Irone

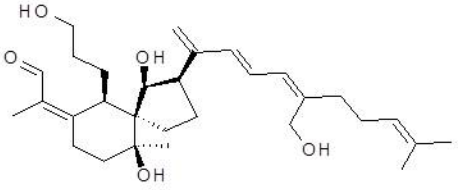
Irone skeletons



Iridal (mono-cyclic)

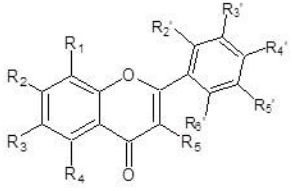


Iripallidal (bi-cyclic)



Spiroiridal-1 (spiro-cyclic)

Triterpenoid skeletons



Flavonoid

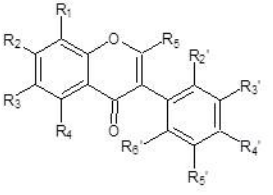
As examples: Iso-swertijaponin

R_1 = Glu

R_2 = OMe

R_3,R_5,R_2',R_3',R_6' = H

R_4,R_4',R_5' = OH



Isoflavonoid

Irigenin

R_1,R_5,R_2',R_6' = H

R_2,R_4,R_3' = OH

R_3,R_4',$R^{5'}$ = OMe

Flavonoid and isoflavonoid skeletons

Figure 1

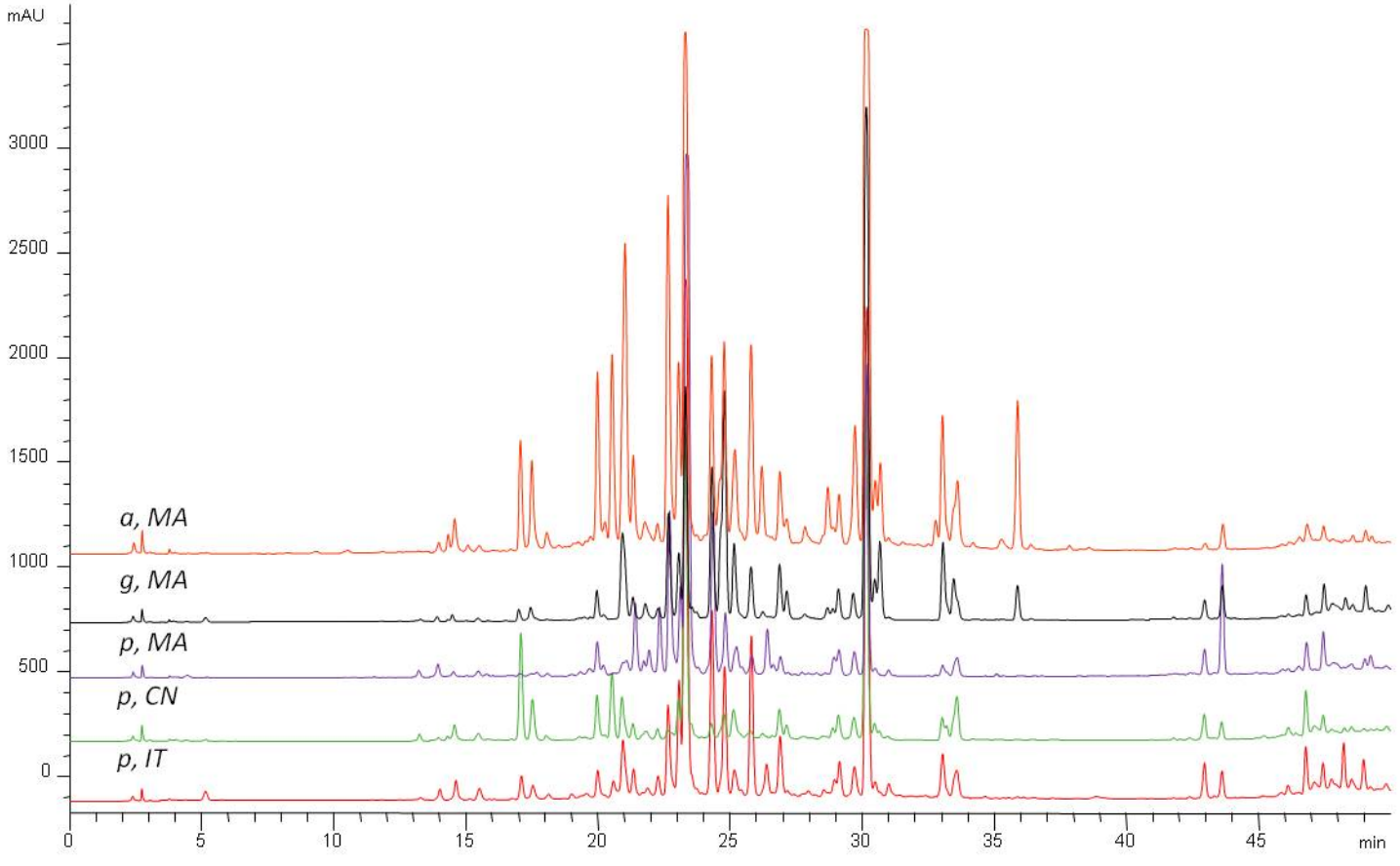
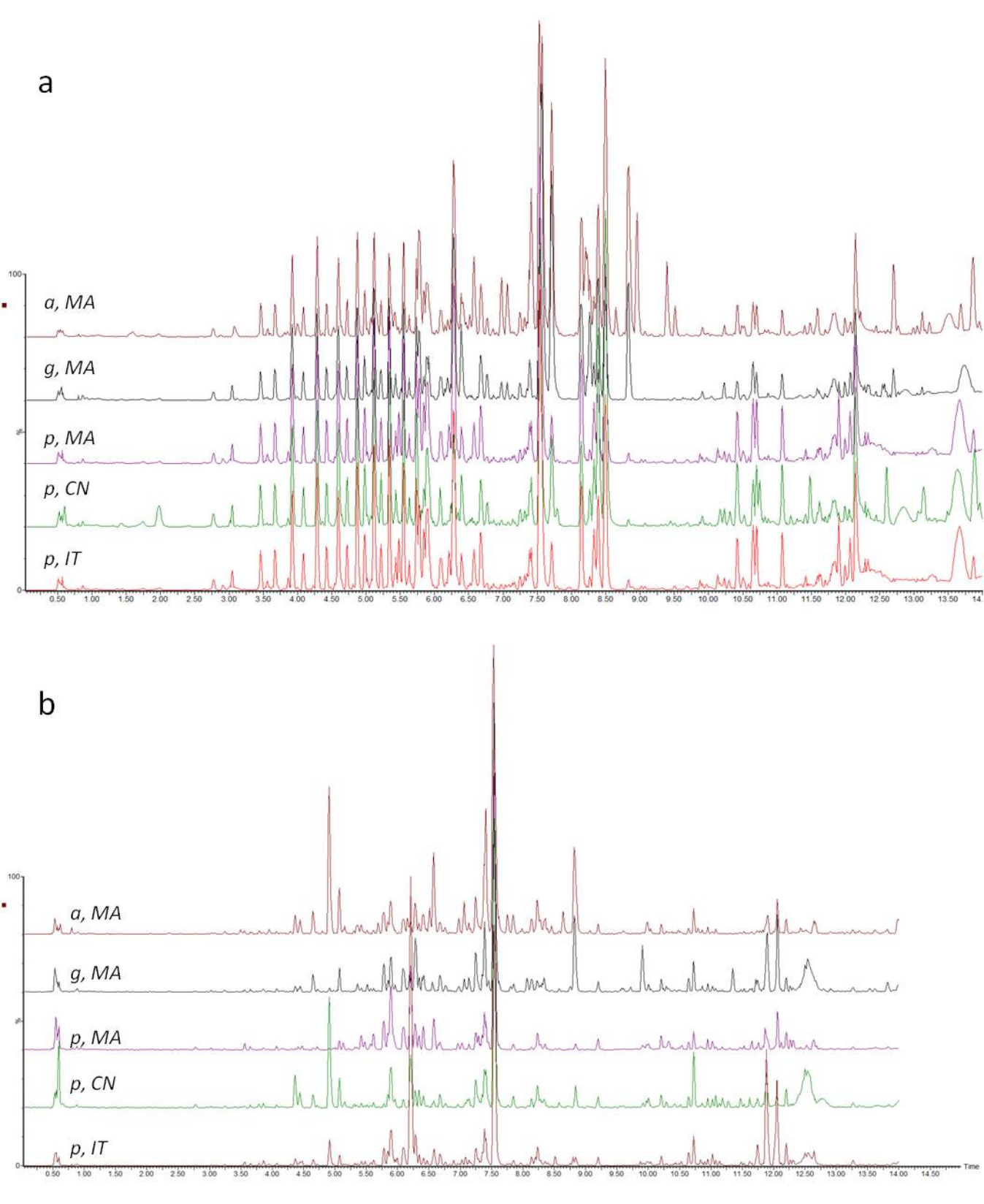

mAU
3000
2500
2000
1500
1000
500
0
a, MA
g, MA
p, MA
p, CN
p, IT
0
5
10
15
20
25
30
35
40
45
min

Figure 2



Figures 3

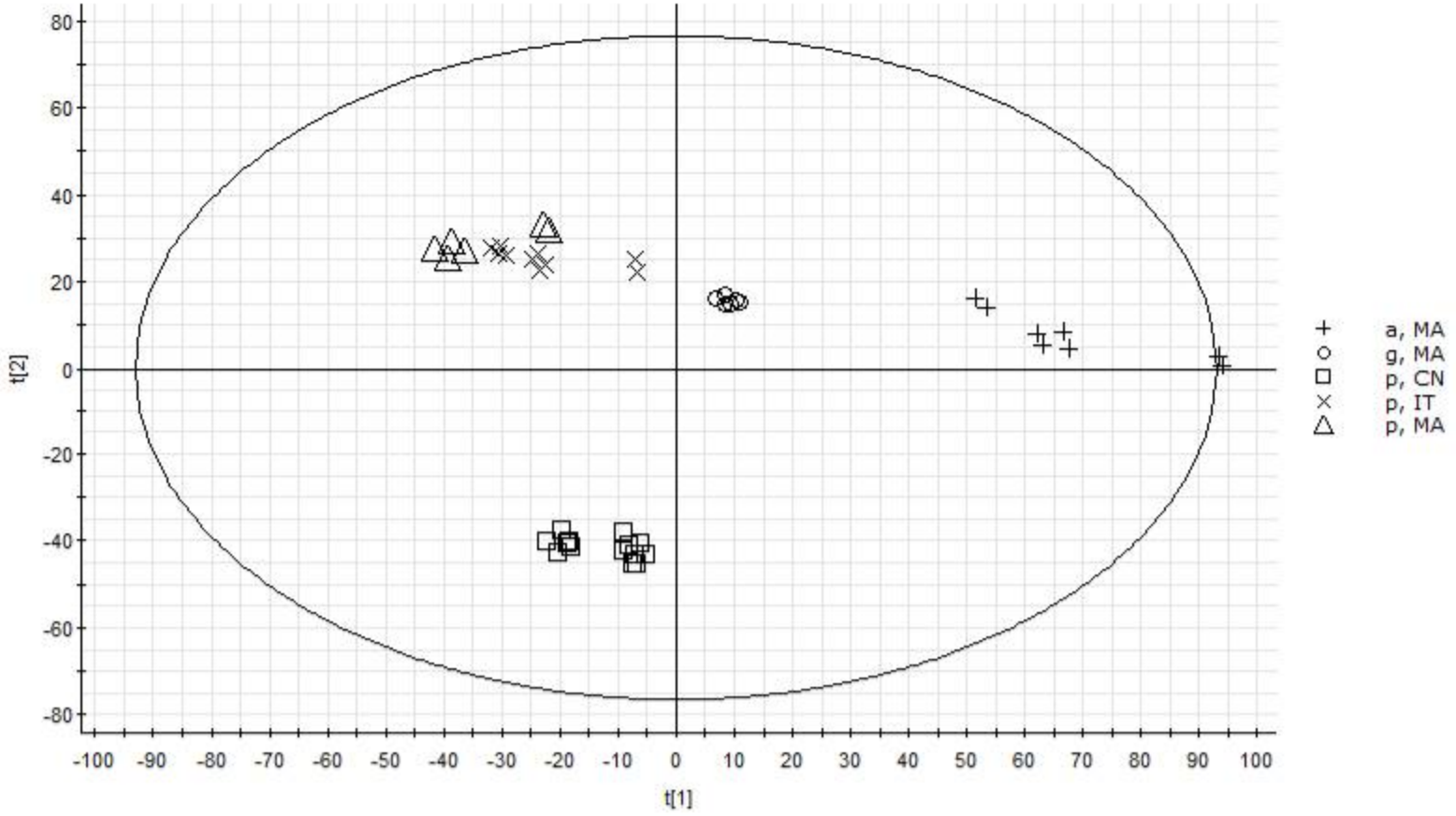
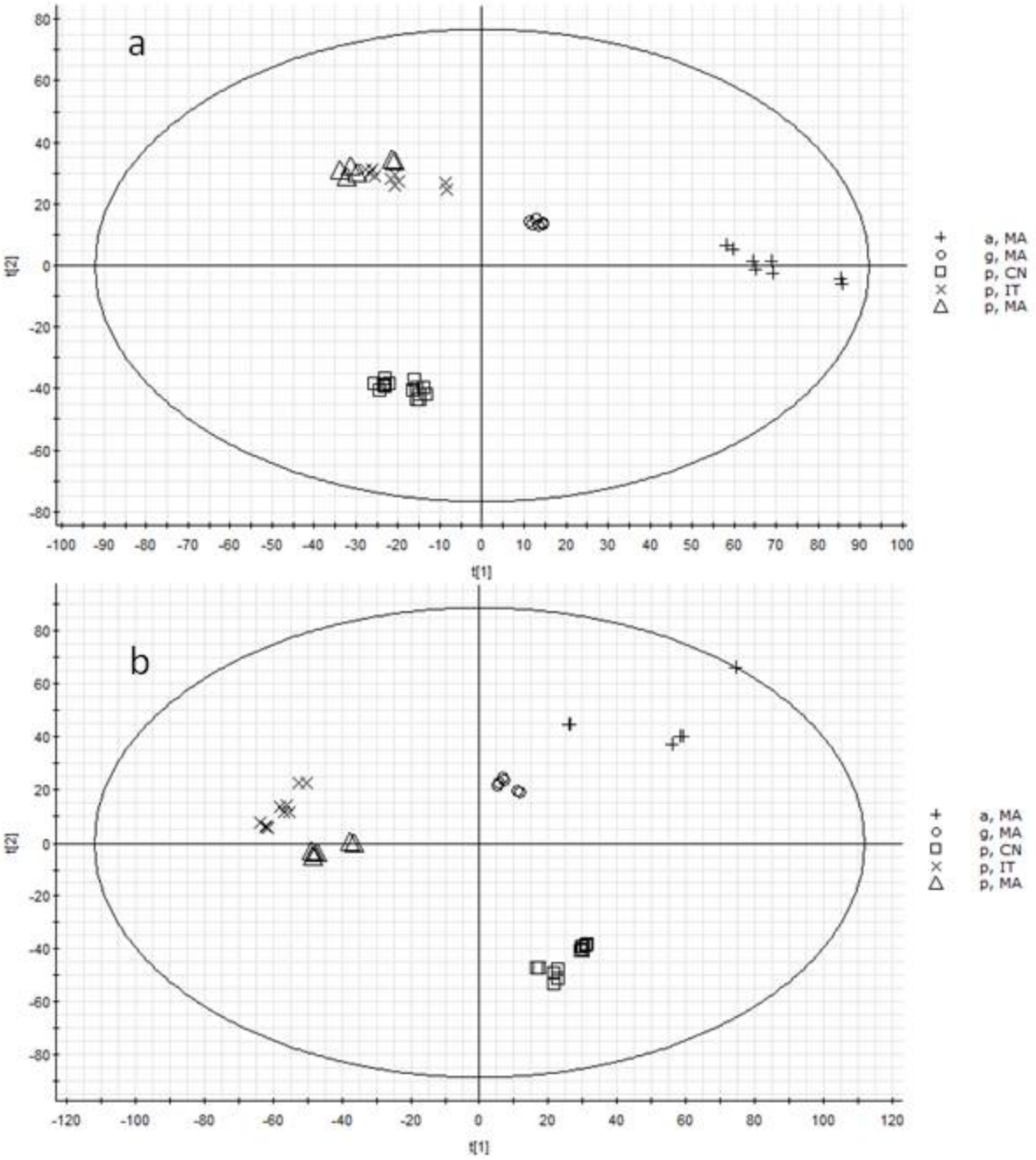
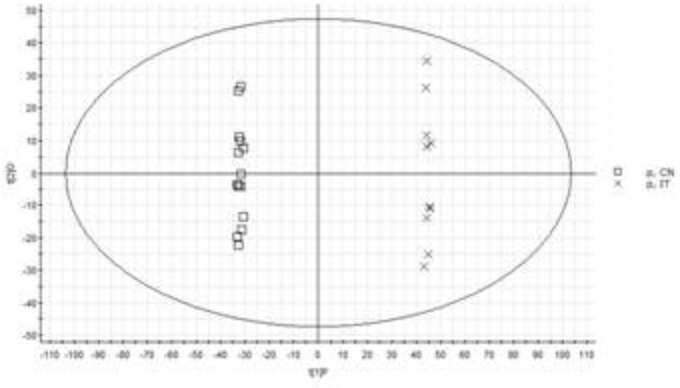

80
60
40
20
0
-20
-40
-60
-80
t[2]
-100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60 70 80 90 100
t[1]
a, MA
g, MA
p, CN
p, IT
p, MA

Figure 4



Figures 5


p, CN
p, IT
t[1]P

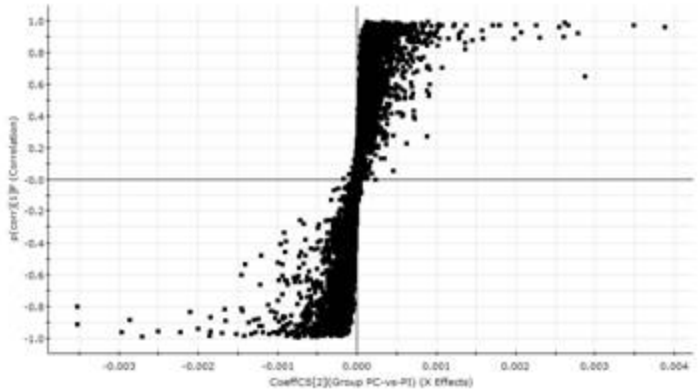

p(corr)[1]P (Correlation)
CoeffCS[2](Group PC-vs-PI) (X Effects)

Figure 6

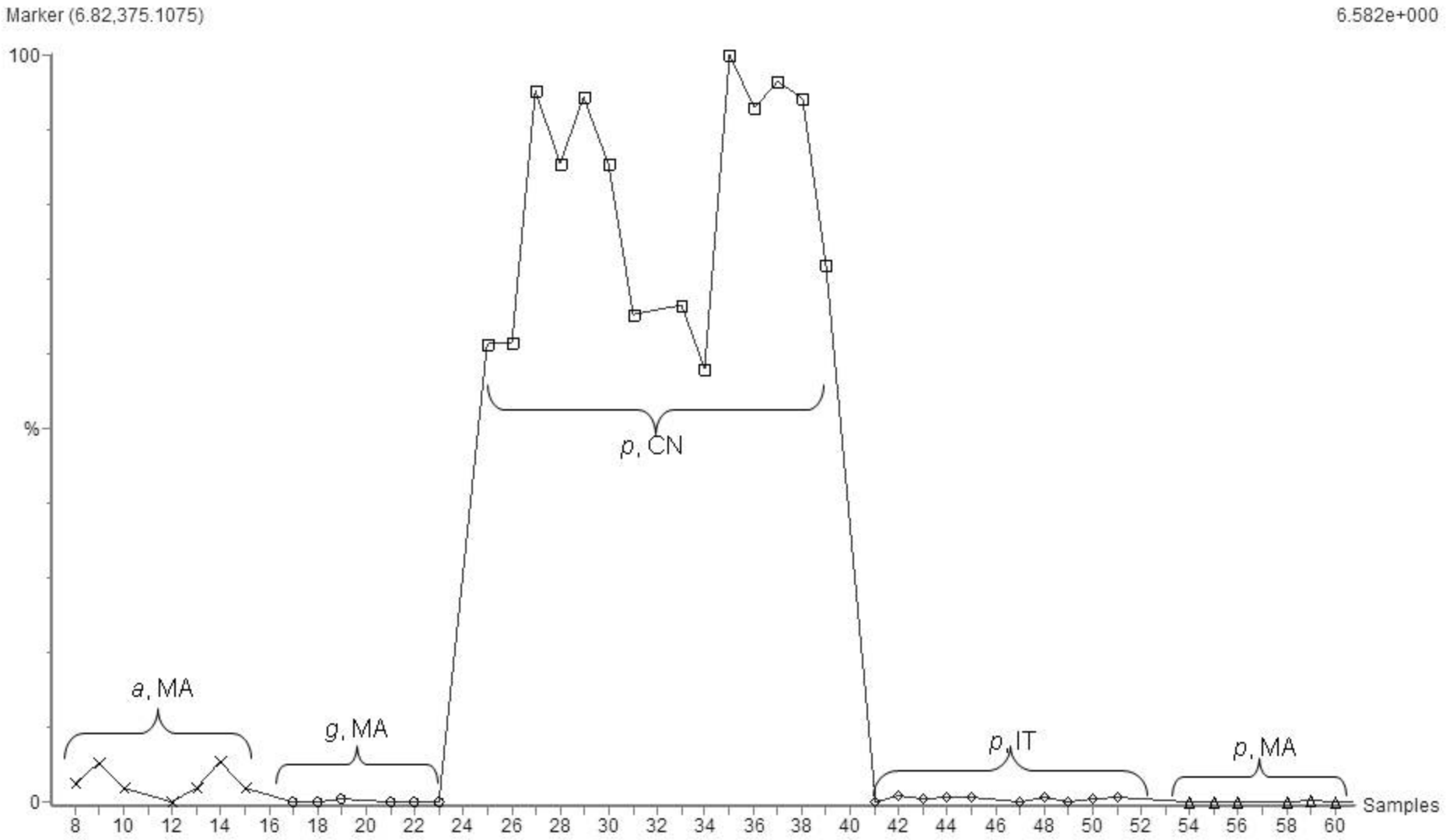
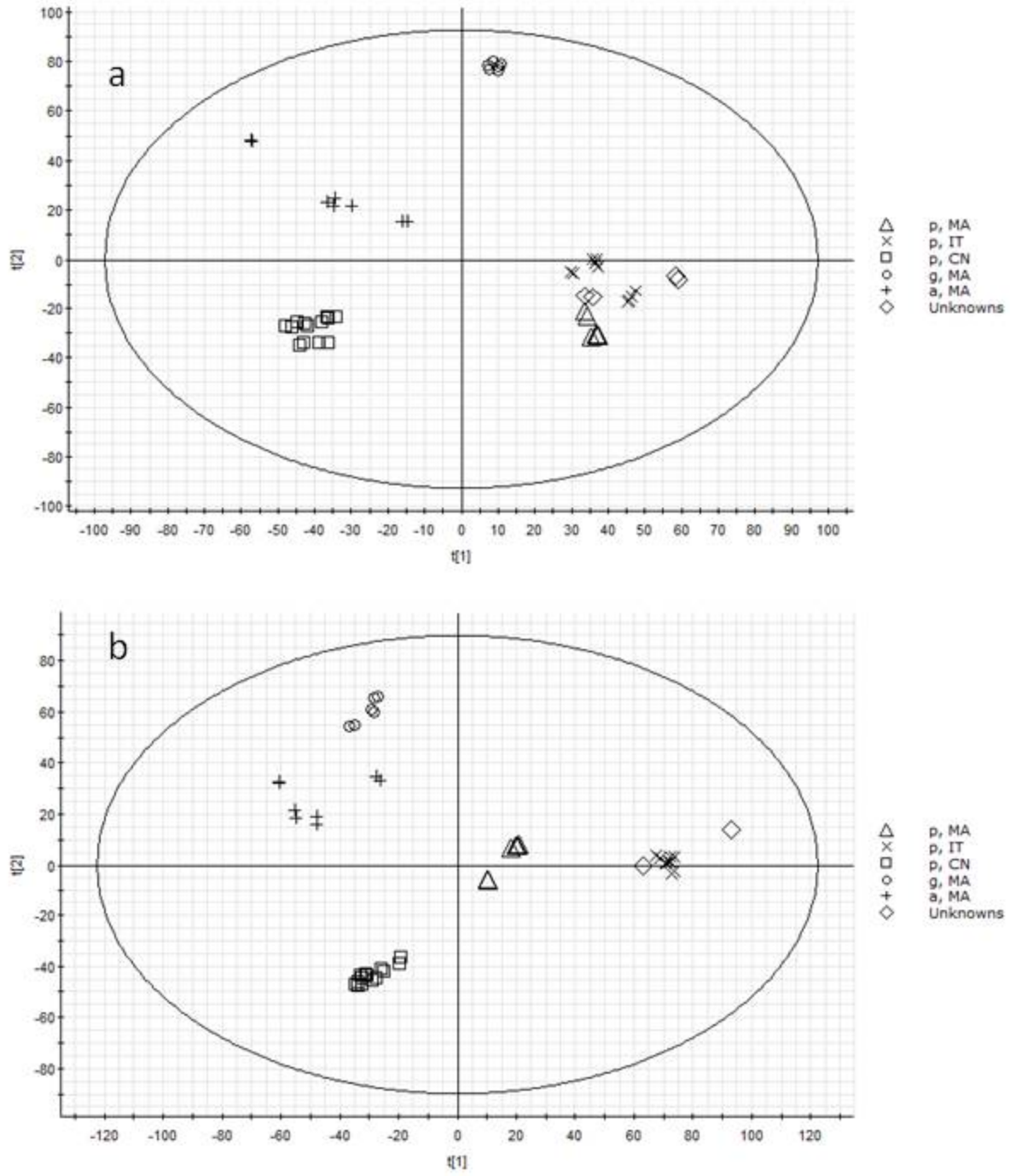

Marker (6.82,375.1075)
6.582e+000
100
%
0
a, MA
g, MA
p, CN
p, IT
p, MA
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
Samples

Figure 7



Figures 8