Interdonato lemon from Nizza di Sicilia (Italy): chemical composition of hexane extract of lemon peel and histochemical investigation

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**ABSTRACT**

Considering that the determination of authenticity and of the geographical origin of food is a very challenging issue, in this study we studied by means of histological and histochemical analyses the famous Sicilian lemon known as ‘Interdonato Lemon of Messina PGI’. Since the protected geographical indication Interdonato lemon of Messina possesses high organoleptic properties, the composition of the hexane extract of lemon peel was determined by HRGC and HRGC–MS analyses and compared with that of lemon of different cultivars. The results obtained are informative of the oil’s quality and explain the variation of the lemon essential oil composition. Given the fundamental economic implications of any fraud, the aim of this study was to determine a fingerprint able to evaluate the authentication of the geographic origin in such way to prevent frauds in national and international markets.

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1. **Introduction**

*Interdonato* lemon, also traditionally known as *‘limone fino’, ‘limone speciale’ or ‘liscone’*, is a hybrid between a lemon and a cedar that matures early (Production Regulations Limone
Interdonato CEE Reg. N° 1081/2009). The yield in fresh product is between 80 and 130 lb per plant. Historically, in Sicily it is only spread along the eastern coast, the traditional production area includes the whole district of Messina, and its incidence on the surface of the Sicilian cultivation has been decreasing in recent years.

Interdonato lemon is generally a medium–large size fruit, between 80 and 350 g; it has an oval shape with pronounced umbo and style scar little depressed; the epicarp is thin and smooth with distended oil glands. The colour, at the beginning of the commercial maturity, is dull green and then it turns to yellow; the yellow flesh is deliquescent with medium texture and very few or no pips. Finally, the juice, with a yield of not less than 25% and total acidity less than 5%, is rich in particular substances, such as vitamin C, that play an important role in many metabolic activities of the human body.

Given its singular traits, its nutritional and organoleptic properties, on 12 November 2009 the lemon obtained the protected geographical indication (PGI) with the name ‘Limone Interdonato Messina Jonica’ according to CEE Regulation n. 1081 (published in GUCE L. 295 on 12 November 2009). For its protection and development, in 2002 in Nizza di Sicilia (Messina) the ‘Consorzio di tutela del limone Interdonato di Sicilia IGP’ (www.limoneinterdonatoigp.it) was formed.

For these reasons, the production of Interdonato lemon essential oil is very poor and there are few scientific papers on this lemon (Cotroneo et al. 1984, 1985; Dugo & Di Giacomo 2002; Cicero et al. 2015). Thus, the main objective of our study was to obtain more information about the chemical variability of lemon oils. Moreover, since lemon (Citrus limon (L.) cv. Interdonato) has never been the subject of anatomo-histological and histochemical studies, this research was designed to highlight the histological characteristics of epicarp and mesocarp, to locate secretor tissues and to identify histochemically the essential oils. These components with antimicrobial, insecticide and fungicide activity are important for perfumers, food industries, pharmaceutical, cosmetics and for their potency for treating different pathologies of relevant social impact such as cancer (Manassero et al. 2013). Identifying these secondary metabolites in Interdonato lemon could provide data on the quality of the fruit and establish itself more of its importance in agriculture of Messina.

2. Results and discussion

2.1. Histological and immunohistochemical analyses

The flavedo includes some layers of cells of which the first, the epidermis, presents some sinuosity. The epidermal cells are in mutual contact, small in size with outer ring road cutinised and with nails cuticle highlighted by Sudan IV (Figure S1.1); the inside radial and tangential walls are made of cellulose–peptic, positive more to Red Ruthenium than to Fast-Green. These cells are vacuolated and certain are positive to Schiff’s reagent, thus showing the presence of terpenes and precisely citral.

Under the skin, there are a few collenchymatic and parenchymatic layers whose cells, vacuolated, have thick pectic walls highlighted by Red Ruthenium (Figure S1.2). The first layer of the above layers is made up of smooth cells with no intercellular spaces, which indeed seems to constitute a collenchymatic tissue, i.e. pectic, angular and lacunate; the second that followed is irregular in outline, forming parenchyma even chlorophyllic. In green lemons, these cells are rich in chloroplasts that dwindle in yellow lemons. In these layers, we observed that nucleated cells are larger than the other. The cells, positive to various
histochemical tests, seem attributable to the oil-bearing cells differentiating both lemon green and yellow ones. We have also observed glands differentiated with a very pronounced cavity with innates, whose epithelium presents crescentic cells whose wall is pectic and positive to Red Ruthenium (Figure S1.2). The glandular epithelium has also terpenic innates, its cytoplasmic content seems to be poured into the above-mentioned cavity and contains various compounds which react with Sudan III (Figure S1.5). These innates, subjected to lipid tests (Blue Nile, Figure S1.6), were seen exclusively in preparations made by cryo-dissection or cut by hand. The oil glands and, presumably, oil-bearing or glandular cells have been detected in various stages of differentiation (Figures S1.2, S1.7, S1.8). The parenchyma of the glands contains lipid droplets (Figures S1.9, S1.10) attributable to terpenes highlighted by Sudan III, especially in lemons still green (Figure S1.17). The staining with Schiff’s reagent showed the presence of citral in the flavedo cells (Figures S1.3, S1.4). Droplets of citral are also observed in some epidermal cells as already described.

The albedo is made from layers of irregular cells with a few intercellular space, the walls are made of cellulose–pectic, but poor in cellulose, vacuolated with some starch granule positive Lugol’s. Starch is present in green lemon (Figure S1.16) and disappears in the yellow ones. We observed that many bundles of conductors that innervate the albedo are also present between a gland and the other. The bundles, side (Figure S1.12) and concentric (Figure S1.13), have been highlighted by Safranin for the woody portion, constituted by tracheal elements and parenchyma, and by Fast-Green for that cribriform (Figure S1.13).

The tannin test, carried out for both the flavedo and for the albedo, was negative. According to Mauseth (2006), in citrus fruits there are aggregates of oil-bearing cells, embedded in parenchyma, that die at maturity, and as a result, it forms an oil cavity or lysigenous pouch. In Interdonato lemon, we observed many oleaginous aggregates with the presence of droplets testing positive for essential oils (Figure S1.14), but did not notice schizogenous duct as observed by Liang et al. (2006) in Citrus medica L. v. sarcodactylis (probably lemons had already been differentiated).

The anatomical and histological observations revealed peculiarity such as essential oils in flavedo that are very rich in glands with the presence of several components. In fact, tests showed both total lipids (Sudan Black, Figure S1.15) and terpenes. According Bennici and Tani (2004), the sub-epidermal tissue creates the glands that are in the first schizogenous and then lysigenous. It seems plausible that the sub-epidermal strata give rise to the glands, as in examined differentiated lemons, we observed many dividing cells that could give rise to glandular structures, or better, secretory cells rich in oil, a feature that is also known to have complete maturity. Epidermal secreting cells, oil rich, highlighted by us with Schiff’s reagent (Figure S1.11), confirmed the research carried out by Tomasello (Tomasello & Palmieri 1977) on Citrus volkameriana that at maturity showed ultra-structurally epidermal cells very vacuolated. Presumably this meant the glandular characteristic. At maturity, in Interdonato lemon, we found various stages of differentiation of the glands, as observed by Knight et al. (2001), but we have not found epidermal ducts.

The observations made after performing histochemical tests reveal the nature of the cell walls, the location of terpenes, sugars and lipids. The secondary metabolites, including citral and essential oils, which are important for the interaction between the plant and the biotic and abiotic factors, are responsible for the fragrance of the Interdonato lemon. Although the epicarp and the mesocarp are not very thick, they are rich in fragrant oils that characterise them. The fragrance is due to components, such as citral and other volatile substances as
Table 1. Percentage composition of 75 compounds detected in essential oil from Interdonato lemon.

| Compound                        | A   | B   |
|---------------------------------|-----|-----|
| Tricyclene                      | tr  | tr  |
| α-Thujene                       | 0.3 | 0.2 |
| α-Pinene                        | 1.1 | 1.2 |
| Camphene                        | tr  | tr  |
| Sabinene                        | 1.3 | 10.8a |
| β-Pinene                        | 8.1 | 6.1 |
| 6-Methyl-5-hepten-2-one         | 0.1 | tr  |
| Myrcene                         | 1.4 | 1.4 |
| Octanal                         | tr  | tr  |
| α-Phelladene                    | tr  | 0.1 |
| δ-3-Carene                      | 0.1 | tr  |
| α-Terpine                       | 0.2 | 0.1 |
| p-Cimene                        | 0.2 | 0.1 |
| Limomene                        | 72.5 | 70.6 |
| (Z)-β-Ocimene                   | 0.1 | tr  |
| (E)-β-Ocimene                   | 0.2 | 0.1 |
| e-Terpinene                     | 11.0 | 7.0 |
| cis-Sabinene hydrate            | 0.2 | 0.1 |
| Terpinolene                     | 0.4 | 0.3 |
| Linalol                         | 0.1 | 0.1 |
| Octanol                         | tr  | tr  |
| cis-Sabinene hydrate            | 0.1 | 0.1 |
| Nonanal                         | tr  | tr  |
| cis-Limonene oxide              | tr  | tr  |
| trans-Limonene oxide            | 0.1 | tr  |
| Camphor                         | tr  | tr  |
| Citronellal                     | tr  | 0.1 |
| Borneol                         | n.d. | tr  |
| Terpinen-4-ol                   | 0.1 | tr  |
| α-Terpineol                     | tr  | 0.1 |
| Decanal                         | tr  | tr  |
| Octyl acetate                   | tr  | tr  |
| Citronellol                     | tr  | tr  |
| Nerol                           | tr  | tr  |
| Neral                           | 0.2 | 1.3b |
| Carvone                         | tr  | 2.2c |
| Geraniol                        | 0.1 | tr  |
| Piperitone                       | tr  | tr  |
| Geranial                        | 0.2 | tr  |
| Perillaldehyde                  | tr  | tr  |
| Bornyl acetate                  | n.d. | tr  |
| Undecanal                       | 0.1 | tr  |
| Nonyl acetate                   | n.d. | tr  |
| Methyl geranate                 | 0.1 | tr  |
| Citronellyl acetate             | 0.1 | tr  |
| Neryl acetate                   | 0.3 | 0.3 |
| Linalol isobutanoate            | tr  | n.d. |
| Geranyl acetate                 | 0.2 | 0.2 |
| 1-Tetradecene                   | tr  | n.d. |
| Tetradecane                     | 0.1 | n.d. |
| Dodecanal                       | tr  | tr  |
| Sesquijene                      | tr  | n.d. |
| β-Caryophyllene                 | 0.2 | 0.2 |
| trans-α-Bergamottene            | 0.2 | 0.4 |
| cis-β-Farnesene                 | tr  | n.d. |
| α-Humulenel                     | tr  | tr  |
| β-Santalene                     | tr  | tr  |
| Geranyl propanoate              | tr  | n.d. |
| Germacrene D                    | tr  | tr  |
| Valencene                       | tr  | tr  |
| Biciclogermacrene               | 0.1 | tr  |
| β-Bisabolene                    | 0.3 | 0.5 |
terpenes which have pharmaceutical and nutraceutical properties. These ones secreted in droplets, or accumulated in the glands or in the intercellular spaces, cannot be highlighted as the techniques adopted by cutting with a cryostat and/or by hand, followed by staining, prevent its extraction. In fact, the studies carried out on samples embedded in paraffin after treatment with fixatives and dehydrating failed for terpenics innates, which histochemically are very difficult to identify entirely, because they are extremely volatile.

### 2.2. Lemon oil composition

Once the analytic conditions for the separation and detection were optimised, the procedure was used to determine 75 compounds, listed in Table 1. The peaks were identified by comparing the retention time obtained for the standards mixture and the hexane extract of lemon peel spiked with the standards under identical conditions; HRGC–MS data were used for unambiguous detection and to eliminate misidentification. The data here presented are obtained as average values of triplicate analysis. The coefficient of variation (CV%) of the three analyses was always lower than 5%. The compounds were listed according to their retention time on SE–52 column.

A first analysis of our data with those reported by Verzera et al. (2001) for Interdonato lemon reveals that the n-hexane extracted oils have a slightly lower content of esters, carbonyl compounds and alcohols than that of oils isolated by applying manual pressure, due to the different isolation processes. The obtained data show that n-hexane extract of Interdonato lemon peels shared a few common characteristics: the presence of high concentration of limonene, γ-terpinene and β-pinene that are the main compounds detected and this was

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**Table 1. Continued**

| Compound                  | A   | B   |
|---------------------------|-----|-----|
| (Z)-α-Bisabolene          | 0.1 | tr  |
| (E)-α-Bisabolene          | tr  | tr  |
| trans-Sesquisabine hydrate| tr  | n.d.|
| 2-Norbornarolo            | tr  | n.d.|
| Tetradecanol              | tr  | tr  |
| Camphor                   | tr  | tr  |
| β-Bisabol                 | tr  | n.d.|
| Nootkatone                | tr  | tr  |
| Total                     | 99.9| 99.7|

Note: n.d., not detected; tr, trace. A = Interdonato (our results regarding this study) (mass fraction rate); B = Interdonato (Verzera et al. 2001) (mass fraction rate).

*In Verzera et al. (2001) sabinene and β-pinene were not separated.

*In Verzera et al. (2001) neral and carvone were not separated.

*In Verzera et al. (2001) geranial and perillaldehyde were not separated.

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**Table 2. Occurrence and comparison of particular essential oil compounds in different types of lemons reported in the literature.**

| Reference                  | Oils isolation | mg/100 g of peel | α-Pinene | β-Pinene | Myrcene | Limonene | γ-Terpinene |
|----------------------------|----------------|------------------|----------|----------|---------|----------|-------------|
| Our results                | With n-hexane  | Interdonato lemon| 118.9    | 856.2    | 143.3   | 7252.5   | 1110.6      |
| Verzera et al. (2001)      | Manual pressure| Interdonato lemon| 141.1    | 844      | 154     | 7020     | 961         |
| Di Vaio et al. (2010)      | With EtOH      | Sfusato Amalfitano| 24.42    | 254.47   | 19.17   | 1426.38  | 176.62      |

Note: n.d., not detected; tr, trace. a = Interdonato (our results regarding this study) (mass fraction rate); B = Interdonato (Verzera et al. 2001) (mass fraction rate).

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*In Verzera et al. (2001) geranial and perillaldehyde were not separated.
in accordance with other studies on the chemical composition of Interdonato lemon essential oil (Verzera et al. 2001). As already observed by Verzera et al. (2001), among the other compounds identified, there is a prevalence of myrcene and α-pinene, while the amount of geranial and perillaldehyde is rather low in our extracted oil. Lower levels were detected also for neryl acetate, geranyl acetate, \(\text{trans-}\alpha\)-bergamottene, \(\beta\)-bisabolene and citronellal. Although the other compounds detected in \(\text{n}\)-hexane extract of peel from Interdonato lemon are numerous, their amounts are not particularly high; the calculated values are shown in Table 1. With regard to the content of the identified compounds, the data presented here were in agreement with those previously reported by Verzera et al. (2001) for Interdonato lemon.

Particular attention deserves instead the sabinene amount, in fact Verzera et al. (2001) determine it together \(\beta\)-pinene that, generally, results among the monoterpene hydrocarbons quantitatively dominant, also in other lemon varieties. Comparing our results with those of others, it was found that sabinene amount of Interdonato lemon is slightly higher than Femminello Santa Teresa and Femminello Continella lemons (Settanni et al. 2014).

It is not easy to rationalise our results, especially inserting more comparative data with respect to volatile profiles of lemon of different geographic origin, because the chemical composition can be influenced not only by genotype, geographic origin and extraction methods but also by harvest time, cultural techniques and ecological conditions (Settanni et al. 2014). Moreover, the determination not always involved the same analytes. However, the comparison of the chemical composition of some common analytes that not resulted remarkably quantitatively different for collection time evidenced that many oxygenated compounds, as before observed by Verzera et al. (2001), and other compounds, such as linalol, \(\alpha\)-terpineol, terpinen-4-ol and geraniol, reached a minimum value in Interdonato samples in comparison with Femminello Santa Teresa and Monachello varieties (Settanni et al. 2014).

In order to evaluate different extraction capacities of processes that extract oils without heating, we compared our data with those reported in the literature for a manual and an ethanolic extract (Verzera et al. 2001; Di Vaio et al. 2010), given that different isolation processes and solvents have a different extraction capacity. The results of this comparison show that manual isolated oils have a slightly higher content of the compounds listed in Table 2, while the same chemicals are extracted better in \(\text{n}\)-hexane than in ethanol. These differences cannot be attributable to the cultivar since \(\alpha\)-pinene, \(\beta\)-pinene, \(\gamma\)-terpinene, myrcene and limonene are among the chemicals quantitatively dominant in all essential oils. Rather, it should be underlined that \(\text{n}\)-hexane shows an increased extracting capacity in respect of these specific compounds, if compared to EtOH.

3. Materials and methods

3.1. Geological framework of the areas

The Interdonato lemon is a citrus fruit cultivated in the Sicilian Ionian slope stretched from Messina to Casalvecchio Siculo. The studied lemon plantation (owner: Attilio Interdonato) is located at about 30 m a.s.l. (geographic coordinates: 38°00’03.45” N–15°24’37.29” E) in the Ali Terme territory, a village situated 25 km SW of Messina (Figure S2). Under a pedological and geological point of view, this plantation grows on an alluvial soil
developed on Holocene deposits. These latter are related to the hydrographic basin of the Fiumedinisi stream, one of the most important streams of the Ionian side of the Peloritani Alpine chain. This hydrographic basin, characterised by alluvial plains and terraces, developed on a territory characterised by extensive outcrops of Palaeozoic high-grade metamorphic rocks (Mela Unit) exposed at the most elevated altitudes, and of Palaeozoic low-grade metamorphic rocks (Mandanici-Piraino Unit,) situated at lower height. The alluvial soil of the studied plants is a dusky yellowish brown (10YR 2/2) silty loam with a pH of 7.3. The topsoil consists of gravels with sands or silty sands deriving from alluvial sediments. Gravels are mainly formed by rounded clasts of grey phyllites of the Mandanici-Piraino Unit. The sandy or silty sandy matrix is composed of silicate minerals, such as quartz, muscovite, chlorite and albite, deriving from weathering of metamorphic rocks present in the Fiumedinisi hydrographic basin.

3.2. Samples and extraction process

*Interdonato* lemon samples were obtained from fruits harvested in February 2015 and analysed at the same ripening stage. The fruits were picked in February since the Italian winter lemon fruits give the most valuable oils (Verzera et al. 2001).

Fresh samples of *Interdonato* lemon were obtained from four local different growers belonging to the ‘Consorzio di tutela del limone Interdonato di Sicilia IGP’. All the fruits were collected in the same experimental field in order to avoid possible differentiation.

The extraction of the oil was carried out as follows: 50 g of fresh skins were reduced to a homogeneous size and extracted three times with 150 mL of n-hexane. Each extraction was carried out in a screw-cap flask with magnetic stirring in the dark at 4 °C for 3 h. Anhydrous sodium sulphate was added to the final extracts in order to remove the water able to be extracted using hexane as extractive solvent. Then, the combined solvent was filtered and removed under vacuum at room temperature; the oil obtained was flushed with a stream of dry nitrogen to volatilise the solvent residues and then was weighed. The extraction procedure was repeated twice for each sample. The qualitative and quantitative volatile fraction composition was studied by HRGC and HRGC/MS.

3.3. HRGC analysis

The HRGC analysis of the hexane extract of lemon peels was performed on a Gas Chromatograph 5160 Mega series (Fisons Instrument, Milan, Italy) equipped with a Shimadzu data processor C-R3A. The determination was executed according to a method reported in a previous study by Verzera et al. (2001) using the following experimental conditions: SE-52 fused silica capillary column, 30 m × 0.32 mm; 0.40–0.45 μm, film thickness (Mega, Legnano, MI, Italy); column temperature, 45 °C (6 min) to 250 °C at 3 °C/min; injection mode, split; split ratio, 1:100; detector, FID; injector and detector temperature, 250 °C; carrier gas, He 100 kPa; injected volume, 1 μL of net whole oil. The quantitative data for each compound were calculated with the external calibration method. For all components, a calibration graph was obtained using linear regression of the least squares method and the peak response of each standard injection plotted against standard concentrations. The quantitative composition for each component was calculated by measuring peak areas at corresponding retention time and by comparing them with a calibration curve.
3.4. HRGC–MS analysis

HRGC/MS (EI) analysis of the samples was carried out on a Fisons MD 800 (Fisons Instrument, Milan, Italy) system coupled with Adams’ library (Adams 1995) as previously described (Verzera et al. 2001). The column used was Megawax fused silica capillary column, 30 m x 0.32 mm; 0.40–0.45 μm, film thickness (Mega, Legnano (MI), Italy); carrier gas, He 90 kPa; linear velocity, 34.7 cm/s at 40°C; column temperature, 40°C (6 min) to 220°C at 2°C/min. For both columns: injector temperature, 250°C; injection mode, split; split ratio, 1:30; volume injected, 1 μL of a solution 1:100 in pentane of the oil; interface temperature, 250°C; acquisition mass range, 41–300 amu; solvent cut, 2 min. Linear retention indices of the sample components were determined on the basis of homologue n-alkane hydrocarbons analysed under the same GC conditions. The compound identification was confirmed by comparison of mass spectra of compounds with published spectra and of retention indices with published index data (Verzera et al. 2001).

3.5. Histochemical experiments

The research was carried out on mature Interdonato lemons approximately 8–12 cm in diameter. Some portions of flavedo and albedo were fixed in Immunofix 2% in phosphate buffer 0.05 M pH 6.9, dehydrated in alcohol and finally embedded in paraffin and dissected with a microtome-type Minot (Leitz); other samples were dissected with a cryostat 1720 Digital Leitz (Lauda), or manually. All samples thus prepared were subjected to various histochemical tests (toluidine blue, Sudan III, Sudan IV, Sudan Black B, Safranin and Fast-Green, phloroglucinol and HCl, Lugol and ferric chloride, Blue Nile, Red Ruthenium) (Gahan 1984), Nadi’s Reagent (David & Carde 1964), Schiff’s Reagent (Lewinsohn et al. 1998). The observations were made using an optical microscope Leica DMLS 52 provided with ICCA telecamera connected to the PC.

4. Conclusion

The histological and histochemical studies indicate the presence of both secretory cells and of glands with abundant essential oils. We found sinuous epidermis, with outer ring road cutinised, sometimes with secretory cells; tissues are collenchymatic and parenchymal with pectic cell walls with chloroplasts in green lemons; presence of oil-bearing cells rich of lipid; oleiferous aggregates and glands in various stages of differentiation; glandular epithelium with innates positive to terpenes tests; glandular lumen rich in terpenes; presence of terpenes in most cells of the epicarp and the mesocarp and in the intercellular spaces. Finally, we found the presence of bundles of conductors and glandular pouch with essential oils.

The data obtained for the n-hexane extract of lemon peel showed that sabinene amount of Interdonato lemon is slightly higher than Femminello Santa Teresa and Femminello Continella lemons. Moreover, the comparison of the chemical composition of some analytes that not resulted remarkably quantitatively different for collection time evidenced that many oxygenated compounds, and particularly linalol, α-terpineol, terpinen-4-ol and geraniol reached a minimum value in Interdonato samples in comparison with Femminello Santa Teresa and Monachello varieties.
Disclosure statement
No potential conflict of interest was reported by the authors.

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