Food and Chemical Toxicology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The problem of misidentification between edible and poisonous wild plants: Reports from the Mediterranean area

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ARTICLE INFO

Keywords:
Poisonous plants
Edible plants
Plant leaves
Accidental ingestion
Intoxication
Discriminant analyses

ABSTRACT

Today, in many European countries, people are looking for wild edible plants to experience new tastes and flavors, by following the new trend of being green and environmentally friendly.

Young borage and spinach leaves can be easily confused by inexpert pickers with those of other plants, including poisonous ones, such as *Mandragora autumnalis* Bertol. (mandrake) or *Digitalis purpurea* L. (foxglove), common in southern and northern Italy respectively. In the last twenty years, several cases of intoxication by accidental ingestion of mandrake and foxglove have been reported. The purpose of this work was to perform a pharmacognostic characterization of young leaves from borage, mandrake, foxglove and spinach, by micro-morphological, molecular and phytochemical techniques.

The results showed that each of the three techniques investigated could be sufficient alone to provide useful information for the identification of poisonous species helping the medical staff to manage quickly the poisoned patients. However, the multi-disciplinary approach proposed could be very useful to asses the presence of poisonous plants in complex matrices, to build a database containing morphological, molecular and phyto-chemical data for the identification of poisonous species or in forensic toxicology, given their increasingly frequent use due to their low cost and relatively common availability.

1. Introduction

Nowadays in many European countries a new trend is spreading among people: the search for wild edible plants aimed at experiencing new tastes and flavors, but also to be green (Colombo et al., 2010). However, the cases of poisoning due to plant ingestion are growing worldwide, as reported by emergency rooms and poison control centers, and one of the main causes of this phenomenon is plant misidentification (Mezzasalma et al., 2017). In the Mediterranean region, different wild and semi-cultivated plant species, as well as species escaped from cultivation, are traditionally collected for culinary uses. Two common examples are *Borago officinalis* L. (borage) and *Spinacia oleracea* L. (spinach). However, the young leaves of borage and spinach may be easily confused by inexpert pickers with those of poisonous plants.

Borage is an annual herb originating in the Mediterranean region, but naturalized and widely cultivated throughout most of Europe. This is traditionally used for culinary and medicinal purposes and has a commercial value as oilseed. Due to a cucumber-like taste, borage leaves are also mixed in salads and used as vegetable in several European countries, such as Germany, Spanish (Aragón and Navarra), and Greece (Crete). In Italy, especially in the region of Liguria, borage is commonly used as filling of the traditional pasta named *ravioli* and *pansoti*, and as an ingredient of soups and vegetable pies (Cornara et al., 2009). Young leaves of borage are sometimes confused with those of other plants, such as the very poisonous *Mandragora autumnalis* Bertol. (mandrake) in Southern Italy and in Sicily, and *Digitalis purpurea* L. (foxglove) in Northern Italy. Patients who unintentionally eat leaves of

Abbreviations: LM, Light microscopy; SEM, Scanning electron microscopy; GC-MS, Gas chromatography-mass spectrometry; FID, Flame ionization detector; EDS, Energy dispersive system; BLAST, Basic Local Alignment Search Tool

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https://doi.org/10.1016/j.fct.2018.04.066
Received 24 March 2018; Received in revised form 27 April 2018; Accepted 29 April 2018

Table 1
List of analysed samples and sampling details.

Specimen	Species	Collection site	Collection year	A.N. rbcL	A.N. matK
FEM_001_BO	*Borago officinalis* L.	Agrigento, Sicilia	2017	LT992827	LT992716
FEM_002_BO	*Borago officinalis* L.	Botanic garden, Genova, Liguria	2017	LT992828	LT992715
FEM_003_BO	*Borago officinalis* L.	Botanic garden, Struppa Genova, Liguria	2017	LT992829	LT992717
FEM_004_BO	*Borago officinalis* L.	Carpi, Genova, Liguria	2017	LT992830	LT992718
FEM_005_BO	*Borago officinalis* L.	Botanic garden, Genova, Liguria	2016	LT992831	LT992719
FEM_006_DP	*Digitalis purpurea* L.	Agrigento, Sicilia	2017	LT992832	LT992615
FEM_007_DP	*Digitalis purpurea* L.	Botanic garden, Genova, Liguria	2017	LT992833	LT992616
FEM_008_DP	*Digitalis purpurea* L.	Botanic garden, Genova, Liguria	2016	LT992834	LT992617
FEM_009_MA	*Mandragora autumnalis* Bertol.	Agrigento, Sicilia	2017	LT992819	LT992812
FEM_010_MA	*Mandragora autumnalis* Bertol.	Natural reserve, Trapani, Sicilia	2017	LT992820	LT992811
FEM_011_MA	*Mandragora autumnalis* Bertol.	Botanic garden, Genova, Liguria	2017	LT992821	LT992813
FEM_012_MA	*Mandragora autumnalis* Bertol.	Favignana, Sicilia	2017	LT992822	LT992814
FEM_013_MA	*Mandragora autumnalis* Bertol.	Favignana, Sicilia	2017	LT992823	LT992815
FEM_014_MA	*Mandragora autumnalis* Bertol.	Botanic garden, Genova, Liguria	2016	LT992824	LT992816
FEM_015_SO	*Spinacia oleracea* L.	Agrigento, Sicilia	2017	LT992825	LT992817
FEM_016_SO	*Spinacia oleracea* L.	Market, Genova, Liguria	2017	LT992826	LT992818

mandrake (the most common case), or foxglove (less frequently), mistaking them for the edible *B. officinalis*, turn to the Italian Poison Control Centers or Hospital Emergency Service showing anticholinergic symptoms. In the period 1995–2007, 50 cases of intoxication by accidental ingestion of mandrake, and 6 cases due to an accidental ingestion of foxglove, were reported in Italy (Colombo et al., 2009). Other cases were also reported in the island of Crete, where two patients consumed accidentally mandrake instead of the eatable borago (Tsiligianni et al., 2009).

By the end of 2017, cases of poisoning, due to the presence of mandrake leaves mixed with spinach in commercial frozen vegetable, were reported in Italy. In one case, the hallucinogen-tainted frozen spinach caused the hospitalization of 4 family members in Milan, but the presence in the batch of the poisonous mandrake, containing tropane alkaloids, was not proven (http://www.ansa.it/). In another circumstance, 7 people were hospitalized with symptoms of mental confusion, amnesia and nausea, after eating a vegetable soup (*minestrone*). Also in this case, the suspect of vegetables contaminated with madrake leaves was advanced (http://genova.repubblica.it/). Analyses performed on soup samples by a Public Health Service laboratory confirmed the presence of three hallucinogenic substances, i.e. atropine, scopolamine and norscopolamine. The same substances were also detected in biological samples from the same patients analysed at the Anti-Poison Center (Pavia, Italy). In the light of these findings, food poisoning was attributed to leaves of mandrake or of some other infesting plant (http://www.lastampa.it/).

In this study, we show pharmacognostic characterizations of borage, spinach, mandrake and foxglove, in their young stage of development, based on micro-morphological, phytochemical and molecular analyses. This study provides a multidisciplinary approach to the problem of misidentification among edible and poisonous wild plants. The reported protocols provide an integrate and reliable identification systems to identify poison plant species in complex matrices, which could be useful to different stakeholder categories involved in the diagnostics of poisonous plants, thus allowing a quick management of patients.

2. Material and methods

2.1. Plant material

Representative samples of young leaves from *Borago officinalis* L. (Boraginaceae), *Digitalis purpurea* L. (Schrophulariaceae), *Mandragora autumnalis* Bertol. (Solanaceae), and *Spinacia oleracea* L. (Chenopodiaceae), were obtained from plants growing in two different Italian regions (Liguria and Sicily), collected directly in the field or in botanical gardens. One of us (LC) taxonomically identified plant specimens collected in the field, following standard analytical keys (Pignatti, 1982). In the case of borage and spinach, samples from the Municipal Market of Genova (Italy) were also examined.

2.2. Macro- and micromorphologycal analyses

2.2.1. Light microscopy (LM)

Unprocessed plant material was observed by a Leica M205C stereomicroscope, coupled with EC3 camera and LAS EZ V1.6.0 image analysis software. A sample-clearing process was carried out on small leaf portions by using 5% aqueous hypochlorite for 15–30 min, followed by rinsing in distilled water and immediate microscopic examination. Sections mounted on glass slides were then observed by a Leica DM 2000 transmission-light microscope, coupled with a computer-driven DFC 320 camera (Leica Microsystems, Wetzlar, Germany).

2.2.2. Scanning electron microscopy (SEM)

Small pieces of approximately 1 cm of leaves from each species, representative of the median portions, were sectioned with a razor blade. Samples were fixed in FineFIX working solution (Milestone s.r.l., Bergamo, Italy) with 70% ethanol, left overnight at 4 °C (Chieco et al., 2013), dehydrated for 1 h through a graded series of ethanol: 80, 90, 95 and 100%, and finally dehydrated in CO_2 using a Critical Point Drier processor (K850 CPD 2M Strumenti S.r.l., Roma, Italy). Dried specimens were mounted on aluminium stubs using double stick tape, and coated with 10 nm gold. SEM analysis was carried out using a Vega3 Tescan-type LMU microscopy equipped with the X-ray energy dispersive system (EDS) Apollo XSD (Tescan USA Inc., Cranberry Twp, PA, USA) at an accelerating voltage of 20 kV.

2.3. Molecular analysis and DNA barcoding

Sixteen samples of *B. officinalis*, *D. purpurea*, *M. autumnalis* and *S. oleracea* (average number of samples per species: 4 ± 1.58, range 2–6) were collected at different Italian geographically distant sites. For each species, young leaves were collected directly in the field or in botanical gardens (Table 1), and the samples were used to identify species by DNA barcoding and evaluate genetic distances.

DNA was isolated from young leaves by using the Eurogold plant DNA Mini Kit (EuroClone s.p.a., Milan, Italy). Purified DNA concentration and quality for each sample were estimated with Eppendorf BioPhotometer®. The Consortium for the Barcode of Life-Plant Working Group (CBOL, 2009) recommended the two-locus combination of rbcL + matK (RuBisCo large subunit + maturase K) as standard plant barcodes. Each sample was analysed by sequencing these two coding plastidial regions. PCR amplification for each candidate marker was performed using Wonder taq Polymerase (EuroClone s.p.a., Milan, Italy) in a 25 μL reaction volume, according to the manufacturer's

instructions. PCR cycles consisted of an initial denaturation for 5 min at 95 °C, 35 cycles of denaturation (45 s at 95 °C), annealing (45 s at 52 °C), extension (1 min at 72 °C), and final extension at 72 °C for 7 min. Primer 1F and 724 R primers were used (Fay et al., 1998; https://www.eurofinsgenomics.eu/) to amplify rbcL region while 3F_KIM (5-CGTACAGTACTTTTGTGTTTACGAG-3′) and 1R_KIM (5-ACCCAGTCCATCTGGAAATCTTGGTT-3′) were used to amplify matK region. Products were submitted for sequencing to Eurofins genomics (https://www.eurofinsgenomics.eu/). The DNA strands were bidirectionally sequenced. All obtained sequences were manually edited, aligned, and used as query in an individual Basic Local Alignment Search Tool (BLAST) in GenBank to verify samples identity. K2P molecular distance (converted into percentage), calculated with MEGA (version 7.0.26), was used to evaluate the genetic distances among the investigated species.

2.4. Phytochemical analyses

A preliminary phytochemical analysis, based on classes of compounds contained in the poisonous plants (alkaloids for mandrake and digital glycosides for foxglove), was carried out using standardized methods reported in the USP NF (2006) with some modifications.

2.4.1. Alkaloid identification

Aliquots of 1 g of grounded fresh leaves of each plant species were treated with 25 mL of 10% HCl (v/v) and boiled for 2–3 min. The solution was filtered by qualitative filter paper grade 1, 90 mm (Whatman^{tm}, GE Healthcare Life Sciences, Buckinghamshire, UK), and tested using general (Bouchardat, Mayer, Dragendorff, tannic acid 5%) and characterizing (Hager, picric acid) alkaloid reagents. The formation of dark, beige, reddish brown and white amorphous precipitates for general reagents, and crystalline yellow precipitate for characterizing reagents, respectively, was indicative of the presence of alkaloids (Fig. 1a).

2.4.2. Digital glycoside identification

Samples of each plant species as above were treated with 20 mL of 70% ethanol (v/v) and 20 drops of basic lead acetate, boiling for 2–3 min. Ethylic ether was added to the filtered solution (1:1, v/v). The ether phase, dried s in a test tube, was suspended with 3 mL glacial acetic acid and 2–3 drops of iron (III) chloride. Concentrated sulphuric acid was stratified along the test tube wall. The formation of a red-brown ring (Fig. 1b, red arrow) and a blue-green upper layer on the solution is indicative of the presence of digitalis glycosides (Fig. 1b, black arrow).

2.5. GC-FID and GC-MS analysis

An additional common discriminant analysis of leaf wax compounds of the four plant species under investigation by GC-FID and GC-MS analysis was setup according to the Maffei method (1994), with some modifications. Briefly, 5 g of grounded fresh leaves was extracted by sonication with 5 mL pentane-hexane (5:1, v/v) for 60 s. The extract was concentrated through a gentle stream of nitrogen, passed through a column of anhydrous $MgSO_4$ and then analysed. GC analysis was performed on an Agilent gas chromatograph, Model 7890A, equipped with a flame ionization detector (FID) (Agilent Technologies Santa Clara, CA, USA), using a HP-5MS capillary column (30 m, 0.25 mm coated with 5% phenyl methyl silicone, 95% dimethyl polysiloxane, 0.25 µm film thickness) and helium as carrier gas (1 mL/min). Injection was done in split mode (50:1), injected volume was 1 µL, and the injector and detector temperature were 250 °C and 280 °C, respectively. The oven temperature was held at 35 °C for 2 min, increased to 250 °C at 10 °C/min, 300 °C at 20 °C/min and held at 300 °C for 23 min. Percentages of compounds were determined from their peak areas in the GC-FID profiles. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out by the above instrument, coupled with an Agilent 5975C mass detector, with the same column and the same operative conditions used for the analytical GC. The ion source and detector temperature were 200 °C and 180 °C, respectively. Mass Spectra data were acquired in scan mode within the *m/z* range 30–600. The compounds were identified based on literature-reported GC retention index (relative to C_7-C_{40} *n*-alkanes on the HP-5MS column), computer matching of recorded mass spectral data with MS library (NIST 08), comparison of recorded MS fragmentation patterns with those reported in literature, and, whenever possible, co-injection with authentic standards (β-sitosterol, linolenic acid, oleic acid, phytol).

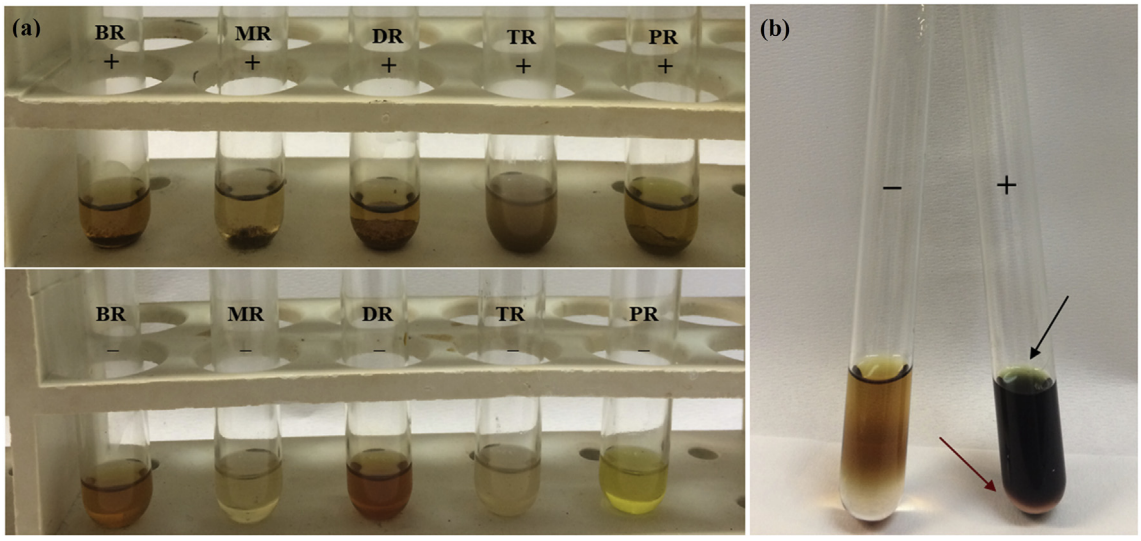

Fig. 1. Representative picture of preliminary *in vitro* phytochemical screening. Fig. 1 (a) represents the negative (−) and the positive results (+) of alkaloids determination with dark, beige, reddish brown and white amorphous precipitate for general reagents and crystalline yellow precipitate for characterizing reagent of alkaloids, respectively. The Fig. 1 (b) represents the negative (−) and positive (+) results of digitalis glycosides determination with red-brown ring (red arrow) and blue-green upper layer (black arrow). BR, Buchardat reagent; MR, Mayer reagent; DR, Dragendorff reagent; TA, Tannic acid reagent; PA, Picric acid reagent. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

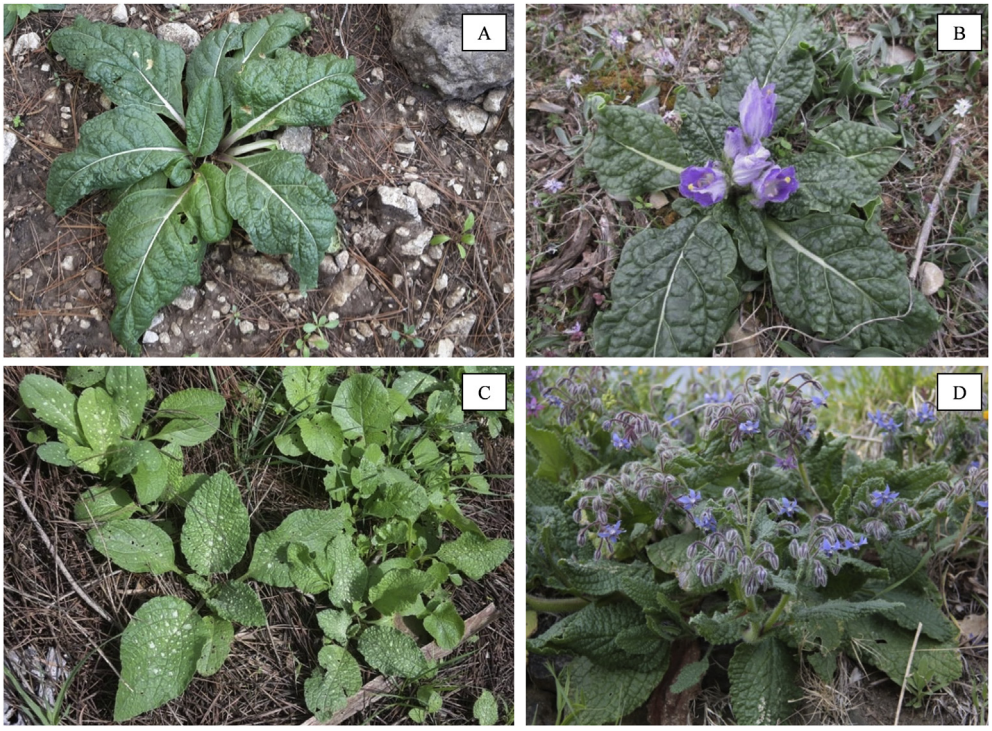

Fig. 2. A–D: Comparison between mandrake and borage plants. (A–B) *Mandragora autumnalis*, young leaves in rosette (A) and flowering plant (B); (C–D) *Borago officinalis*, young leaves in rosettes (C) and flowering plant (D).

3. Results and discussion

3.1. Macro- and micromorphologycal analyses

Among the four studied species, most easily confused by inexpert pickers are those of mandrake, which in Southern Mediterranean coasts are frequently collected in place of those of borage (Fig. 2A-D). Both borage and mandrake grow in sunny areas and their young leaves form similar basal rosettes (Fig. 2 A and C). In addition, both plants have small, blue violet flowers with five lobes, although with different stem and scape. In borage, a flower stalk grows directly from the ground whereas in mandrake the stalk is lacking (Fig. 2 B and D). The leaves of floxglove can be confused with those of borage only at the young stage, since in the second year the plant produces a tall, leafy flowering stalk with many tubular, bell-shaped flowers that vary in color from white to purple.

The leaves of spinach and mandrake can be confused with one another only when young, as both form a rosette of thick, fleshy leaves. In fact, spinach is a dioecious species, showing inflorescences of inconspicuous male flowers, and greenish-yellow female flowers, very different from the hermaphrodite, blue violet flowers of mandrake.

The leaves of *M. autumnalis* are ovate-lanceolate, oblanceolate or spatulate, rather large, with obtuse apex and acute base, showing an elongated petiole and wavy, sometimes ciliate margins. The leaf color is dark green, shiny above, paler beneath, while scattered trichomes are present. The epidermal cells have wavy cells and stomata are of anysocitic type (Fig. 3A). A few covering hairs are present, distributed along the leaf margins and on the veins, mainly in the lower leaf surface. Hairs are very long, uniseriate and multi-celled (8–20 cells), with some cells sometimes collapsed (Fig. 3 B-C and F). Glandular trichomes showing a short pedicel and a multicellular glandular head are scattered on both leaf surfaces (Fig. 3 D and E-F). In the spongy mesophyll, some cells filled with microspheroidal crystals of calcium oxalate can be found (Berry, 1970).

The leaves of *B. officinalis* are large, obovate, with rounded apex, undulate margins and an attenuate base that gradually transforms into an elongated petiole. Stomata are numerous and mainly of anomocytic type (Fig. 4 A). Both lower and upper leaf surfaces and the entire petiole are pubescent (Fig. 4D–E), with leaves densely covered by different types of trichomes, both covering and glandular ones (Fig. 4 B-C and D-G). Covering trichomes are short to long, typically one-celled, but sometimes showing 2–3 cells, swollen at the base, straight and/or sickle-shaped, with elongated and acuminate tips, with or without subtending 'rosettes' (Fig. 4 B and D-F). These latter are formed by neighbouring epidermal cells and can be composed of up to 30 cells, one-to multi-layered (Fig. 4 F). Cystolith bodies of calcium carbonate can be found, located at the base of one-celled hairs and in epidermal 'rosette' cells (Rimma and Vitaly, 2016). Small glandular trichomes are also present, mainly located along the veins (Fig. 4C–E), and show a unicellular stalk and one-celled head (Fig. 4 C and G). In our specimens, glandular trichomes occur on either side of the blades and not only on the abaxial side, along large protruding veins, as previously observed by Rimma and Vitaly (2016).

The leaves of *D. purpurea* are usually ovate-lanceolate and petiolate, with decurrent lamina at the base, and crenate margins. The leaf upper surface, bearing many short hairs, is rugose and dull green, while the lower surface is paler and densely hairy. The mid-rib prominent on the lower surface shows lateral veins leaving it at an acute angle, and then curving towards the apex. Both surfaces show epidermal cells with thin wavy walls, anomocytic type of stomata (Fig. 5 A), and both covering and glandular trichomes (Fig. 5B–F). The uniseriate covering trichomes consist of 3–5 elongated cells with finely warty cuticle and acute apex (Fig. 5 B and D-F). These trichomes are frequently broken off, leaving a characteristic scar (Fig. 5 B). Different types of glandular trichomes are present: short with a unicellular stalk and a bicellular head (Fig. 5 C and E-F), short with a 1-2-celled stalk and unicellular head (Fig. 5 B and E), and long with a uniseriate stalk (3–5 cells) and unicellular head (Fig. 5 B–D and F). In all types of glandular trichomes, ahemispheroidal protuberance on the top of the secretory cell can be found (Fig. 5 G). Calcium oxalate crystals are absent (Serrano et al., 2014).

The leaves of *S. oleracea* are ovate to triangular at the base, variable in size, with apex oval in shape. The upper surface leafs is smooth,

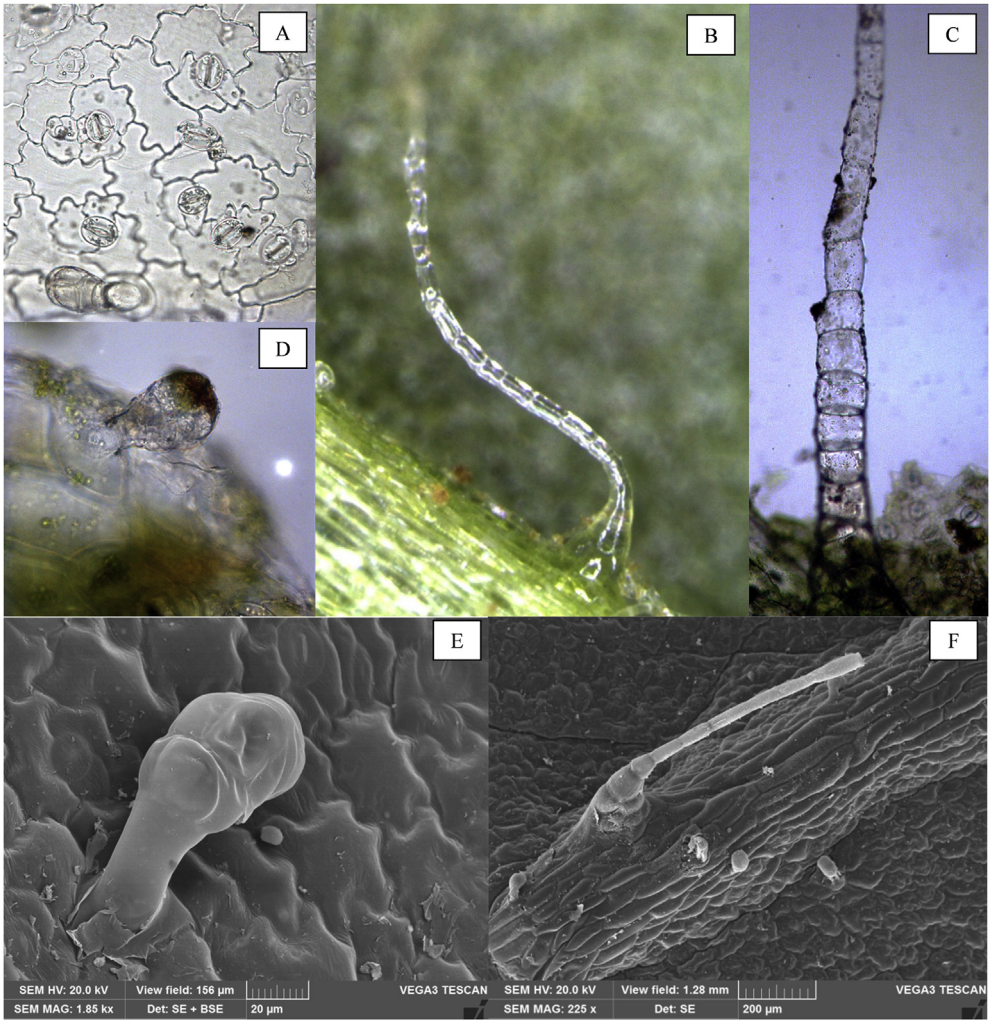

Fig. 3. A–F: Micromorphological characteristics of mandrake leaves. A-D, Light microscopy: anysocitic stomata (A); long covering trichomes (B–C) and a small glandular trichomes (D). E-F, Scanning Electron Microscopy, showing the typical feature of a glandular trichome, with its multicellular glandular head (E) and the distribution of both glandular and covering trichomes on the main vein, in the lower side of the leaf (F).

while the lower one is ruff. Anomocytic stomata are present (Fig. 6 A) and many idioblasts containing calcium oxalate druses are visible in the mesophylls (Fig. 6 B). It is well known that the density of stomata and trichomes types can vary in different spinach cultivar (Lopez-Velasco et al., 2011). In our samples, we found vesicular hairs (Fig. 6 C and E), consisting of a uniseriate stalk bearing an enlarged head containing water and often oxalates in solution or in form of crystals, common in many Chenopodiaceae species (Metcalfe and Chalk, 1950). These vesicular hairs are mainly located along the vein on both leaf surfaces and at maturity dry up forming a white powder on the leaf surface. Glandular trichomes are also present with a short uniseriate stalk and an ellipsoidal head, bending toward the epidermis (Fig. 6 D–F). These trichomes are similar to a type described in different species of Chenopodiaceae by Bonzani et al. (2003).

Microscopic data show that, even though the macromorphological features of the 4 species can be confused by inexpert pickers at the stage when only young leaves are present (Fig. 2), the analysis of leaf fragments leads to certain identification, mainly due to the different kinds and distribution of covering and glandular trichomes (Figs. 3–6).

3.2. Molecular analysis

The *rbcL* and *matK* sequences were subjected to an individual Basic Local Alignment Search Tool (BLAST) in GenBank to verify their taxonomic identity. All samples returned 100% maximum identity (with 100% query coverage) with all the declared species regardless of their origin. Our analysis also showed a complete lack of genetic intraspecific variability at the tested loci. Conversely, high interspecific variability was detected. All the species showed consistent genetic distances, with values ranging between 7.75% ± 0.012 (*B. officinalis* vs *M. autumnalis*) and 9.90% ± 0.015 (*M. autumnalis* vs *S. oleracea*) in the case of *rbcL*. Concerning *matK*, the species showed values ranging between 16.6% ± 0.020 (*D. purpurea* vs *M. autumnalis*) and 28.52% ± 0.027 (*B. officinalis* vs *S. oleracea*). Between the two core barcode markers the highest genetic variability was observed for *matK*, with a mean K2P value of 21.36%. The genetic distances are shown in Table 2A and B. These data show that the DNA analysis allows to easily distinguish all the species, due to the high genetic interspecific variability.

3.3. Phytochemical analysis

The preliminary fast phytochemical analysis allowed not only to discriminate immediately between poisonous and non-poisonous plants, but also between *M. autumnalis* and *D. purpurea,* the first one containing tropane alkaloids (mainly atropine, hyoscyamine and scopolamine) (Wagner and Keim, 2009), and the second one containing cardiac glycosides (cardenolides) (Kuroda et al., 2013).

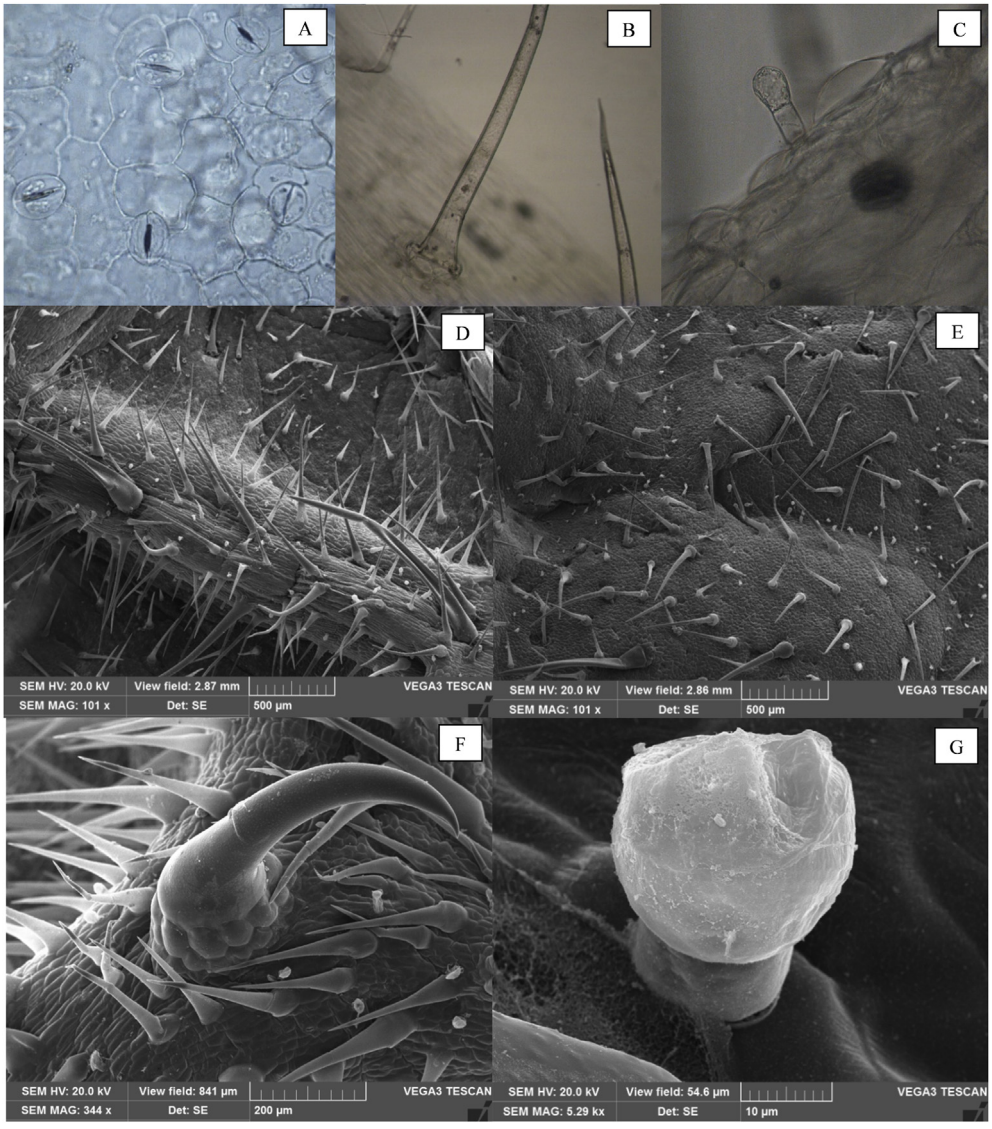

Fig. 4. A–G: Micromorphological characteristics of borage leaves. A-C, Light microscopy: anomocytic stomata (A); covering trichomes (B) and a small glandular trichome (C). D–G: Scanning Electron Microscopy, showing pubescence of both lower (D) and upper (E) leaf surfaces; a sickle-shaped covering trichomes, subtended by a rosette of many epidermal cells (F); a small glandular trichome with unicellular stalk and one-celled head (G).

This fast phytochemical screening is a very useful tool especially in cases of poisoning when rapid determination of ingested matrices is needed. Another advantage is that this test can be carried out quickly on fresh leaves, and that even a single leaf is enough to do it. Advantages include fast execution, easy to prepare and inexpensive reagents, no expensive equipment, and small amounts of sample. However, in better equipped laboratories a GC-FID and GC-MS analysis, are certainly useful tools to better discriminate the different species investigated.

Our phytochemical analyses, revealed a typical chemical fingerprint of each plant and a particularly interesting difference between poisonous and non-poisonous plants. In fact, *M. autumnalis* and *D. purpurea* leaf extracts contain α-tocopherol, n-hexadecanoic acid, and n-docosane, while in *B. officinalis* and *S. oleracea* these metabolites are absent. Therefore, these metabolites are discriminant among poisonous and edible plants, and could be used as phytochemical markers.

Even more discriminant at species level were wax compounds. Although these species showed similarities in the profile of these chemicals, the relative abundances of each metabolite were very different (see bold numbers in Table 3). Epicuticular wax compounds represent one of the most studied class of secondary metabolites in terms of chemotaxonomy, so the distribution pattern of these compounds could be a potential marker for discriminations among plant species or genera (Cordeiro et al., 2011). This fraction includes linear long-chain aliphatic compounds, like alkanes, alcohols, acids, fatty acids, aldehydes, ketones and esters. In addition, triterpenoids, tocopherols, or aromatic compounds can be present in some species (Buschaus et al., 2007). The composition of epicuticular wax is influenced by ecological and genetic relationships but, although the relative abundance of the wax compounds is influenced by environmental factors, the chemical composition remains preserved (Sharma et al., 2018).

D. purpurea is the only species, between those investigated, which contain benzophenone, octadecanone-1-chloro-, linolenic acid, squalene, n-pentacosane, n-heptacosane, n-octacosane, n-nonacosane and β-sitosterol. Furthermore, with the other species, *D. purpurea* showed the highest content of n-hexacosane and the lowest content of phytol.

M. autumnalis is the only species containing linolenic acid-methyl ester and n-triacontane. Among shared metabolites, this species showed a higher content in n-docosane and phytol, and a lower content of α-tocopherol with respect to *D. purpurea*. Therefore, even though the two poisonous plants have many common metabolites, it is possible to discriminate well between the two species.

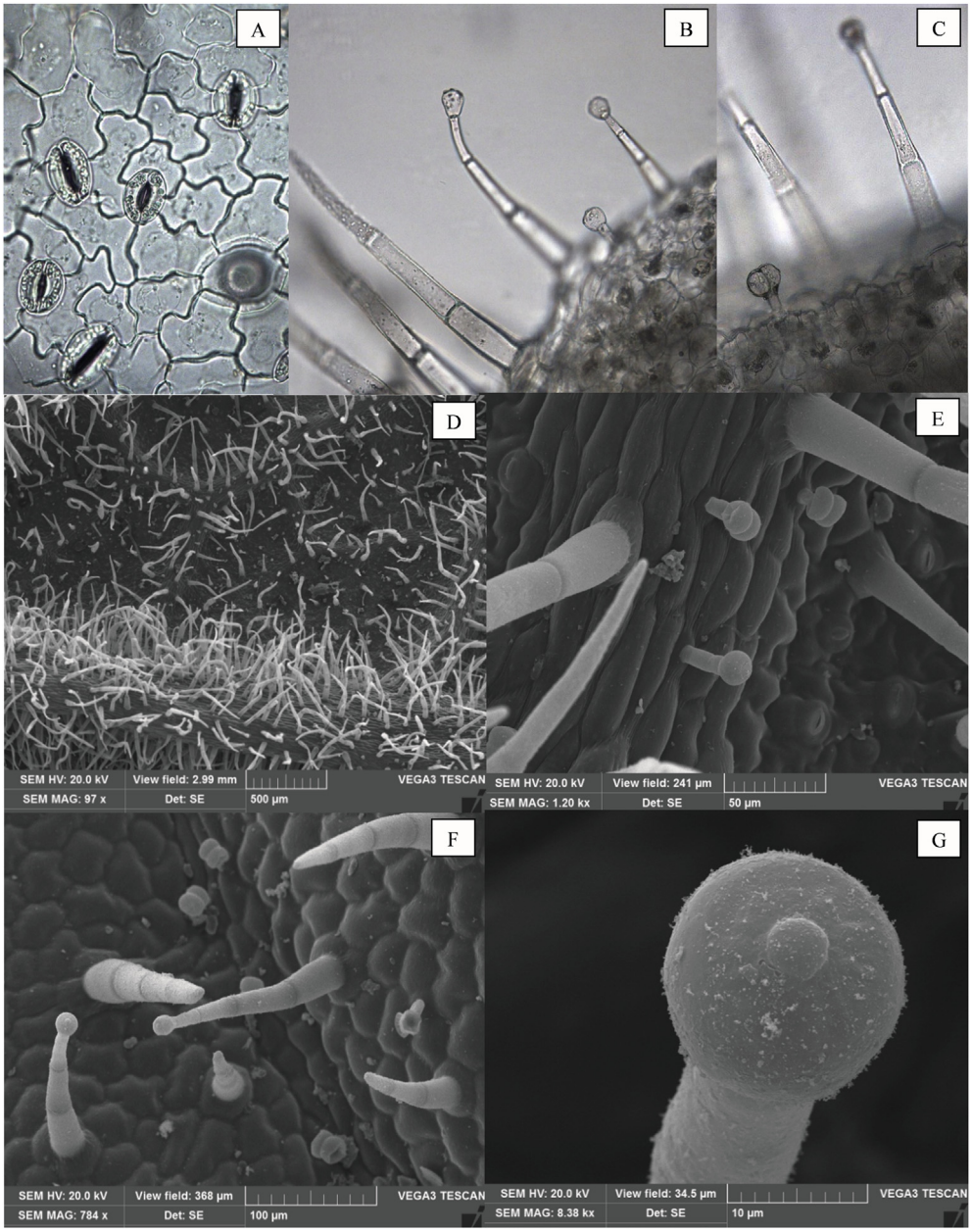

Fig. 5. A–G: Micromorphological characteristics of foxglove leaves. A-C, Light microscopy: anomocytic type of stomata (A); covering and glandular trichomes (B–C). D-G, Scanning Electron Microscopy, showing pubescence of both lower and upper leaf surfaces (D–F); different types of glandular trichomes (E–F); one hemispheroidal protuberance on the unicellular head of a long glandular trichomes is visible (G).

B. officinalis is the only species that contains n-tricosane, n-eicosane and methyl dihydrojasmonate. Furthermore, among metabolites shared with the other species, *B. officinalis* showed the highest content of phytol and the lowest content of n-hexacosane.

Interestingly, the phytochemical profile of *S. oleracea* was completely different in respect to the other plant species investigated. Therefore, leaf wax compounds analysis is a valid tool to identify the species investigated.

4. Conclusions

Many people are currently interested in harvesting edible plants from the wild or domestic garden. However, in many cases, inexpert pickers can easily confuse the young leaves of edible species with those of poisonous ones. As a result, in the last few decades, several cases of intoxication by accidental ingestion of wild plants, sometimes with fatal outcomes, have been reported around the world.

In the case of poisoning caused by misidentification among the plants considered in the present study, the analysis of leaf fragments by light microscope is useful to identify the species, due to the different kinds and distribution of covering and glandular trichomes. In addition, DNA analysis confirms the possibility of distinguishing species, due to high interspecific genetic variability. Also, phytochemical screening reveals the chemical fingerprint of each plant species, useful in the distinction between poisonous and non-poisonous plants. The presence or absence of some metabolites such as α-tocopherol, n-hexadecanoic acid, and n-docosane are typical of *M. autumnalis* and *D. purpurea* leaf extracts, while these compounds are lacking in *B. officinalis* and *S. oleracea*. Our data show that also the kind and distribution pattern of epicuticular wax compounds could be a potential marker to discriminate between these species.

The results show that each of the three techniques alone used in the

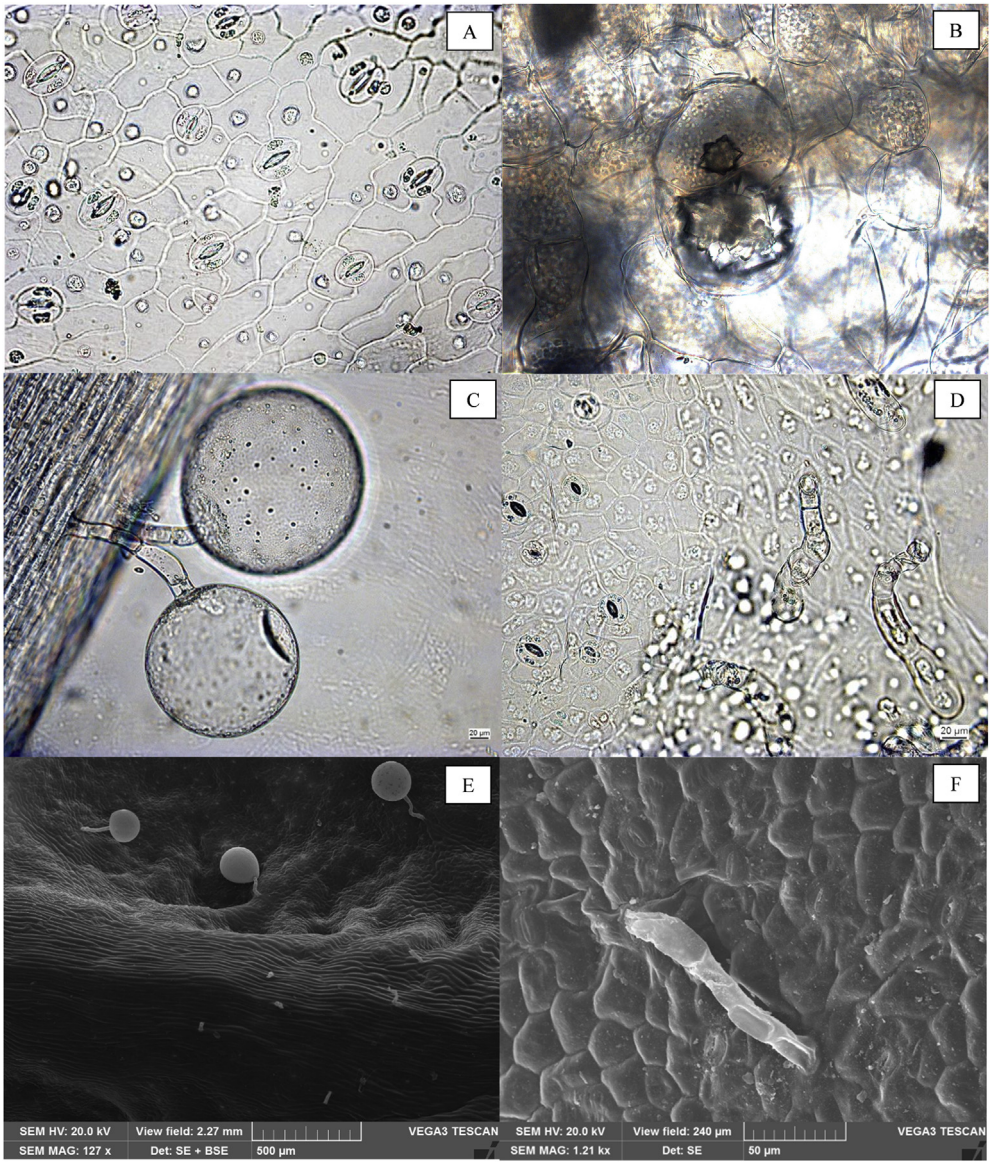

Fig. 6. A–F: Micromorphological characteristics of spinach leaves. A-D, Light microscopy: anomocytic type of stomata (A); a crystalline idioblast containing a calcium oxalate druse is visible (B); vesiculose trichomes (C); glandular trichomes (D). E-F, Scanning Electron Microscopy, showing a few scattered glandular and vesiculose trichomes on the epidermis in the lower leaf surfaces.

Table 2
Genetic K2P distances with MEGA (version 7.0.26) for *matK* (a) and *rbcL* (b) respectively.

	1	2	3	4
(a)				
1. *Spinacia oleracea* L.		**0.027**	**0.026**	**0.021**
2. *Borago officinalis* L.	0.285		**0.024**	**0.019**
3. *Digitalis purpurea* L.	0.254	0.215		**0.020**
4. *Mandragora autumnalis* Bertol.	0.194	0.167	0.166	
(b)				
1. *Spinacia oleracea* L.		**0.013**	**0.012**	**0.014**
2. *Borago officinalis* L.	0.084		**0.013**	**0.014**
3. *Digitalis purpurea* L.	0.075	0.078		**0.015**
4. *Mandragora autumnalis* Bertol.	0.092	0.089	0.099	

present work could be sufficient to provide useful information for the identification of poisonous species. This equipotency could simplify the research for an expert who could help the medical staff, providing useful information for the appropriate treatment of the patient. However, the multidisciplinary analysis presented in this study could find various other applications. This approach could be useful for assessing the presence of poisonous plants in complex matrices, such as bags of pre-frozen vegetables or ready meals, where plant identification is extremely difficult. It could even lead to the construction of a database containing morphological, molecular, and phytochemical data for the identification of poisonous species that may contaminate forage, or to be used in toxicological crimes. Forensic analyses could especially benefit from these data since toxic plants are often used by criminals in rape, burglary and murder cases, due to their low cost and relatively common availability.

Conflicts of interest

The authors declare no competing financial interest.

Table 3
Comparative GC-FID and GC-MS analysis of *M. autumnalis*, *D. purpurea*, *B. officinalis* and *S. oleracea* fresh leaves extracts. Results were expressed as relative peak area percentage with respect to the total compounds identified. Bold numbers refers to the marker metabolites of each species.

#	KI[a]	Compound	*M. autumnalis*	*D. purpurea*	*B. officinalis*	*S. oleracea*
			Area[b] (%)			
1	727	Benzophenone	–	**0.30**	–	–
2	855	Methyl dihydrojasmonate	–	–	**0.49**	–
3	938	Cinnamaldehyde, α hexyl	–	3.42	4.59	–
4	1124	Hexahydrofarnesyl acetone	0.93	–	0.48	–
5	1132	Versalide	–	0.40	0.56	–
6	1223	n-Hexadecanoic acid	2.48	1.23	–	–
7	1234	Octadecanone-1-chloro-	–	**0.13**	–	–
8	1252	Phytol	27.76	0.75	55.93	–
9	1275	Linolenic acid	–	**5.35**	–	–
10	1280	Linolenic acid-methyl ester	**9.29**	–	–	–
11	1308	Oleic acid	–	–	–	**42.82**
12	1391	6-Octadecenoic Acid, (Z)-	–	–	–	**0.06**
13	1416	1-Nonadecene	–	–	–	**0.39**
14	1475	Oleamide	–	–	–	**0.52**
15	1528	α-Monoolein	–	–	–	**0.47**
16	1572	Olealdehyde	–	–	–	**19.36**
17	1584	1-Docosene	–	–	–	**0.10**
18	1585	n-Eicosane	–	–	**0.73**	–
19	1604	β-Monopalmitin	–	–	–	**0.80**
20	1647	n-Docosane	8.02	0.45	–	–
21	1657	n-Tricosane	–	–	**14.54**	–
22	1660	Behenic alcohol	–	–	–	**10.41**
23	1730	Erucylamide	–	–	–	**0.39**
24	1756	Cyclotetracosane	–	–	–	**0.34**
25	1758	Squalene	–	**1.15**	–	–
26	1770	Spinacene	–	–	–	**0.60**
27	1815	n-Tetracosane	8.29	3.45	9.45	–
28	1831	1-Hexacosene	–	–	–	**5.95**
29	1872	n-Pentacosane	–	**6.84**	–	–
30	1922	n-Hexacosane	27.01	28.05	4.43	–
31	1954	n-Tetratetracontane	–	–	–	**17.79**
32	1979	α-tocopherol	2.67	17.12	–	–
33	2091	n-Heptacosane	–	**1.94**	–	–
34	2146	n-Octacosane	–	**11.43**	–	–
35	2152	n-Nonacosane	–	**14.72**	–	–
36	2183	n-Triacontane	**4.13**	–	–	–
37	2191	β-Sitosterol	–	**3.45**	–	–
38	2221	γ-Sitosterol	9.42	–	8.80	–

#: Components are listed in their elution order from HP-5-MS column; [a]: Retention index (KI) relative to standard mixture of *n*-alkanes on HP-5MS column; [b]: values (relative peak area percentage) represent averages of three determinations.

Acknowledgments

We wish to thank Mrs. Laura Negretti (DISTAV) for SEM technical assistance.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2018.04.066.

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