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XXIV CICLO

Monica Scordino

FOOD CONTROL: QUALITY ASSURANCE AND PROTECTION AGAINST ADULTERATION TECHNIQUES

PhD Thesis

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To my beautiful family

(Alla mia “famiglia bella”)

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LIST OF PAPERS

This thesis is based on the following papers, referred to by the following numbers in the text:

1) Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano. **Preliminary study on bioactive compounds of *Citrus x myrtifolia Rafinesque* (Chinotto) to its potential application in food industry.** Food and Nutrition Sciences, 2011, 2 (7), 685-691.

2) Monica Scordino, Leonardo Sabatino, Antonio Muratore, Adalgisa Belligno, Giacomo Gagliano. **Molecole bioattive ed attività antiossidante in *Citrus x myrtifolia Raf.* (Chinotto).** Ingredienti alimentari, Chiriotti ed., *in press*

3) Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano. **Flavonoids and furocoumarins distribution of unripe chinotto (*Citrus x myrtifolia Rafinesque*) fruit: beverage processing homogenate and juice characterization.** European Food Research and Technology, 2011, 233 (5), 759-767.

4) Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano. **Characterization of polyphenolic compounds in unripe chinotto (*Citrus myrtifolia*) fruit by HPLC/PDA/ESI/MS-MS.** Natural Product Communications, 2011, 6 (11).

5) Leonardo Sabatino, Monica Scordino, Rosario Caruso, Elena Chiappara, Pasqualino Traulo, Adalgisa Belligno, Giacomo Gagliano. **LC/MS^n detection of short-chain aliphatic amines in glazing agents for fruit coating**. *Under review.*

6) Leonardo Sabatino, Monica Scordino, Maria Gargano, Adalgisa Belligno, Pasqualino Traulo, Giacomo Gagliano. **HPLC/PDA/ESI-MS evaluation of saffron (*Crocus sativus*) adulteration.** Natural Product Communications, 2011, 6 (11).

7) Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano. **Phenolic characterization of Sicilian yellow-flesh peach (*Prunus persica L.*) cultivars at different ripening stage**. *Under review.*

8) Muratore Antonio, Mazzaglia Agata, Monica Scordino, Leonardo Sabatino, Lanza Carmela Maria, Adalgisa Belligno, Giacomo Gagliano. **Caratterizzazione fisico-chimica e valutazione sensoriale di pesche di Leonforte (*Persica Vulgaris cv tardiva di Leonforte*) a maturazione commerciale e fisiologica.** Ingredienti alimentari, Chiriotti ed., *in press*.

9) Monica Scordino, Leonardo Sabatino, Rosario Caruso, Adalgisa Belligno, Giacomo Gagliano. **Caratterizzazione di ammine biogene dansil derivate mediante HPLC-UV-ESI/MS.** *In progress to be submitted.*

INTRODUCTION AND AIM OF THE PHD WORK

Effective food control systems are essential to protect the health of consumers. They are also vital in enabling countries to assure safety and quality of food products for international trade and to verify that imported food products meet national requirements. The legal framework for food safety in the European Union Member States is currently in process. The European Union has established a specific legislation to harmonize official control procedures in the Member States. It also regulates the provisions that countries outside the EU should fulfil for entering the EU markets with their food products. Although control procedures are largely harmonized, the structure and set-up of control organizations is quite different across the European Union. The variety in national political and economical conditions have led to differences in the way Community legislation was transposed in national law and in operational systems for food production and inspection. Therefore, there is a large variety of food safety control systems throughout the Member States. In certain countries the responsibility of food control is decentralized and mandated to regions or provinces, whereas in other countries food safety control is in the hands of just one central organization. In recent years many countries in the European Union have established a National Food Safety Authority.

1. FOOD CONTROL IN THE EUROPEAN UNION

1.1 THE EU WHITE PAPER ON FOOD AND FEED: PRINCIPLES OF FOOD CONTROL

White papers are documents containing proposals for Community action in a specific area. They sometimes follow a green paper published to launch a consultation process at European level. While green papers set out a range of ideas presented for public discussion and debate, white papers contain an official set of proposals in specific policy areas and are used as vehicles for their development.

Assuring that the EU has the highest standards of food safety is a key policy priority for the European Union. The White Paper on Food Safety reflects this priority. A radical new approach has been proposed. This process is driven by the need to guarantee a high level of food safety. Greater transparency at all levels of food safety policy is the thread running through the whole White Paper and will contribute fundamentally to enhancing consumer confidence in EU Food Safety policy.

The writing of a white paper on food safety was mainly driven by events and developments during the 1990s. The well-known dioxin and BSE crises have led to a whole new approach of risk management. The risks associated with the contamination of foods were brought into sharp focus by the dioxin crisis. Steps will be taken to address those areas where existing legislation needs to be improved to provide adequate protection. But also developments in the rulemaking process have led to new ways of dealing

with food safety control. The experience of the Commission's own Food and Veterinary Office, which visits Member States on a regular basis, has shown that there are wide variations in the manner in which Community legislation is being implemented and enforced. This means that consumers cannot be sure of receiving the same level of protection across the Community, which makes it difficult to evaluate the effectiveness of national measures.

The White Paper proposes that, as a general principle, all parts of the food production chain must be subject to official controls. Responsibility for safe food production and control is shared between operators, national authorities and the European Commission.Operators are responsible for compliance with legislative provisions, and for minimizing risk on their own initiative. National authorities are responsible for ensuring that operators respect food safety standards. They need to establish control systems to make sure that Community rules are being respected and, if required, enforced. To ensure that these control systems are effective, the Commission, through the Food and Veterinary Office (FVO), carries out a programme of audits and inspections. These controls evaluate the performance of national authorities against their ability to deliver and operate effective control systems, and are supported by visits to individual premises to verify that acceptable standards are actually being met.

One of the action points in the White paper is to develop a regulation on official food and feed safety controls.The Regulation 882/2004/EC (of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules) was published. This Regulation sets a Community framework of national control systems,

which will improve the quality of controls at Community level, and consequently raise food safety levels across the European Union. The operation of such control systems remains a responsibility of the individual Member States.

1.2 THE EU GENERAL FOOD LAW, THE LEGAL BASIS OF EUROPEAN UNION FOOD CONTROL

An important need mentioned in the White Paper was to create a coherent and transparent set of food safety rules. With the publication of the General Food Law (GFL), the European Union has made a new legal framework laying down the principles to ensure a coherent approach and to fix the principles, obligations and definitions that apply in the field of food safety. It defines the common principles underlying food legislation and the establishment of a food safety policy as a primary objective of EU food law. This Regulation also provides the general frame for those areas not covered by specific harmonized rules but where the functioning of the Internal Market is ensured by mutual recognition. Under this principle, in the absence of Community harmonization, Member States may only restrict the placing on the market of products lawfully marketed in another Member State when and to the extent that this can be justified by a legitimate interest such as the protection of public health and only when the measures taken are proportionate.

The General Food Law consists of three parts. The first part lays down the general principles and requirements of food legislation, the second part defines the establishment of the European Food Safety Authority and the

last part lays down procedures in matters of food safety. Let us focus on the first part.

A general principle of food law is that operators in the feed and food business have the primary responsibility for food safety. Competent authorities monitor, enforce and verify this responsibility through the operation of national surveillance and control systems at all stages of production, processing and distribution. Member States are also obliged to lay down rules on measures and penalties applicable to infringements of food and feed law. They shall be effective, proportionate and dissuasive. The Commission concentrates on evaluating the ability of competent authorities to deliver these systems through audits and inspections at the national level.

A successful food policy demands the traceability of feed and food and their ingredients. This is an important requirement of the GFL. It includes the obligation for feed and food businesses to ensure that adequate procedures are in place to recall products that might pose a possible health risk. Operators should also keep adequate records of suppliers of raw materials and ingredients so that the source of a problem can be identified.

1.3 National implementation of the General Food Law

Regulations and directives in the framework of the General Food Law have to be transposed into national legislation of individual EU Member States regarding enforcement, sanctioning and the designation of the competent authority. Regulations are imposed directly on countries and need no further interpretation, while directives may be implemented according to national policies. For instance, in the national implementation of the General Food Law the penalties must be laid down to be applied if an

operator does not have an adequate traceability system and the competent authorities for inspections and controls.
National implementation of EU law must fit into national structures, such as centralized and decentralized control structures. Therefore most EU food safety legislation focuses on criteria and procedures rather than on detailed regulations for control.
In addition to the legal implementation of EU legislation it is also necessary to put a national performance policy in place and to explain the meaning of this policy to the public. This process may give rise to questions that must be checked with other EU Member States and the European Commission.
In recent years many countries in the European Union have chosen to establish a National Food Safety Authority to contribute to higher food safety standards and more effective food safety control. These authorities comply with the requirements of the General Food Law but their establishment has never been obligatory. The responsibilities and tasks of these organizations may be quite different per Member State. In some countries their mandate is limited to risk assessment and scientific advice to the Government. In other cases their mandate includes risk communication and enforcement of food control regulations. Risk management tasks are usually kept in the hands of the responsible ministries.

1.4 FOOD SAFETY CONTROL POLICIES IN THE EU AND ITS MEMBER STATES

1.4.1 GENERAL FRAMEWORK AND COVERAGE

Feed and food should be safe and wholesome. European Community legislation comprises a set of rules to ensure that this objective is attained.

These rules on hygiene and safety extend to the production, processing and the introduction of food products on consumer markets.
The basic rules with regard to feed and food law are laid down in Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002, often referred to as the "General Food Law". This law lays down the general principles and requirements of food legislation, the establishment the European Food Safety Authority, and procedures in matters of food safety.
In addition to these basic rules, more specific feed and food laws cover different areas such as animal nutrition including medicated feeding stuffs, feed and food hygiene, zoonoses, animal by-products, residues and contaminants, control and eradication of animal diseases with a public health impact, feed and food labelling, pesticides, feed and food additives, vitamins, mineral salts, trace elements and other additives, materials in contact with food, quality and compositional requirements, drinking water, ionization, novel foods or genetically modified organisms (GMOs).

1.4.2 FOOD SAFETY RESPONSIBILITIES

Community feed and food law is based on the principle that feed and food business operators at all stages of production, processing and distribution within the businesses under their control are responsible for ensuring that products and manufacturing processes satisfy the requirements of feed and food law which are relevant to their activities.
This principle of placing the main responsibility on producers can only work adequately, when effective and efficient government controls are in place. As a consequence all relevant information on the application of process controls, essential for safe food production, should be fully

available for government control purposes. The same applies to information that is of importance for proper traceability.

The EU-Members enforce feed and food law and monitor and verify that the relevant requirements of law are fulfilled by business operators at all stages of production, processing and distribution. Official controls should be organized for that purpose.

The organization of the official controls differs to a large extent throughout the European Union, as a result of different historical backgrounds and traditions. The differences vary from a completely centralized system (the Netherlands, Denmark, Belgium) to decentralized systems where the competent authorities are working according to a regional (Spain, Germany) or local system (United Kingdom, Ireland).

1.4.3 HARMONIZATION AND COHERENCE

To achieve equivalent official feed and food control systems across Member States, the European Commission has chosen to establish a harmonized framework of general rules at Community level for carrying out such controls. For this reason Regulation No. 882/2004/EC on the official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules and Regulation No. 854/2004/EC laying down specific rules for the organization of official controls on products of animal origin intended for human consumption have recently been adopted.

1.4.4 NATIONAL IMPLEMENTATION AND COMMUNITY VERIFICATION

In order to have a global and uniform approach with regard to official feed and food controls, EU Member States must establish and implement

national control plans in accordance with broad guidelines elaborated at Community level. These guidelines should promote coherent national strategies, and identify risk-based priorities and the most effective control procedures. After developing these guidelines a Community strategy for a comprehensive, integrated approach to the operation of controls can be reached. Moreover, each EU-Member State has to present an annual report to the European Commission covering information on the implementation of the national control plans. This report is meant to provide:

•the results of the official controls and audits carried out during the previous year and,

•where necessary, an update of the initial control plan in response to these results.

The national control plans and the yearly reports will establish a solid basis for the European Commission Food and Veterinary Office to carry out controls in the EU Member States. The control plans will enable the Food and Veterinary Office to verify whether the official controls in the EU Member State are organized in conformity with the criteria laid down in these Regulations. If appropriate and in particular if the audit of a EU Member State against the national control plans shows weaknesses or non-compliances, detailed inspections and audits will be carried out.

Eventually Community controls in the EU-Member should allow the Food and Veterinary Office to verify whether feed and food law, and the legislation on animal health and animal welfare are implemented in a uniform and correct way throughout the European Union.

1.4.5 IMPORTED GOODS

Regulation 882/2004/EC gives rules for the official control on the import of products from third countries. For this reason, Community controls in third

countries are required in order to verify compliance or equivalence with Community feed and food law. Third countries may also be requested to establish control plans similar to those intended for Member States, in respect of the feed and food exported by them. These plans, which must be established on the basis of Community guidelines, should form the basis for subsequent Commission controls, which should be carried out within a multidisciplinary framework covering the main sectors exporting to the Member States. This development should allow a simplification of the current control regime, enhancing effective control co-operation, and consequently facilitating trade flows.

To help developing countries in setting up official feed and food control systems equivalent to the control systems in the European Union, it is appropriate to identify and consider the special needs of those countries. The EU is committed under Regulation 882/2004 to support developing countries with regard to feed and food safety, which is an important element of human health and trade development.

Besides this Regulation, there is a specific Regulation No. 854/2004/EC for the organization of official controls on products of animal origin intended for human consumption. The purpose for a specific official control procedure for these products is that specific sanitary rules must be complied with here (Regulation No. 853/2004/EC).

The Member States are aware of the need to harmonize control procedures of imported goods across the European Union. There is a need for setting detection limits of certain residues in food products in order to facilitate uniformity of procedures and sanctioning at all European border posts.

1.5 THE EUROPEAN FOOD SAFETY AUTHORITY

Following a series of food safety crises in the 1990s (e.g. BSE, dioxins) which undermined consumer confidence, the European Union concluded that it needed to establish a new scientific body charged with providing independent and objective advice on food safety issues associated with the food chain. Its primary objective as set out in the White Paper on Food Safety would be to: "... contribute to a high level of consumer health protection in the area of food safety, through which consumer confidence can be restored and maintained". After the publication of the General Food Law this resulted in the establishment of the European Food Safety Authority (EFSA).

Set up provisionally in Brussels in 2002, EFSA provides independent scientific advice on all matters linked to food and feed safety - including animal health and welfare and plant protection - and provides scientific advice on nutrition in relation to Community legislation. The Authority communicates to the public in an open and transparent way on all matters within its remit. EFSAs risk assessments provide risk managers (consisting of EU institutions with political accountability, i.e. European Commission, European Parliament and Council) with a sound scientific basis for defining policy-driven legislative or regulatory measures required to ensure a high level of consumer protection with regards to food safety.

EFSA was born from Parliament and Council regulation (EC) No 178/2002 of 28 January 2002. The new Authority quickly found its feet, organizing its first Management Board meeting nine months later. Shortly afterwards it nominated its first Executive Director and created the Advisory Forum, made up of representatives from food safety bodies in the EU Member States. Momentum grew in 2003 as EFSA developed into a fully-fledged

independent European Union agency. EFSA truly opened for business in May with the establishment of its Scientific Committee and Panels. World-class scientists from all over Europe were appointed to eight Panels, covering everything from food additives to animal health, and to a Scientific Committee with oversight of these panels.

2. FOOD SAFETY CONTROL IN ITALY

Matters pertaining to food safety and quality involve several Ministries and the current system is the product of a recent reorganization. The reform of the 2001 Constitution, in particular article 117 (Constitutional Law No 3, 18 October 2001), introduced concurrent legislative powers for both the central and regional governments. So, human and animal health in general and food and feed safety, in particular, have become matters of shared responsibility at the national and regional levels, while remaining the competence of the State in international relations and international maritime, aerial and border prophylaxis, including veterinary matters, as well as controlling the importation of products destined for nutrition. The regions are gradually becoming the main channel through which EU directives and regulations are implemented in the country. However, structural differences between regions and the political choices of the local governments cause some difficulties when receiving and implementing EU legislation, resulting in a varied impact throughout the country. While awaiting new national European-based regulations, national laws still remain in force if they do not contradict new European laws.

2.1 THE MINISTRIES

At least four Ministries in Italy, are related to feed and food safety controls:

- Ministry of Health, in particular the Veterinary Public Health, Nutrition and Food Safety Department and the associated bodies at central level: Experimental Zoo Prophylaxis Institutes (IZS), the Border Inspection Posts (PIF), the Veterinary Offices for Compliance with Community Requirements (UVAC), the Port, Airport and Border Health Offices (USMAF), the Military Police (Carabinieri) Headquarters for Public health, the National Health Institute (ISS); at local level: the Local Health Units (A.U.S.L.) with Food Hygiene and Nutrition Service (S.I.A.N.) and Local Veterinary Service (S.V.L.);
- Ministry of Agriculture Foodstuffs and Forestry Policies with the Central Inspectorate for Quality Control of Agricultural and Food Products, the State Forestry Corps, the Agricultural Policies Command of the Carabinieri;
- Ministry of the Economy and Finances with the Customs Agency;
- Ministry for the Environment and Protection of the Territory and Sea with the Regional Agencies for Environmental Protection.

2.2 MINISTRY OF AGRICULTURE FOODSTUFF AND FORESTRY POLICIES

The Ministry of Agriculture and Forestry, established in 1946, subject to organizational reforms several times, most recently by Presidential Decree No 129 of 22/7/2009, develops and coordinates the lines of agriculture, forestry, food and fisheries at the national, European and international level.

The MIPAAF consists of: the Department of European and International Policy (DIPE), The Department of competitive policies and the quality of the rural world (DICOR), the Central Inspectorate Department of Protection and Prevention of Fraud quality of food products (ICQRF), the State Forestry Department (CFS), the National Council of Agriculture (CNA), the Carabinieri Agriculture and Food command, Department of Marine Fishery and Port Authority.
The General Directorate for Agricultural and Food Development, Quality and Consumer Protection is involved in activities concerning products with a protected geographical indication (IGP), protected designation of origin (DOP) (Regulation 2006/510/EC) and with the recognition of guaranteed traditional specialities (STG) (Regulation 2006/509/EC). In general it is responsible for applying Law No 164, 10 February 1992, which concerns the designation of wine origin and geographic indications; organic agriculture and the certification of environmentally friendly agriculture; the application of the Codex Alimentarius/Food Standards.

2.3 MINISTRY THE CENTRAL INSPECTORATE DEPARTMENT OF PROTECTION AND PREVENTION OF FRAUD QUALITY OF FOOD PRODUCTS

Its statutes follow those of the Central Inspectorate for Fraud Suppression, with Law No 462, 7 August 1986. It is the technical arm of the state for preventing and suppressing fraud in the preparation and commerce of agricultural and food products, and in agricultural and forestry consumables. It reports directly to the Minister (Legal Decree No 1, 11 January 2001, converted into Law No 49, 9 March 2001) and operates with

complete autonomy. Its operating structure was reorganised for the first time by Ministerial Decree No 44, 13 February 2003, subsequently by Law No 231, 11 November 2005 when it assumed a departmental structure with two general directorates (both located in Rome) and recently by Presidential Decree No 18, 9 January 2008. As a consequence of the 2007 Italian Finance Law (Law No 296, 27 December 2006 subs 1047), the Central Inspectorate for Fraud Suppression changed its name to the Central Inspectorate for the Quality Control of Agricultural and Food Products (ICQ). It is in charge of the accreditation and surveillance of public and private bodies involved in certification of DOP, IGT and STG products. The Presidential Decree July 22, 2009, No 129, which target the "Regulations for the reorganization of the Ministry of Agriculture and Forestry", has planned for The Central Inspectorate for Quality Control of Agricultural and Food Products, the name of “Central Inspectorate Department of Protection and Prevention of Fraud quality of food products” and the acronym “ICQRF”, and determines its new competence in the field of prevention and repression of fraud in the preparation and trade of food products and substances used for agriculture and forestry, quality products for the supervision of registered descended from European and national regulation.

The ICQRF oversees the labelling and market presence, DOP, IGP and STG products, winemaking, olive oil, dairy products, organic food, cereal derivatives (pasta, flour, durum wheat flour, pastry products), honey, vegetable preserves, eggs, seeds, animal feed, fertilisers and bio-stimulants, phytosanitary products. New norms have extended their competencies (Law No 231/2005) to include checking for irregular trading of agricultural and food products from EU extra-EU countries.

There are twelve managing inspection offices throughout Italy, with seventeen local units. There is also a network of five chemical laboratories for analysing the samples collected by local inspection offices. A central laboratory for second level analyses is located in Rome and its mission is to assess the quality of chemical analysis, and improve methods.

3. AIM OF THE WORK

The quality assessment of agro-food typical of the member states is one of the primary objectives of economic policy and agriculture in the EU. The check on overall quality, according to the specifications and typical products, however, is subject to information gaps that are subject to continuous development of methods of analysis. This has resulted in multiple attempts at adulteration and counterfeiting, which allowed the use of poor quality products and / or different geographical origin and / or non-typical tree species, with consequences for consumers, local producers on "honest" on the preservation of local ecotypes. In this context, this research is a meaningful goal to promote local quality products and protection from the illegal adulteration techniques, providing analytical parameter control from the local reference for the consumer protection.

This PhD research was conducted almost entirely in agreement with the laboratory of Catania of ICQRF, considering its growing interest in organic production controls, as well as typical products of denomination of protected and controlled origin, determined by the new supervisory functions assigned to it by the rules mentioned above. In this context, this research has the meaningful objective of health-promoting typical quality

products and protection against illegal adulteration techniques, providing analytical control parameter for the protection of the consumer.

The work has developed on several fronts, deepening different research topics as individual cases of study:

- Characterization of Sicilian peach cultivars with different ripening stages.
- Evaluation of bioactive compounds of chinotto extract used in the preparation of commercial soft drinks.
- Development of an analytical method for evaluating the adulteration of saffron with the less valuable spices.
- Detection of illegal use of short-chain aliphatic amines in the wax coating for fruit for consumption.

The analytical results of this PhD thesis have been the subject of communications from different conferences have produced many scientific papers and are currently applied at the ICQRF Laboratory of Catania in food official controls.

Paper No 1

Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano.

Preliminary study on bioactive compounds of *Citrus x myrtifolia Rafinesque* (Chinotto) to its potential application in food industry.

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Preliminary study on bioactive compounds of *Citrus x myrtifolia Rafinesque* (Chinotto) to its potential application in food industry

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ABSTRACT

The present study investigated for the first time some physical quality attributes of unripe Citrus × myrtifolia Rafinesque which is the ingredient of the popular soft drink Chinotto. Samples for analysis were processed to better reproduce the crude materials used for industrial chinotto extract, discarding part of the juice. Fruit bioactive compounds such as ascorbic acid, carotenoids, chlorophylls, flavonoids and antioxidant capacity were estimated. An important nutritional aspect arose from the data presented was the high concentration of flavonoids (780 mg/100g FW) and vitamin C (42 mg/100g FW). A good antioxidant capacity (5872 µM Trolox equivalents/100g FW) was estimated by oxygen radical absorbing capacity (ORAC). This matrix could be considered as a good nutraceutical source, giving new opportunity to citrus industry.

***Keywords*:** *ascorbic acid, antioxidants, chinotto, citrus, flavonoids, ORAC.*

1. Introduction

The *Citrus × myrtifolia Raf.* is a citrus fruit of the genus Citrus. Native of southern China, its origin has not been exactly ascertained; probably it is mutation of sour orange that eventually evolved into the species known today [1]. The unripe fruits look like small green aromatic tangerines, while mature fruits are bigger and orange painted. The flesh is bitter and sour and divided into 8-10 segments. The plant was cultivated for centuries in France and Italy [1], especially in Liguria, Calabria and Sicily where the fruits are used in sweets for candies and jams as well as flavoring syrups, soft drinks and spirits. In Italy the plant has given its name to a very popular Italian drink Chinotto, flavored with *Citrus × myrtifolia* extract. The chinotto is set up as a classic soda, but no producer has been revealed details about its preparation. Its ingredients are regulated at the legislative level by a Decree of President of the Republic (DPR n.719 of 1958 May 19, Article 5 and subsequently amends) which specifies that the soft drinks sold under the name of a not juice fruit, including cedar and chinotto, should be prepared with substances derived from the fruit or the plant. The *Citrus × myrtifolia* extract for chinotto drink is a aqueous-alcoholic solution made up from infusion of softly pressed unripe fruits with partially discarding of the juice; it was then flavored with spices such as rhubarb, gentian, cinchona, cinnamon, cloves, sweet orange, thyme, tamarind. The first Chinotto soda was produced in 1932 by San Pellegrino®.

Despite the growing distribution of this popular soft drink, the few literature papers about the *Citrus × myrtifolia* [2, 3] are related only to the juice and no informations are available about bioactive compounds in the whole fruit. The have of interest in the Italian citrus industry for chinotto production could be increased by studies on its composition.

The possible beneficial effects of fruit consumption are due to micronutrients such as vitamins and to functional food ingredients and antioxidant nutraceuticals (phytochemicals) [4]. Phytochemicals can be defined as substances found in edible fruits and vegetables that, daily ingested, may exhibit a potential for modulating human metabolism to the prevention of chronic and degenerative diseases. An increased consumption of fruit and vegetables, typical Mediterranean diet foods, may protect against degenerative pathologies, such as cancer and atherosclerosis [5]. Over the past decades, a large number of studies have been carried out with the aim of identifying the bioactive components present in citrus fruits, in an attempt to gain a deeper understanding of the correlation between diet, health benefits and reduced risk of diseases [6-8]. Among the phytochemicals, flavonoids are widely contained in Citrus fruits, especially in Sicilian cultivars, but are also good source of other bioactive components such as carotenoids and vitamin C [9].

The aim of the present work was to characterize for the first time unripe *Citrus × myrtifolia* raw materials used for beverage industrial purposes. The main bioactive compounds such as carotenoids, ascorbic acid, flavonoids and chlorophylls were quantified. Furthermore, the radical scavenging activity was screened using ORAC method.

2. Materials and methods

2.1. Fruits

Fruits used in this study were grown in Castiglione di Sicilia (Catania, Italy). Immature *Citrus x myrtifolia Raf.* (chinotto) fruits were collected with the degree of fruit maturity determined from the surface color, mean diameter, total soluble solids (TSS), titratable acidity (TA) and pH. Unripe fruits picked on 2010 October were green, had a mean diameter of 2.5 (±0.3) cm and a mean weight of 27g (±2). The peel and the pulp represented the 39% and the 55% of the fresh weigh (FW) respectively, while the remaining weigh were constituted by seeds. The TSS were 8.4 Brix, TA of 0.83% citric acid and pH 3.87; the evaluated ratio (TSS/TA) for maturity requirements was of 10.1. Samples for analysis were prepared from 25 fruits and processed to better reproduce the crude materials used for industrial chinotto extract; the fruits were slightly squeezed to discard part of the juice and reduced in small pieces with Turbo Homogenizer HMHF (PBI International). The

samples were stored at -20 °C until needed for the study.

2.2. Extraction and analysis of flavonoids

Two grams of sample was extracted for 2 h with 10 mL of 80% methanol containing 1% hydrochloric acid at room temperature on an orbital shaker. The mixture was centrifuged at 1000 rpm for 15 min and the supernatant decanted. The pellets were extracted under identical conditions. Supernatants were combined and used for total flavonoid assay. The flavonoid content was determined in all of the samples as indicated by Association of the Industry of the Juices and Nectars (AIJN) according to the colorimetric Davis method [10]. Ten milliliters of diethylene glycol were added to 0.2 ml of water/methanolic (50:50, v:v) extract and mixed. Thereafter 0.2 ml of approximately 4N sodium hydroxide is added and the increase in color is read at 420 nm after 5 minutes with Perkin-Elmer LAMBDA 35 UV/VIS spectrophotometer. The observed color increases were compared with a standard curve prepared from the ACS grade pure naringin (Sigma Aldrich, Italy). The values were expressed as mg naringin/100 g FW (fresh weight).

2.3. Extraction and analysis of chlorophylls

The extraction of pigments were performed in subdued light in 90% acetone and the content of chlorophylls a and b were calculated in according with formulas proposed by Jeffrey and Humphrey in 1975 [11] from the absorption spectra recorded at 645 and 663nm:

chlorophyll a $=11.93 \times A663-1.93 \times A645$

chlorophyll b $=20.36 \times A645-5.50 \times A663$

The values were expressed as µg/g FW.

2.4. Extraction, saponification and analysis of carotenoids

A 25g amount of sample were accurately weighed and transferred to a 250 ml amber Erlenmeyer flask. Then, 0.5 g of ascorbic acid (Sigma Aldrich, Italy), 50 ml of absolute ethanol and 10 ml of 60% potassium hydroxide (Carlo Erba, Italy) solution were added, under a stream of nitrogen. Saponification was performed overnight with slow constant stirring at room temperature. Then, the saponified mixture was transferred to a 250 ml amber separatory funnel, rinsed with 30 ml water and extracted five times (shaking for 2 min) with three fractions containing 50 ml of n-hexane and two fractions containing 25 ml of n-hexane. The combined n-hexane extract was washed with 50 ml fractions of water, which were added with some drops of phenolphthalein, until the aqueous layer appeared colorless. A 1 g amount of BHT butilidrossitoluene (BHT) (Sigma Aldrich, Italy) was added as the antioxidant and the mixture was then passed through a Whatman No. 1 filter containing 20 g of anhydrous sodium sulfate (Carlo Erba, Italy) and was collected into a 250 ml amber volumetric flask. The extract was concentrated by rotatory evaporation at 40°C. Finally, the evaporated residue was reconstituted with n-hexane and the absorbance at 470 nm of extracts was determined. The pigment concentration was expressed in µg β-carotene per g FW using an external calibration prepared from the pure β-carotene (Sigma Aldrich, Italy). The corresponding content of provitamin A was calculated in term of Retinol Equivalent (R.E.) per gram of FW (1 µg R.E.=6 µg β-carotene).

2.5. Total and reduced ascorbic acid content

The used procedure is based on the method of Kampfenkel et al [12] for the spectrophotometric determination of ascorbic acid as ascorbate (AsA) and dehydroascorbate (DAsA). The assay is based on the reduction of $Fe3+$ to $Fe2+$ by AsA and the spectrophotometric detection of complexed $Fe2+$ with 2,2-dipyridyl (Sigma Aldrich, Italy).

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DAsA was reduced to AsA by preincubation of the sample with dithiothreitol (Sigma Aldrich, Italy). Subsequentely the excess dithiothreitol was removed with N-ethylmaleimide (Sigma Aldrich, Italy), and the total AsA was determined from the difference of total AsA and AsA (without pretreatment with dithiothreitol). The Vitamin C content was expressed as mg/100 g FW, by comparison with a standard curve of AsA (Sigma Aldrich, Italy).

2.6. Ascorbate peroxidase (APX) assay

The APX activity was assayed following Wang et al. [13]. APX extraction was performed with a 50 mM potassium-phosphate buffer (pH 7.0) in presence of 1 mM ascorbic acid to avoid the enzyme inactivation during extraction. APX activity was evaluated on a reaction mixture made up of 0.5 ml enzyme extract in 50 mM potassium-phosphate buffer (pH 6.6), 1 mM ascorbic acid, 4 mM H_2O_2, 0.4 mM Na_2EDTA, following the extinction rate of ascorbic acid due to its oxidation by H_2O_2. The reaction was started with the addition of H_2O_2 and the ascorbic acid degradation was followed monitoring the decrease of absorbance at 290 nm at 25°C. Ascorbate peroxidase specific activity was expressed in U mg^{-1} of enzymatic proteins.

2.7. Enzymatic protein determination

The enzymatic proteins content was determined according to the method of Bradford [14], using Coomassie® Brilliant Blue R (Sigma Aldrich, Italy) which shows, in the free form, a maximum absorbance peak at 465 nm. The reagent, which binds mainly to the residues of arginine and, to a less extent, to lysine, histidine, tyrosine, tryptophan and phenylalanine of the enzyme, shows a maximum absorbance peak at 595 nm. One ml Coomassie® was added to variable aliquots of enzyme. After 15 minutes the absorbance at 595 nm was read and the protein amounts were calculated using a calibration curve obtained with bovine serum albumine (Sigma Aldrich, Italy) at concentrations ranging from 2 to 10 µg.

2.8. Determination of the total antioxidant capacity

Aliquots of sample were extracted with phosphate buffer 75 mM/l (pH 7.4) and after sonicated for 60 s. The extract was centrifuged at 10000 rpm for 10 min at 4°C. The total antioxidant capacity was assayed on the supernatant according to ORAC (Oxygen Radical Absorbance Capacity)-fluorescein assay [15]. Trolox standards (10–100 µM/l), fluorescein (7.0 µM/l), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (63 mM/l) solutions (Sigma Aldrich, Italy) were prepared prior to use in phosphate buffer (75 mM/l, pH 7.4). Chinotto samples and different concentrations of trolox (10-100 µM/l) were pipetted into 96 well of assay plates and the total volume was adjusted to 40 µl by the addition of buffer. Further, 200 µl of working fluorescein solution was added. The plate was then allowed to equilibrate by incubating for 3 min at 37 °C in the PerkinElmer VICTOR3 V Multilabel Counter 1420. Reaction was initiated by the addition of 20 µl of AAPH solution using the microplate reader's injector. The fluorescence was then monitored kinetically with data taken every three min. The plate was top read at excitation and emission wavelengths of 485 and 535 nm, respectively at 37 °C and at 3 min intervals for 60 min. The standard curve was obtained by plotting trolox concentrations against the average net area under the curve (AUC) of the three measurements for each concentration. The net AUC corresponding to a sample was calculated by subtracting the AUC of the blank. A standard curve was generated from the net AUC of the trolox standards and used to assign trolox equivalence values to the samples. Final ORAC values were calculated using the

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regression equation between trolox concentration and the net AUC and are expressed as micromol trolox equivalents per 100 gram of FW.

2.9. Statistical analysis

To verify the statistical significance of all parameters the values of means and standard deviation (SD) were calculated. Where it was appropriate, the data by two-way ANOVA were tested. The $P_{value}<0.05$ were adopted as statistically significant. All data are means of five measurements. Average amounts of bioactive compounds and antioxidant activity in *Citrus x myrtifolia Raf.* FW basis were reported in **Table 1**.

3. Results and discussion

Flavanone glycosides are the most abundant phenolic compounds present in citrus fruit, but significant concentrations of other flavonoids such as methoxylated flavones and flavonols have also been found [16, 17]. Among them, literature data available for chinotto juice demonstrated that flavanone 7-O-neohesperidoside components neoeriocitrin, naringin, and neohesperidin predominate in *Citrus x myrtifolia* [2, 3]. Therefore the total flavonoids content determined by Davis method was reported as the main flavonoids naringin [2], referring to standard curve. The total flavonoids found in *Citrus x myrtifolia* extract were 780 mg/100g FW (Table 1). It's well known that phenolic contents were usually higher in peels [18] and in immature fruits [3], thus it is not entirely surprising that this flavonoids amount was more and more high to what reported for chinotto juice; literature data reported values of total juice flavonoids ranging from 57.4 mg/L [3] and about 2000 mg/L [2].

Carotenoids in citrus fruits are localized in plastids present in both the flavedo (containing about 70% of the total in fruit) and in the vesicles that contain the juice. When the fruit is immature their color is masked by chlorophylls, with the progress of ripening yellow appears in various shades from pale yellow to deep orange due to variations in type and quantity of different carotenoids [19]. Due to the presence of chlorophylls, immature fruits are capable of photosynthesis but cannot make significant contribution to own nutrition. There is a rapid synthesis of carotenoids in the chromoplast during ripening, which is accompanied by a simultaneous loss of chlorophylls [19]; in many citrus there is no further synthesis of colored carotenoids during ripening after all chlorophylls had disappeared [20]. The analyzed samples contained in fact a high amount of total chlorophylls (41.4 µg/g FW) (Table 1).

Table 1. Average amounts of bioactive compounds and antioxidant activity in *Citrus x myrtifolia Raf.* on fresh weigh (FW) basis.

Parameter	***Mean Value***[a]
Total flavonoids (mg/100g)[b]	780 (13)
Total chlorophylls (µg/g)	41.4 (1.8)
chlorophyll a (µg/g)	*36.8 (1.5)*
chlorophyll b (µg/g)	*4.7 (0.2)*
Total carotenoids (µg/g)[c]	1.7 (0.1)
Provitamin A (µg R.E.[d]/g)	0.28 (0.01)
Ascorbic acid (mg/100g)	41.8 (3.1)
ascorbate (mg/100g)	*35.0 (1.3)*
dehydroascorbate (mg/100g)	*6.8 (1.8)*
APX[e] (U/mg enzymatic proteins)	12.88 (0.05)
ORAC[f] total antioxidant activity (µM T.E.[g]/100 g)	5872 (351)

[a] mean value of five determination, standard deviation in parenthesis ($P_{value}<0.05$). [b] expressed as naringin; [c] expressed as β-carotene; [d] retinol equivalents; [e] ascorbate peroxidase activity; [f] Oxygen Radical Absorbance Capacity; [g] trolox equivalents.

Studies about effect of ethylene application on chlorophylls in Navelate fruit harvested [21]

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demonstrated that a decrement of chlorophylls occurred during maturation from about 115 μg/g to 84 or 7 μg/g depending on air or ethylene post-harvest. Chlorophylls in citrus consist mainly of two pigments: chlorophyll a and chlorophyll b; chlorophyll c, d and chlorophyll e are not reported in citrus and are mainly present in algae and certain sea weed [19]. Chlorophyll a and b were present in the sample in the ratio 7:1. Chlorophyll a/b ratio is an indicator of the functional pigment equipment and light adaptation of the photosynthetic apparatus; chlorophyll b is found exclusively in the pigment antenna system, whereas chlorophyll a is present in the reaction centers of photosystems I and II and in the pigment antenna [22].

On the other hands, total carotenoids were present only in the alkaline saponified extract to demonstrate that at this maturation stage carotenoids are present bonded form. The saponification of the extract of carotenoids has been traditionally an important step in the protocol for the determination of these pigments. Xanthophylls are usually esterified in fruits, the degree of esterification depending on the number of hydroxyl groups in the molecule [7]. Thus, monol carotenoids like β-cryptoxanthin may be found either free or as monoesters; diol carotenoids, such as lutein or zeaxanthin, may be free or esterified by one or two fatty acids, and so forth. Furthermore, the saponification reaction leads to the elimination of the chlorophylls present in the samples. Chinotto total carotenoids content of 1.7 μg/g FW (Table 1) is quite low; the published total carotenoid content in the peels of unripe oranges is about ten times higher [21, 23]. Carotenoids with an unsubstituted b-ring with an 11-carbon polyene chain such as β-cryptoxanthin and β-carotene are nutritionally important because of the vitamin A activity, since they are converted to retinal by mammals. This role is of particular importance, especially in developing countries where the dietary deficiency of vitamin A can lead to blindness and premature childhood mortality [24]. The provitamin A activity estimated from total carotenoids content corresponded to 0.28 μg R.E./g (Table 1). Aside from the nutritional relevance of some carotenoids owing to their vitamin A activity, these pigments are increasingly drawing the interest of researchers as they may be somehow implicated in the prevention and/or protection against major human diseases [7].

Citrus fruits are rich in vitamin C; the abundance of citrus in the Mediterranean diet may provide the main dietary source for natural this vitamin [19]. Daily intake of 5 mg is sufficient to prevent symptoms of scurvy in an adult. The intake of 30-60 mg is estimated to be required for full grown adults. The limiting step for vitamin C absorption in humans is transcellular active transport across the intestinal wall where AsA may be oxidized to dehydroascorbic acid (DAsA), which is easily transported across the cell membrane and immediately reduced back to AsA by two major pathways [25]. Quantitative analyses of the chinotto sample are in agreement with literature references for citrus fruits [19] showing a content of 41.8 mg/100g, which 84% biologically active reduced form (AsA) (Table 1). AsA bioavailability in the presence of flavonoids has yielded controversial results. Whereas flavonoids seem to inhibit intestinal absorption of AsA, some studies have shown that AsA in citrus extract was more available than synthetic ascorbic acid alone [25]. DAsA is reported to possess equivalent biological activity to AsA, so recent studies often consider the vitamin C activity in the diet as the sum of AsA plus DAsA [25]. Oxidative damage has many pathological implications in human health, and AsA may play a central role in maintaining the metabolic antioxidant response.

In whole fruit the enzymatic system of oxidation of AsA is intact, while when the fruit is processed some losses of vitamin C may occur [19]. Because steady-state levels of e reactive oxygen species (ROS) depend on the balance between ROS -producing and -

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scavenging reactions, we measured levels of both AsA and DAsA together the activity of ascorbate peroxidase (APX); the latter plays a major role in scavenging H_2O_2 in plants, because can oxidize AsA [19]. The value estimated in fresh sample was of 12.88 U/mg enzymatic proteins (Table 1).

The measurement of the antioxidant capacity of food products is a matter of growing interest because it may provide a variety of information, such as resistance to oxidation, quantitative contribution of antioxidant substances, or the antioxidant activity that they may present inside the organism when ingested [26]. The studied chinotto showed high antioxidant effect (5872 µM Trolox equivalents/100g FW), measured with ORAC method (Table 1). The antioxidant activity industrially processed chinotto fruit was determined for the first time in this study and therefore no data are available in the literature to compare with our results. On the basis of the of edible portion of fruit, it was demonstrated that strawberry had the highest ORAC activity (about 1500 µM Trolox equivalents/100g FW), followed by plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear, and melon (about 100 µM Trolox equivalents/100g FW) [27].

The elevate antioxidant activity may be mostly attributed to their high content of polyphenols. The correlation between total phenol contents and antioxidant activity has been widely studied in different foodstuffs such as fruit and vegetables [28-30]. As reported, antioxidant activity of fruits and vegetables significantly increases with the presence of high concentration of total polyphenols content [31-33]. It has been demonstrated flavonoids have 2–6 times the antioxidant activity of common antioxidants, such as ascorbic acid [34].

The absence of correlation between ORAC and ascorbic acid values was found by Rapisarda et al. [35] on different citrus hybrids. The same results were obtained by Wang et al. [36] for orange juice and Prior et al. [34] for Vaccinium species. Shahidi and Marian [37] reported that differences in antioxidant activities of fruits could be due to their different structures from phenolic acids and flavonoid compounds as well as their derivatives. Results of Rababah et al. [33] showed that the concentrations of antioxidants in strawberry, peach, and apple mixed with 0.1% ascorbic acid were very similar to those without addition of ascorbic acid.

4. Conclusions

Our results indicated that the *Citrus* × *myrtifolia* raw materials used for beverage industrial purposes is a good source of phytochemicals, mainly vitamin C and flavonoids, and may therefore provide health benefits to consumers. The overall amount of bioactive compounds in whole fruit are in fact higher than in the juice. Nowadays these aspects are considered to be highly valuable for the commercial valorization of chinotto as a citrus with high potential as nutraceutical source.

The studied matrix contain a group of natural antioxidants that have not only a high antioxidant activity, but also a good antioxidant quality that could enrich lower density lipoproteins, thereby protecting them from oxidation and preventing development of atherosclerosis and other diseases. The supplementation of natural antioxidants through a balanced diet containing enough fruits could be much more effective and economical than the use of individual antioxidants for protecting of the body against various oxidative stresses. Neither chlorophylls nor carotenoids can be synthesized by animal tissues, though animal cells can chemically modify them for assimilation. Thus, these molecules must be obtained from food.

Moreover recent research has demonstrated the possibility of recovering anthocyanins, flavanones and hydroxycinnamic acids from blood orange juice or citrus byproducts and

using this extract as an antioxidant ingredient for dietary supplements [38, 39]. Carotenoid and chlorophyll molecules could be also extracted and used as natural colorants and antioxidants [40]. This approach on chinotto matrix could represent a new and important strategy in the citrus industry.

Further works are in progress in our laboratory to elucidate the identity of compounds responsible for nutraceutical source.

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Paper No 2

Monica Scordino, Leonardo Sabatino, Antonio Muratore, Adalgisa Belligno, Giacomo Gagliano.

Molecole bioattive ed attività antiossidante in *Citrus x myrtifolia Raf.* (Chinotto).

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MOLECOLE BIOATTIVE ED ATTIVITA' ANTIOSSIDANTE IN *Citrus x myrtifolia Raf.* (CHINOTTO)

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ABSTRACT
Studi epidemiologici suggeriscono che un elevato consumo di frutta può ridurre il rischio di tumori e malattie cardiovascolari grazie all' attività antiossidante delle vitamine e dei composti fenolici in essa contenuti. Il presente studio analizza alcuni attributi di qualità del frutto acerbo di Citrus ×myrtifolia Raf., ingrediente della popolate bevanda Chinotto. Sebbene non siano noti i dettagli della preparazione dell'estratto industriale di chinotto, esso proviene dall'estrazione per infusione in soluzione idroalcolica del frutto immaturo privato del succo, assieme ad erbe officinali. Per la preparazione del campione per le analisi è stata pertanto simulata la metodologia impiegata per la preparazione dell'estratto del chinotto industriale, sottoponendo i frutti ad una lieve spremitura e scartandone il succo.
La nostra attenzione è stata rivolta alla valutazione del contenuto di alcune molecole bioattive (acido ascorbico, carotenoidi, clorofille e flavonoidi) e della capacità antiossidante (ORAC). I risultati hanno mostrato un apprezzabile contenuto di molecole ad attività antiossidante, tra cui prevalgono i flavonoidi e la vitamina C, confermando le proprietà salutistiche dei frutti di agrume, indiscussi protagonisti della dieta Mediterranea.

Keywords:
acido ascorbico, antiossidanti, chinotto, agrumi, flavonoidi, ORAC.

INTRODUZIONE

Il Citrus × myrtifolia Raf. è un agrume del genere Citrus. Nativo della Cina meridionale, la sua origine non è stata esattamente accertata; probabilmente è stato generato da una mutazione dell'arancio amaro che alla fine si è evoluta nella specie conosciute oggi [1]. I frutti acerbi hanno l'aspetto di piccoli mandarini verdi molto aromatici, mentre i frutti maturi sono più grandi e colorati d'arancione. La polpa, amara ed acida, è suddivisa in 8-10 segmenti. La pianta è stata coltivata per secoli in Francia e in Italia [1], in particolare in Liguria, Calabria e Sicilia, dove i frutti sono utilizzati per la preparazione di dolci, caramelle e nella produzione di marmellate, sciroppi, bibite e liquori. In Italia la pianta ha dato il nome alla popolare bevanda Chinotto, aromatizzata con estratto di Citrus × myrtifolia, sebbene nessun produttore abbia rivelato i dettagli sulla sua preparazione. Gli ingredienti utilizzati per la sua preparazione sono regolamentati a livello legislativo con il decreto del Presidente della Repubblica (DPR n.719 del 19 maggio 1958, articolo 5, e successive modifiche) che specifica che le bevande analcoliche vendute con il nome di un frutto non a succo, tra cui il chinotto, devono essere preparate con sostanze provenienti dal frutto o della pianta. L'estratto di Citrus × myrtifolia è il risultato dell'infusione idroalcolica dei frutti acerbi parzialmente pressati, aromatizzata con erbe come il rabarbaro, la genziana, la china, la cannella, i chiodi di garofano, l'arancio dolce, il timo e il tamarindo.

Nonostante la crescente diffusione di questa popolare bevanda, i lavori scientifici sul Citrus × myrtifolia [2, 3] sono relativi esclusivamente al succo e nessuna informazione è disponibile sui composti bioattivi presenti nel frutto intero. Il crescente interesse dell'industria italiana per la produzione del chinotto potrebbe essere incrementato da studi sulla sua composizione.

I possibili effetti benefici del consumo di frutta sono dovuti alla presenza di nutraceutici [4]. Un aumento del consumo di frutta e verdura, cibi tipici dieta mediterranea, può proteggere contro le patologie degenerative come il cancro e l'aterosclerosi [5]. Negli ultimi decenni, un gran numero di studi sono stati effettuati con lo scopo di individuare i componenti bioattivi presenti negli agrumi, nel tentativo di ottenere una più profonda comprensione della correlazione tra dieta, benefici per la salute e riduzione del rischio di malattie [6-8]. Tra i nutraceutici, i flavonoidi sono ampiamente contenuti negli agrumi, soprattutto in cultivar siciliane, ma sono anche fonte di altri componenti bioattivi, quali carotenoidi e vitamina C [9].

Lo scopo del presente lavoro è stato quello di caratterizzare il frutto acerbo di Citrus x myrtifolia in termini di composti bioattivi quali carotenoidi, acido ascorbico, flavonoidi e clorofille. Inoltre, l'attività antiradicalica è stata valutata con il metodo ORAC.

MATERIALI E METODI

Frutti. I frutti di Citrus x myrtifolia Raf. (Chinotto) utilizzati in questo studio sono stati coltivati nella proprietà "castrorao" a Castiglione di Sicilia (Catania, Italia) (Figura 1). I frutti sono stati raccolti immaturi ad Ottobre 2010 e caratterizzati in funzione del colore della buccia e della polpa, il diametro medio, i solidi solubili totali, l'acidità titolabile ed il pH (Tabella 1). La buccia e la polpa rappresentavano il 39% e il 55% del peso fresco (PF) rispettivamente, mentre la restante parte era costituita da semi. I campioni per l'analisi sono stati preparati da 25 frutti, processati in modo da riprodurre al meglio i materiali grezzi utilizzati per estrarre il chinotto industriale: i frutti sono stati leggermente spremuti per eliminare una parte del succo e ridotti succesivamente in piccoli pezzi con un Turbo Omogeneizzatore HMHF (PBI International). I campioni sono stati conservati a -20 ° C fino al momento dell'analisi.

Estrazione e analisi dei flavonoidi. Due grammi di campione sono stati estratti per 2 h con 10 mL di una soluzione acquosa di metanolo 80% contenente l'1% di acido cloridrico, a temperatura ambiente e sotto agitazione. La miscela è stata centrifugata a 1000 giri/min per 15 min e il surnatante decantato. I peletts sono stati riestratti in condizioni identiche. I surnatanti sono stati combinati ed utilizzati per il dosaggio dei flavonoidi. Il contenuto di flavonoidi totali è stato determinato in tutti i campioni come indicato dall'Associazione dell'industria dei succhi e nettari (AIJN) secondo il metodo colorimetrico Davis [10]. I valori sono stati espressi come mg naringina/100 g PF (peso fresco). La valutazione qualitativa del pattern polifenolico è stata condotta utilizzando un sistema Thermo Finnigan LCQ DECA XP MAX equipaggiato di autocampionatore, LC/Pump e detector PDA montato in serie; è stata utilizzata una colonna Gemini C18 150 x 2,1 mm (Phenomenex), 3µm termostatata a 30°C iniettando 10 µL di campione. Come fase mobile è stato impiegato un gradiente di acetonitrile acidificato con 0.3% di acido formico (A) ed acqua acidificata con 0.3% di acido formico (B) per 80min con un flusso di 0,2 mL/min. Gradiente: dal 5 al 28% di A in B in 50 min, quindi incremento di A al 57% fino a 60 min, seguito da 5 min di isocratica e ritorno alle condizioni iniziali in altri 5 min. Scan range PDA 190-700 nm.

Estrazione e analisi delle clorofille. L'estrazione dei pigmenti è stata eseguita in luce attenuata con acetone 90% ed il contenuto di clorofille a e b è stato calcolato dagli spettri di assorbimento registrati a 645 e 663nm, in accordo con le formule proposte da Jeffrey e Humphrey nel 1975 [11]:

clorofilla a =11,93*A663-1,93*A645

clorofilla b =20,36*A645-5,50*A663

I valori sono stati espressi come mg/g di PF.

Estrazione, saponificazione e analisi dei carotenoidi. 25 g di campione sono stati accuratamente pesati e trasferiti in un pallone ambrato da 250 ml. Quindi, 0,5 g di acido ascorbico (Sigma Aldrich, Italia), 50 ml di etanolo assoluto e 10 ml di idrossido di potassio 60% (Carlo Erba, Italia) sono stati aggiunti sotto corrente di azoto. La saponificazione è stata eseguita durante la notte in agitazione ed a temperatura ambiente. La miscela saponificata è stata trasferita in un imbuto separatore ambrato da 250 ml, risciacquato con 30 ml di acqua ed estratta cinque volte con tre frazioni contenenti 50 ml di n-esano e due frazioni contenenti 25 ml di n-esano. L'estratto è stato lavato con 50 ml di acqua aggiungendo qualche goccia di fenolftaleina, finché lo strato acquoso è apparso incolore. 1 g di butilidrossitoluene (BHT) (Sigma Aldrich, Italia) è stato aggiunto come l'antiossidante e la miscela è stata fatta passare attraverso un filtro Whatman No.1 contenente 20 g di solfato di sodio anidro (Carlo Erba, Italia) ed è stato raccolto in un pallone ambrato da 250 ml. L'estratto è stato concentrato al rotavapor a 40 ° C. Infine, il residuo evaporato è stata ripreso con n-esano ed è stata determinata l'assorbanza a 470 nm. La concentrazione di pigmenti è stata espressa in mg β-carotene per grammo di PF usando una taratura esterna ottenuta con β-carotene (Sigma Aldrich, Italia). Il contenuto corrispondente di provitamina A è stato calcolato in termini di retinolo equivalenti (RE) per grammo di PF (1 μg RE = 6 μg β-carotene).
Contenuto di acido ascorbico totale e in forma ridotta. La procedura utilizzata si basa sul metodo di Kampfenkel et al [12] per la determinazione spettrofotometrica dell'acido ascorbico come ascorbato (ASA) e deidroascorbato (DASA). Il test si basa sulla riduzione della Fe3+ a Fe2+ da ASA e la rilevazione spettrofotometrica di Fe2+ complessato con 2,2-Dipyridyl (Sigma Aldrich, Italia). Il contenuto di vitamina C è stato espresso in mg/100 g PF, per confronto con una curva stardard di ASA (Sigma Aldrich, Italia).
Dosaggio dell'ascorbato perossidasi (APX). L'attività APX è stata determinata seguendo il metodo di Wang et al. [13]. La reazione è stata avviata con l'aggiunta di H2O2 e la degradazione dell'acido ascorbico è stata seguita monitorando il decremento di assorbanza a 290nm a 25 ° C. L'attività di ascorbato perossidasi specifica è stata espressa in mg di U/mg delle proteine enzimatiche.
Determinazione delle proteine enzimatiche. Il contenuto delle proteine enzimatiche è stato determinato secondo il metodo di Bradford [14], con Coomassie Brilliant Blue® R (Sigma Aldrich, Italia). Le proteine sono state dosate utilizzando una curva di calibrazione ottenuta con BSA (Sigma Aldrich, Italia) a concentrazioni da 2 a 10 μg.
Determinazione della capacità antiossidante totale. Aliquote di campione sono state estratte con tampone fosfato 75 mmol /l (pH 7,4) e, dopo sonicazione per 60 s, l'estratto è stato centrifugato a 10000 rpm per 10

min a 4 °C. La capacità antiossidante totale è stata determinata sul surnatante secondo il metodo ORAC (Oxygen Radical Assorbance Capacity) [15]. L'attività antiossidante è stata espressa in μmol Trolox equivalenti/100 g di PF.
Analisi statistica. Per verificare la significatività statistica di tutti i parametri, i valori di media e deviazione standard (SD) sono stati calcolati. $p_{value}<0,05$ sono stati adottati come statisticamente significativi. Tutti i dati sono generati dalla media di cinque misure. Le quantità medie dei composti bioattivi studiati e l'attività antiossidante in Citrus x myrtifolia Raf. sono riportati nella Tabella 2.

RISULTATI E DISCUSSIONE

I flavanoni glicosidi sono i composti fenolici maggiormente presenti nei frutti di agrumi, ma sono stati riscontrati concentrazioni significative di altri flavonoidi quali polimetossiflavoni e flavonoli [16, 17]. Tra questi, dati di letteratura disponibili per il succo di chinotto hanno dimostrato che in Citrus x myrtifolia predominano i flavanoni 7-O-neoesperidosidi neoeriocitrina, naringina, e neoesperidina [2, 3]. Pertanto, il tenore di flavonoidi totali determinato con il metodo Davis è stato espresso in funzione della curva standard del principale flavonoide naringina [2]. I flavonoidi totali riscontrati nell'estratto di Citrus x myrtifolia sono pari a 780 mg/100g PF (Tabella 2). E' ben noto che il contenuto fenolico è generalmente più elevato nelle bucce [18] e nei frutti immaturi [3], pertanto non è sorprendente che tale quantitativo sia molto più elevato di quanto riportato per il succo di chinotto: i dati di letteratura oscillano da 57,4 mg / L [3] a circa 2000 mg / L [2]. Da un punto di vista qualitativo, le singole specie chimiche sono state studiate mediante separazione cromatografica HPLC e successiva caratterizzazione sulla base degli spettri e dei tempi di ritenzione. I risultati mostrano come il prodotto sia caratterizzato dalla presenza dei flavoni C-glucosidi vicetin-2 e lucenin-2-4'metiletere, dei flavanoni O-glucosidi neoeriocitrina, naringina, neoesperidina e brutieridina, e dal flavone O-glucoside roifolina. Da sottolineare anche la presenza di polimetossiflavoni (sinensetina, tangeretina, tetrametossiflavone, nobiletina, eptametossiflavone), composti caratteristici della buccia dei frutti di agrumi, tra i quali predomina la nobiletina (Figura 2).
I carotenoidi negli agrumi sono localizzati nei plastidi presenti nel flavedo (contenente circa il 70% del totale dei carotenoidi) e nelle vescicole che contengono il succo. Quando il frutto è immaturo il loro colore è mascherato dalla clorofilla, ma con il progredire della maturazione il colore si sviluppa in varie tonalità di arancio in funzione del tipo e della quantità dei carotenoidi presenti [19]. A causa della presenza di clorofille, i frutti immaturi sono capaci di fotosintesi, ma di contro non contribuiscono in modo significativo alla nutrizione. I campioni analizzati

contenevano infatti una elevata quantità di clorofille totali (41,4 μg / g PF) (Tabella 2). Le clorofille dei frutti di agrume sono rappresentate principalmente da due pigmenti: clorofilla a e clorofilla b, presenti nel campione in rapporto 7:1. Tale rapporto è un indicatore dell'adeguamento funzionale del pigmento dell'apparato fotosintetico; la clorofilla b si trova esclusivamente nel sistema di antenna pigmento, mentre la clorofilla a è presente nei centri di reazione dei fotosistemi I e II e nel pigmento antenna.

Per quanto concerne la frazione carotenoidica, tali composti sono stati individuati solo nell'estratto alcalino saponificato, a dimostrare che in questa fase di maturazione i carotenoidi sono presenti in forma legata. Inoltre, la reazione di saponificazione porta all'eliminazione delle clorofille presenti nei campioni. Nell'estratto di chinotto il contenuto totale di carotenoidi di 1,7 μg/g PF (Tabella 2) è molto basso, considerando che dati di letteratura riportano contenuti circa dieci volte superiori nelle bucce delle arance immature [20]. I carotenoidi sono nutrizionalmente importanti a causa dell'attività provitaminica, dal momento che sono convertiti in retina dai mammiferi. Questo ruolo è di particolare importanza, soprattutto nei paesi in via di sviluppo dove la carenza nella dieta di vitamina A può portare alla cecità infantile e la mortalità precoce. L'attività di provitamina A stimata dal contenuto totale di carotenoidi è pari a 0,28 μg RE/g (Tabella 2).

Gli agrumi sono ricchi di vitamina C, l'abbondanza di agrumi nella dieta mediterranea può fornire la principale fonte alimentare di questa vitamina naturale [19]. L'assunzione giornaliera di 5 mg è sufficiente per evitare i sintomi di scorbuto in un adulto. L'assunzione di 30-60 mg è ritenuto necessario per un completo sviluppo. Il passaggio limitante per l'assorbimento della vitamina C negli esseri umani è il trasporto attivo transcellulare attraverso la parete intestinale, dove l'ASA può essere ossidato ad acido deidroascorbico (DASA), che viene facilmente trasportato attraverso la membrana cellulare e subito ridotto di nuovo ad ASA da due vie principali [21]. Le analisi quantitative del campione di chinotto sono in accordo con i riferimenti di letteratura per gli agrumi [19], mostrando un contenuto di 41,8 mg/100g, di cui l'84% nella forma ridotta e quindi biologicamente attiva (ASA) (Tabella 2). La biodisponibilità di ASA in presenza di flavonoidi ha dato risultati controversi. Mentre alcuni sostengono che i flavonoidi sembrano inibire l'assorbimento intestinale di ASA, altri studi hanno dimostrato che l'ASA nell'estratto di agrumi è più disponibile di quello sintetico assunto singolarmente [21]. Il danno ossidativo ha molte implicazioni patologiche nella salute umana, e l'ASA svolge un ruolo centrale nel mantenimento della risposta metabolica antiossidante.

Nei frutti interi il sistema enzimatico di ossidazione di ASA è intatto, mentre quando il frutto viene processato possono verificarsi alcune

perdite di vitamina C [19]. Poiché i livelli di specie reattive all'ossigeno (ROS) dipendono dal rapporto tra la produzione di ROS e le reazioni di scavenging, sono stati misurati i livelli di entrambe le ASA e DASA insieme l'attività dell'ascorbato perossidasi (APX), il quale svolge un ruolo importante nelle piante, perché in grado di ossidare l'ASA [19]. Il valore stimato nel campione è di 12,88 U/mg di proteine enzimatiche (Tabella 2).
La misura della capacità antiossidante dei prodotti alimentari è una questione di crescente interesse perché può fornire una varietà di informazioni, quali la resistenza all'ossidazione, il contributo quantitativo di sostanze antiossidanti, l'attività antiossidante che queste possono presentare all'interno dell'organismo allorché ingerite [22]. Il campione di chinotto ha mostrato un effetto antiossidante (5872 µmol Trolox equivalenti/100g PF), misurato con metodo ORAC (Tabella 2). L'attività antiossidante su un chinotto processato per la trasformazione industriale è stata determinata per la prima volta in questo studio e pertanto non vi sono dati disponibili in letteratura per confrontare tali risultati. Sulla base della valutazione della capacità antiossidante delle parti edibili dei frutti, è stato dimostrato che la fragola presenta l'attività ORAC più alta (circa 1500 µmol Trolox equivalents/100g PF), seguita da prugna, arancia, uva rossa, kiwi, pompelmo rosa, uva bianca, banana, mela, pomodoro, pera e melone (circa 100 µmol Trolox equivalenti/100g PF) [22].
L'elevata attività antiossidante può essere attribuita in gran parte all'alto contenuto polifenolico del campione analizzato. La correlazione tra contenuto totale di fenoli e l'attività antiossidante è stata ampiamente studiata in diversi prodotti alimentari come frutta e verdura [23-24]. Come riportato, l'attività antiossidante di frutta e verdura aumenta significativamente con la presenza di un'elevata concentrazione di polifenoli totali [25]. È stato dimostrato che i flavonoidi sono 2-6 volte più antiossidanti degli antiossidanti comuni, come l'acido ascorbico [26]. Parecchi lavori hanno dimostrato invece l'assenza di correlazione tra i valori ORAC ed il contenuto di acido ascorbico, come riportato da Rapisarda et al. [27] su diversi ibridi di agrumi, gli stessi risultati sono stati ottenuti da Prior et al. [26] per le specie Vaccinium.

CONCLUSIONI

I nostri risultati indicano che i materiali grezzi utilizzati come ingredienti per la produzione della bibita chinotto sono una buona fonte di sostanze fitochimiche, soprattutto vitamina C e flavonoidi, e possono quindi fornire benefici per la salute dei consumatori. Questi aspetti sono da considerare per la valorizzazione commerciale del chinotto come agrume ad elevato potenziale nutraceutico.
La matrice in esame ha mostrato di contenere antiossidanti naturali che hanno non solo una elevata attività antiossidante, ma anche una buona

qualità antiossidante in quanto in grado di arricchire lipoproteine a bassa densità, in modo da proteggere dall'ossidazione e prevenire lo sviluppo di arterosclerosi e altre malattie. L'apporto di antiossidanti naturali attraverso una dieta equilibrata contenente frutta sufficiente potrebbe essere molto più efficace ed economico che l'uso degli antiossidanti individuali per proteggere il corpo contro vari stress ossidativi.
Inoltre, recenti ricerche hanno dimostrato la possibilità di recuperare antociani, acidi idrossicinnamici e flavanoni dal succo di arance rosse o dai sottoprodotti degli agrumi ai fini di utilizzare tale estratto come ingrediente antiossidante per integratori alimentari [28-29]. Carotenoidi e clorofilla potrebbero essere estratti ed utilizzati come coloranti naturali e/o antiossidanti [30]. Né clorofille né carotenoidi possono essere infatti sintetizzati da tessuti animali, anche se le cellule animali possono modificarli chimicamente per l'assimilazione; queste molecole devono essere ottenute da prodotti alimentari. Tale approccio applicato alla matrice chinotto potrebbe rappresentare una nuova strategia per la valorizzazione nel settore agrumario.

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BIOACTIVE MOLECULES AND ANTIOXIDANT ACTIVITY OF *Citrus x myrtifolia Raf.* (CHINOTTO)

ABSTRACT
Epidemiological studies suggest that a high consumption of fruits can reduce the risk of some cancers and cardiovascular disease, and this may be attributable to the antioxidant activity of vitamins and phenolic compounds. The present study investigated for the first time some quality attributes of unripe Citrus × myrtifolia Raf. which is the ingredient of the popular soft drink Chinotto. Although the details of preparing the extract of bitter orange industry is still unknown, it comes from the extraction of water-alcohol solution for infusion of immature fruit juice with herbs. Samples for analysis were processed to better reproduce the crude materials used for industrial chinotto extract, discarding part of the juice. Fruit bioactive compounds such as ascorbic acid, carotenoids, chlorophylls, flavonoids and antioxidant capacity (ORAC) were estimated. The results showed an appreciable content of molecules with antioxidant activity, which are mainly flavonoids and vitamin C, confirming the healthy properties of citrus fruits, the undisputed protagonists of the Mediterranean diet.

Keywords: ascorbic acid, antioxidants, chinotto, citrus, flavonoids, ORAC.

Figura 1. Frutti immaturi di *Citrus x myrtifolia Raf*. (Chinotto) utilizzati.

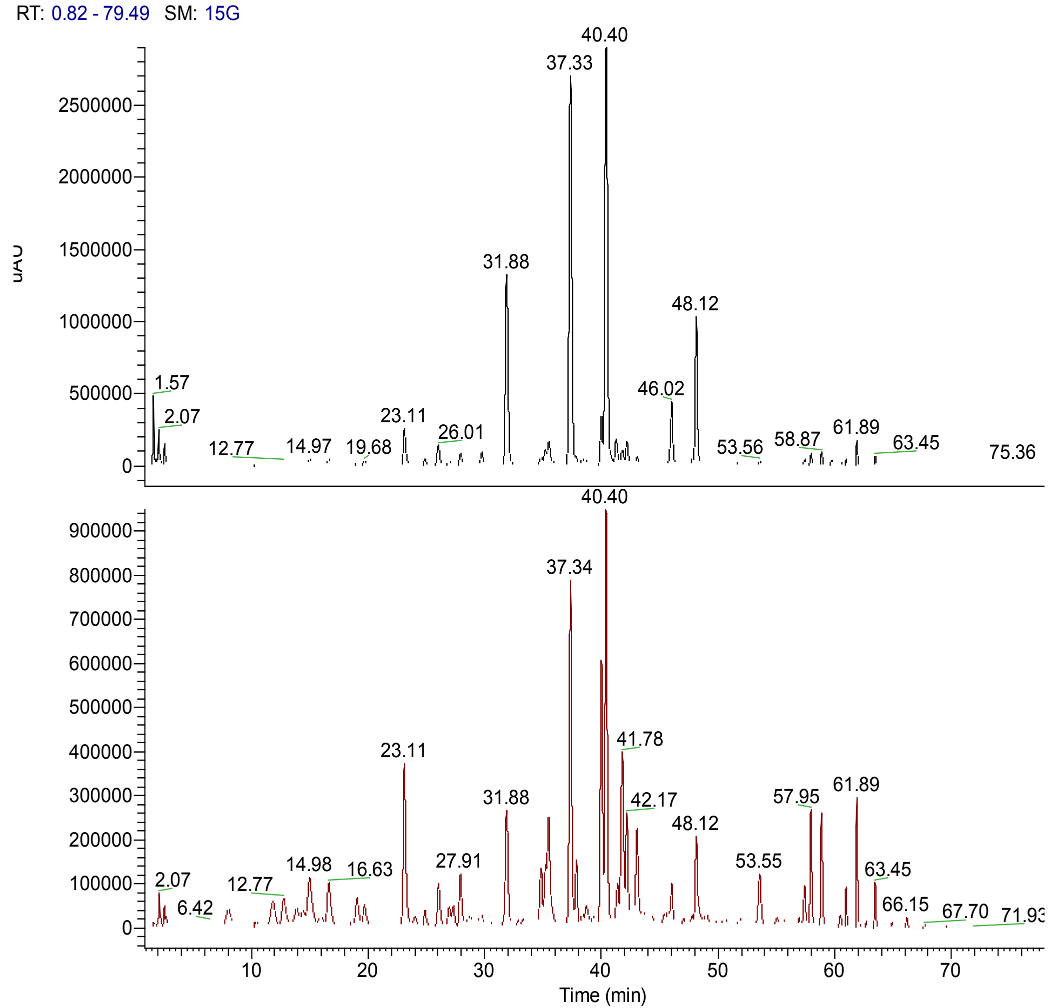

Figura 2. Cromatogramma HPLC/PDA della componente polifenolica a λ 280 nm e λ 325 nm dell'estratto di *Citrus x myrtifolia Raf*. (Chinotto) analizzato. Componenti identificati: vicetin-2 (tr 23,1 min), lucenin-2-4'metiletere (tr 27,9 min), neoeriocitrina (tr 31,8 min), naringina (tr 37,3 min), roifolina (tr 40,0 min), neoesperidina (tr 40,5 min), brutieridina (tr 48,1 min), sinensetina (tr 60,0 min), tetrametossiflavone (tr 60,7 min), nobiletina (tr 62,0 min), eptametossiflavone (tr 62,8) min, tangeretina (tr 63,5 min).

Tabella 1. Caratteristiche del campione di *Citrus x myrtifolia Raf.* (Chinotto) studiato.

Parametro	Valore medio[a]	
Parametri del colore *Hunter*	*Buccia*	*Polpa*
a	36,3 (6,2)	5,4 (1,5)
b	-34,4 (4,2)	-14,3 (2,7)
L	66,3 (2,2)	78,8 (2,4)
Diametro medio (cm)	2,5 (0,3)	
Peso medio (g)	27 (2)	
Solidi solubili totali (Brix)[b]	8,4 (0,1)	
Acidità titolabile (% acido citrico)[b]	0,83% (0,05)	
pH[b]	3,87 (0,21)	
Ratio (Brix/acidità)[b]	10,1	

[a] valore medio di cinque determinazioni, deviazione standard in parentesi.
[b] parametro riferito al succo.

Tabella 2. Risultati delle determinazioni analitiche condotte sul frutto di *Citrus x myrtifolia Raf.* (Chinotto) immaturo.

Parametro	Valore medio[a]
Flavonoidi totali (mg/100g)[b]	780 (13)
Clorofille totali (µg/g)	41,4 (1,8)
Clorofilla a (µg/g)	36,8 (1,5)
Clorofilla b (µg/g)	4,7 (0,2)
Carotenoidi totali (µg/g)[c]	1,7 (0,1)
Provitamina A (µg/g RE[d]/g)	0,2 (0,01)
Attività antiossidante ORAC (µmol TE[e]/100g)	5872 (351)
Acido ascorbico (mg/100g)	41,8 (3,1)
ASA (mg/100g)	35,0 (1,3)
DASA (mg/100g)	6,8 (1,8)
APX[f] (U/mg proteine enzimatiche)	12,88 (0,05)

[a] valore medio di cinque determinazioni, deviazione standard in parentesi.
[b] espresso come naringina; [c] espresso come β-carotene; [d] retinolo equivalenti;
[e] trolox equivalenti; [f] attività dell'ascorbato per ossidasi.

Paper No 3

Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano.

Flavonoids and furocoumarins distribution of unripe chinotto (*Citrus x myrtifolia Rafinesque*) fruit: beverage processing homogenate and juice characterization.

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FLAVONOIDS AND FUROCOUMARINS DISTRIBUTION OF UNRIPE CHINOTTO (*Citrus x myrtifolia Rafinesque*) FRUIT: BEVERAGE PROCESSING HOMOGENATE AND JUICE CHARACTERIZATION

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ABSTRACT:

The present study investigated for the first time the flavonoids and the furocoumarins composition of unripe fruits of *Citrus × myrtifolia Rafinesque*, ingredient of the popular soft drink Chinotto. Samples for analysis were processed to simulate at best the raw material used to produce the industrial extract, discarding part of the juice. The compounds separation was made by HPLC-PDA detector coupled to ESI/MS/MS in positive and negative mode. It has allowed the identification and relative quantification of flavones C-glycosides lucenin-2, vicenin-2 and lucenin-2 4'-methyl ether, the flavanones O-glycosides neoeriocitrin, naringin, neohesperidin, the two 3-hydroxy-3-methylglutaryl flavanone glycosides melitidin and brutieridin, the flavone O-glucoside rhoifolin and the furocoumarins bergapten and epoxybergamottin. Besides Citrus x myrtifolia homogenate was characterized by a great amount of polymethoxylated flavones, among which nobiletin predominated. The flavonoids distribution was compared with that of Citrus x myrtifolia juice. Furthermore, the antioxidant activity of beverage processing homogenate and juice was assessed by ORAC (Oxygen Radical Absorbance Capacity)-fluorescein assay.

Keywords: HPLC-PDA-ESI/MS^2, chinotto, citrus, flavonoids, antioxidant activity, furocoumarins.

INTRODUCTION

The *Citrus* × *myrtifolia Rafinesque* is a citrus fruit of the genus *Citrus*. Native of southern China, its origin has not been exactly ascertained; probably it is mutation of sour orange that eventually evolved into the species known today [1]. The unripe fruits look like small green aromatic tangerines, while mature fruits are bigger and orange painted. The flesh is bitter and sour and divided into 8-10 segments. The plant was cultivated for centuries in France and Italy [1], especially in Liguria, Calabria and Sicily where the fruits are used in sweets for candies and jams as well as flavoring syrups, soft drinks and spirits. In Italy the plant has given its name to a very popular Italian drink Chinotto, flavored with *C. myrtifolia* extract. The chinotto is set up as a classic soda, but no producer has been revealed details about its preparation. Its ingredients are regulated at the legislative level by a Italian Decree of President of the Republic (DPR n.719 of 1958 May 19, Article 5 and subsequently amends) which specifies that the soft drinks sold under the name of a not juice fruit, including cedar and chinotto, should be prepared with substances derived from the fruit or the plant. The *C. myrtifolia* extract for chinotto drink is a aqueous-alcoholic solution made up from infusion of a chinotto homogenate (obtained from chopped unripe whole fruits with partially discarding of the juice) flavored with rhubarb (*Rheum rhabarbarum L.*), gentian (*Gentiana acaulis L.*), cinchona (*Cinchona officinalis L.*), cinnamon (*Cinnamomum zeylanicum*), cloves (*Eugenia caryophyllata*), sweet orange (*Citrus* × *sinensis*), thyme (*Thymus vulgaris*), tamarind (*Tamarindus indica L.*). Despite the growing distribution of this popular soft drink, the few literature papers about the *C. myrtifolia* [2, 3] are related only to the juice and no informations are available about bioactive compounds quantitation and distribution in the homogenate used to obtain beverage extracts. Studies on its composition could increase the importance of the citrus industry for chinotto soft drink production. Recently our research group has characterized the chinotto materials used for beverage industrial purposes in terms of bioactive compounds and antioxidant activity [4]. Results indicated that it is a good source of vitamin C and flavonoids, and may therefore provide health benefits to consumers [4]. Many potentially health promoting effects have been ascribed to the citrus flavonoids [5]. Citrus fruits are among the richest dietary sources of flavonoids, which occur principally in the peel. Flavanone glycosides are the most abundant phenolic compounds present in citrus fruit, but significant concentrations of other

flavonoids such as methoxylated flavones and flavonols have also been found [6, 7]. Among them, literature data available for chinotto juice demonstrated that flavanone 7-*O*-neohesperidoside components neoeriocitrin, naringin, and neohesperidin predominate in *C. myrtifolia* [2, 3].
The aim of the present work was to elucidate by HPLC-PDA-ESI/MS^2 technique the identity and the relative abundance of flavonoids and furocoumarins of industrial chinotto homogenate and to compare their relative amount with that of *C. myrtifolia* juice.

MATERIALS AND METHODS

Chemicals. HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system. Apigenin, bergamottin, diosmetin, neoeriocitrin, naringin, neohesperidin and sinensetin were obtained from Extrasynthèse (Genay, France). Trolox standards, fluorescein and 2,2′-azobis(2-methylpropionamidine) dihydrochloride were from Sigma Aldrich (Milan, Italy).
Fruits. Fruits used in this study were grown in Castiglione di Sicilia (Catania, Italy). Immature *Citrus x myrtifolia Rafinesque* (chinotto) fruits were collected with the degree of fruit maturity determined from the surface color, mean diameter, total soluble solids (TSS), titratable acidity (TA) and pH. Unripe fruits picked on 2010 October were green, had a mean diameter of 2.5 (±0.3) cm and a mean weight of 27g (±2). The peel and the pulp represented the 39% and the 55% of the fresh weigh (FW) respectively, while the remaining weigh were constituted by seeds. The TSS were 8.4 Brix, TA of 0.83% citric acid and pH 3.87; the evaluated ratio (TSS/TA) for maturity requirements was of 10.1. Samples for analysis were prepared from 25 fruits and processed to better reproduce the crude materials used for industrial chinotto extract: the whole fruits were cut in half, seeds removed, softly squeezed discarding the juice (separately analyzed) and homogenated with a Turbo Homogenizer HMHF (PBI International, Milan, Italy). The juice and the homogenate were stored at -20 °C until needed for the study.
Extraction and analysis of flavonoids and furocoumarins. Two grams of homogenate was extracted for 2 h with 10 mL of 50% aqueous methanol containing 0.3% formic acid at room temperature on an orbital shaker. The mixture was centrifuged at 1000rpm for 15 min and the supernatant decanted. The pellets were re-extracted under identical

conditions. Supernatants were combined and used for the LC analyses, after filtration through 0.45µm PTFE filters (LabService Analytica, Bologna, Italy). The juice was analyzed before centrifugation and filtration through 0.45µm PTFE filters. The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Luna C18 250 x 4.6mm, 5 µm i.d. (Phenomenex), the flow rate was 1 mL/min, the column temperature 30°C and the injection volume 20 µL. Flow rate was split 1/10 before MS interface. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43 % B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 190–700 nm, and for quantitative determinations the chromatograms were recorded at 285 nm (flavanones), 310 nm (furocoumarins), 335 nm (polymethoxylated flavones) and 340 nm (flavones). Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative and positive ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 4.0 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 12 (arbitrary units). The MS-MS spectra were obtained using collision energy of 25% of instrument maximum, operating in selected reaction monitoring (SRM). Preliminary positive and negative tunings were carried out with continuous introduction of dilute solutions of sinensetin and naringin respectively, at the flow rate of 5 µL/min and the voltages on the lenses were optimized in TunePlus (Excalibur software).

The chinotto flavonoids were characterized in terms of retention times, lambda max, MS and MS/MS data operating in positive and negative mode as follows:

Compound 1: Rt, 16.9 min; UV, 270, 350 nm. MS, 611 $[M + H]^+$ (100); MS, 609 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 611 $[M + H]^+$ (20), 593 $[M + H-18]^+$ (100), 575 $[M + H - 36]^+$ (10), 545 $[M + H - 66]^+$ (5), 491 $[M + H - 120]^+$ (15), 473 $[M + H - 138]^+$ (15).

Compound 2: Rt, 23.5 min; UV, 270, 335 nm. MS, 595 $[M + H]^+$ (100); MS, 593 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 595 $[M + H]^+$ (20), 577 $[M + H-18]^+$ (100), 559 $[M + H - 36]^+$ (10), 529 $[M + H - 66]^+$ (5), 457 $[M + H - 138]^+$ (10).

Compound 3: Rt, 26.3 min; UV, 270, 350 nm. MS, 625 $[M + H]^+$ (100); MS, 623 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 625 $[M + H]^+$ (70), 607 $[M + H -18]^+$ (100), 559 $[M + H - 66]^+$ (5), 505 $[M + H - 120]^+$ (8), 463 $[M + H -162]^+$ (70), 445 $[M + H -180]^+$ (8).
Compound 4: Rt, 31.8 min; UV, 285, 330 nm. MS, 597 $[M + H]^+$ (100); MS, 595 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 597 $[M + H]^+$ (100), 451 $[M + H - 146]^+$ (65), 289 $[M + H - 308]^+$ (90).
Compound 5: Rt, 37.4 min; UV, 285, 330 nm. MS, 581 $[M + H]^+$ (100); MS, 579 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 581 $[M + H]^+$ (10), 563 $[M + H-18]^+$ (30), 545 $[M + H - 36]^+$ (25), 435 $[M + H - 146]^+$ (65), 419 $[M + H - 162]^+$ (100), 315 $[M + H - 266]^+$ (25), 273 $[M + H - 308]^+$ (10).
Compound 6: Rt, 40.0 min; UV, 265, 335 nm. MS, 579 $[M + H]^+$ (100); MS, 577 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 579 $[M + H]^+$ (50), 432 $[M + H-146]^+$ (50), 273 $[M + H - 308]^+$ (100).
Compound 7: Rt, 40.4 min; UV, 285, 330 nm. MS, 611 $[M + H]^+$ (100); MS, 609 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 611 $[M + H]^+$ (50), 593 $[M + H-18]^+$ (30), 575 $[M + H - 36]^+$ (30), 490 $[M + H - 120]^+$ (15), 449 $[M + H - 162]^+$ (80), 345 $[M + H - 266]^+$ (20), 303 $[M + H - 308]^+$ (70).
Compound 8: Rt, 46.2 min; UV, 285, 330 nm. MS, 725 $[M + H]^+$ (100); MS, 723 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 725 $[M + H]^+$ (5), 707 $[M + H -18]^+$ (100), 461 $[M + H- 264]^+$ (90), 273 $[M + H - 452]^+$ (10). MS-MS focused on $[M - H]^-$, 723 $[M - H]^-$ (100), 661 $[M – H - 62]^-$ (10), 621 $[M – H - 102]^-$ (20), 579 $[M – H - 144]^-$ (80).
Compound 9: Rt, 48.2 min; UV, 285, 325 nm. MS, 755 $[M + H]^+$ (100); MS-MS focused on $[M + H]^+$, 755 $[M + H]^+$ (100), 609 $[M + H- 146]^+$ (30), 301 $[M + H- 452]^+$ (50); MS-MS focused on $[M - H]^-$, 753 $[M - H]^-$ (100); 691 $[M + H - 62]^-$ (10); 651 $[M + H - 102]^-$ (15); 609 $[M + H - 144]^-$ (40).
Compound 10: Rt, 57.4 min; UV, 260, 315 nm. MS, 217 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 217 $[M + H]^+$ (10), 202 $[M + H - 30]^+$ (100).
Compound 11: Rt, 59.3 min; UV, 260 (sh), 310 nm. MS, 355 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 355 $[M + H]^+$ (30), 337 $[M + H - 18]^+$ (100).
Compound 12: Rt, 60.5 min; UV, 335 nm. MS, 373 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 373 $[M + H]^+$ (100), 358 $[M + H - 15]^+$ (50), 312 $[M + H - 61]^+$ (30).

Compound 13: Rt, 61.0 min; UV, 335 nm. MS, 343 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 343 $[M + H]^+$ (100), 328 $[M + H - 15]^+$ (70), 282 $[M + H - 61]^+$ (30).
Compound 14: Rt, 62.0 min; UV, 335 nm. MS, 403 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 403 $[M + H]^+$ (60), 388 $[M + H - 15]^+$ (100), 373 $[M + H - 30]^+$ (50).
Compound 15: Rt, 62.8 min; UV, 335 nm. MS, 433 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 434 $[M + H]^+$ (60), 419 $[M + H - 15]^+$ (100), 403 $[M + H - 30]^+$ (50).
Compound 16: Rt, 63.6 min; UV, 335 nm. MS, 373 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 373 $[M + H]^+$ (40), 358 $[M + H - 15]^+$ (100), 343 $[M + H - 30]^+$ (40).
The external calibrations were obtained using methanolic standard solutions of known concentration (1-100 mg/L). Lucenin-2 and lucenin-2 4'-methyl ether were expressed as diosmetin; vicenin-2 and rhoifolin were expressed as apigenin; meltidin and brutieridin were expressed as naringin and neohesperidin, respectively; bergapten and epoxybergamottin were expressed in terms of bergamottin relative amount; nobiletin, tetramethoxyflavone, heptamethoxylflavone and tangeretin have been determined in terms of sinensetin relative amount.
The total flavonoids content was also determined in all of the samples according to the colorimetric Davis method [8] and the values were expressed as mg naringin/100 g FW (fresh weight).
Determination of the total antioxidant capacity. Aliquots of sample were extracted with phosphate buffer 75 mmol/l (pH 7.4) and after sonicated for 60 s. The extract was centrifuged at 10000 rpm for 10 min at 4°C. The total antioxidant capacity was assayed on the supernatant according to ORAC fluorescein assay [9]. Trolox standards (10–100 µmol/l), fluorescein (7.0 µmol/l), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (63 mmol/l) solutions were prepared prior to use in phosphate buffer (75 mmol/l, pH 7.4). Chinotto samples and different concentrations of Trolox (10-100 µmol/l) were pipetted into 96 well of assay plates and the total volume was adjusted to 40 µl by the addition of buffer. Further, 200 µl of working fluorescein solution was added. The plate was then allowed to equilibrate by incubating for 3 min at 37 °C in the PerkinElmer VICTOR3 V Multilabel Counter 1420 (Milan, Italy). Reaction was initiated by the addition of 20 µl of AAPH solution using the microplate reader's injector. The fluorescence was then monitored kinetically with data taken every three min. The plate was top read at excitation and emission wavelengths of 485 and 535 nm, respectively at 37 °C and at 3

min intervals for 60 min. The standard curve was obtained by plotting Trolox concentrations against the average net area under the curve (AUC) of the three measurements for each concentration. The net AUC corresponding to a sample was calculated by subtracting the AUC of the blank. A standard curve was generated from the net AUC of the Trolox standards and used to assign Trolox equivalence values to the samples. Final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC and are expressed as μM Trolox equivalents per 100 gram of FW.
Statistical analysis. To verify the statistical significance of all parameters the values of means and standard deviation (SD) were calculated. Where it was appropriate, the data by two-way ANOVA were tested. The $P_{value}<0.05$ were adopted as statistically significant. All data are means of five measurements.

RESULTS and DISCUSSION

The *C. myrtifolia* homogenate and juice were investigated by HPLC-PDA-ESI/MS^2 technique. **Figure 1** shows the PDA chromatograms of homogenate and juice at different λ allowing the discrimination of flavanone, furocoumarin and flavone derivatives. All peaks numbered from 1 to 16 were present in both matrixes. The UV spectra, recorded in correspondence with peaks 1, 2, 3 and 6, showed absorptions at 270 and 350 nm (peaks 1 and 3) and 270 and 335 nm (peaks 2 and 6) which can be attributed to bands II and I, respectively, of a flavone structure. The UV spectrum of compounds 4, 5, 7, 8 and 9, showing absorptions centered at 285 and 330 nm, indicated the flavanone nature of the aglycone. Compounds 10 and 11 showed UV absorption maxima at 260 and 310, indicating the probable presence of coumarin structure. The high retention times and UV maxima (335 nm) of peaks from 12 to 16 provided evidence on the assignment of a polymethoxylated flavone skeleton.
The MS/MS spectra recorded in positive and negative mode in correspondence with the studied peaks and the comparison with analytical standards and literature data, when available, permitted to obtain an unambiguous structural assignment for all compounds which were quantified separately in juice and homogenate (**Table 1).**
The results evidenced the presence of the two flavone-*C*-glucosides vicenin-2 and lucenin-2 4’-methyl ether, the flavone-*O*-glycoside rhoifolin, the three flavanone-*O*-glycosides neoeriocitrin, naringin and neohesperidin, the flavanone-3-hydroxy-3-methylglutaryl brutieridin

and the two furocoumarins bergapten and epoxybergamottin (**Figures 2-3-4**). These data are in according with what reported by Barreca *et al.* [3], with the exception of the flavonone-*O*-rutinosides (eriocitrin and narirutin) which were not found in both analyzed juice and homogenate. On the contrary, the flavone-*C*-glucoside luteolin 6,8-di-*C*-glucoside (lucenin-2) and the 3-hydroxy-3-methylglutaryl neohesperidoside of naringenin (melitidin) were identified for the first time in chinotto matrix (peaks 1 and 8) (Figures 2 and 3). The ESI/MS of peak 1 showed ions at m/z 611 and m/z 609 in positive and negative mode respectively, in agreement with the molecular weight of 610 amu of lucenin-2. The further fragmentation of the precursor ion in selected reaction monitoring (SRM) produced a loss of 120 amu, corresponding to the characteristic fragment mass of *C*-glucosyl flavonoids. The positive and negative ESI/MS of peak 8 showed a $[M + H]^+$ ion at m/z 725 and a $[M - H]^-$ ion at m/z 723 respectively, which corresponded to the elemental composition $C_{33}H_{40}O_{18}$. A detailed analysis of peak 8 by tandem mass spectrometry showed highly diagnostic fragment ions. In particular, a structural feature of the negative-ion MS/MS assessed the 3-hydroxy-3-methylglutaryl acid moiety since a peak at m/z 579 was produced by loss of the rhamnose unit and the MS/MS of protonated molecular $[M + H]^+$ showed an ion at m/z 461, which is a typical fragment of flavonoids diglycosides originating from the breakage of the O-C1 and C2-C3 bonds of the hexose directly attached to the aglycon. Moreover, the loss of 3-hydroxy-3-methylglutaryl moiety was confirmed by the positive daughter ion at m/z 273. The discussed MS data and the UV spectra of peaks 1 and 8 are in agreement with those reported in literature for lucenin-2 [10] and melitidin molecules [10, 11]. The *C*-glucosyl flavone lucenin-2 has been recently reported as constituent of the *C. aurantium L.* (sour orange) juice. Recently, Di Donna *et al.* [11] isolated and identified in *C. bergamia* the two 3-hydroxy-3-methylglutaric acid conjugates of neohesperidin and naringin, namely, brutieridin and melitidin. As previously reported, brutieridin was detected for the first time in *C. myrtifolia* by Barreca *et al.* [3]. It is not entirely surprising because both *C. myrtifolia* and *C. bergamia* are taxonomically closed to *C. aurantium L.*: chinotto is regarded as a mutation of sour orange, whereas bergamot is a cross between sour orange and citron (*C. medica L.*) [3].

Moreover, the present paper reported firstly the occurrence of polymethoxylated flavones (PMFs) (Figure 2) in *C. myrtifolia*. PMFs are a class of minor components usually found in the essential oils fraction of citrus peels [12]. Mass spectra generated in positive mode

showed $[M+H]^+$ ions at m/z 343, 403, 373 and 433, well-matched in fragmentation, in UV-vis spectra and retention times with what previously reported by Scordino *et al.* [13]. The parent ions $[M+H]^+$ were further fragmented and the breakage of methyl and methoxy groups were in agreement with the loss of 15 and 30 amu.

Quantitative evaluation. Differently from the juice, the chinotto homogenate was a complex matrix composed of peel, albedo and pulp tissues and consequently a different amount and relative distribution of detected compounds was expected. Neoeriocitrin, naringin and neohesperidin are the flavanone-*O*-glycosides found in the highest amounts in chinotto fruit (**Table 1**), ranging from about 30 to 79 mg/100g in the homogenate and from about 9 to 14 mg/100mL in the juice. The two 3-hydroxy-3-methylglutaryl flavanone glycosides melitidin and brutieridin were found to be present in significant amounts (9.5 and 19.1 mg/100g in the homogenate respectively and about 2.5 mg/100mL in the juice). Small amounts of the flavone-*C*-glucosides lucenin-2, vicenin-2 and lucenin-2 4'-methyl ether were also present in both samples, together with the two furocoumarins bergapten and epoxybergamottin. These data confirmed the relationship of chinotto with bergamot and sour orange, because vicenin-2, lucenin-2 4'-methyl ether, brutieridin, melitidin, bergapten and epoxybergamottin are characteristics of these *Citrus* fruits [10, 11].

The homogenate was also characterized by a small but distinctive amount of polymethoxylated flavones, among which nobiletin predominated; the same components are detectable in traces in the juice. It's well known that flavonoids contents were usually higher in peels [5], therefore the total amount of flavonoids in the homogenate (215 mg/100g) was five times higher than those of the juice (46 mg/100mL), as expected. Literature data for chinotto juice reported values of total juice HPLC flavonoids ranging from 6 mg/100mL [3] to about 200 mg/100mL [2]. The flavonoid contents were also determined in all samples as indicated by Association of the Industry of the Juices and Nectars (AIJN) according to the colorimetric Davis method [10]; data obtained were about 780 mg/100g and 103 mg/100mL for the homogenate and the juice, respectively. Taking into account the relative distribution of the above discussed flavonoids in the two matrixes (**Figure 5**) no considerable changes could be observed between the homogenate and the juice apart from a dissimilar distribution in the flavone-*C*-glucosides and in the neoeriocitrin amounts which predominated in the juice.

The studied chinotto fruit showed high antioxidant effect, measured with ORAC method (**Table 1**). Data obtained were of 5872 µM Trolox equivalents/100g and 506 µM Trolox equivalents/100g for the homogenate and the juice respectively. The different antioxidant activity of the two studied matrixes could be mostly attributed to their different content of flavonoids; the positive correlation between the antioxidant activities of fruits with the presence of high concentration of flavoinoid contents was thoroughly investigated in the last decades [14, 15], although the antioxidative capacity of a phytochemical in vitro does not give reliable information on its in vivo action because many flavonoids have a very low oral bioavailability [16].
In conclusion, our results elucidate for the first time the distribution of flavonoids and coumarins in the *C. myrtifolia* crude homogenate used for beverage industrial purposes comparing the obtained data with those of the juice. Because of the presence of a great quantity of peel and albedo tissues in the homogenate, the polyphenolic amount in the homogenate was higher than in the juice even though their relative distribution did not changed significantly. Nowadays these aspects are considered to be highly valuable for the commercial valorization of chinotto.
Further works are in progress in our laboratory to elucidate the polyphenolic composition of different chinotto tissues [17] and to detect chinotto homogenate in commercial chinotto brands. The matrix effect due to the sweeteners, caramel color and preservatives, together with the sample dilution, require purification and concentration steps before polyphenolics determination in commercial drinks. The results of preliminary studies (data not shown) have evidenced the detection of the main chinotto flavonoids (vicenin-2, neoeriocitrin, naringin, neohesperidin, melitidin and brutieridin) with a total amount ranging from 1 to 5 mg/100mL depending on the analyzed brand.

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Figure Captions

Figure 1. Typical PDA chromatograms of *C. myrtifolia* beverage processing homogenate and juice from immature fruits recorded at 280 (**A**, **B**) and 330 nm (**C**, **D**). Compounds **1-16** were identified as follows: **1**, lucenin-2; **2**, vicenin-2; **3**, lucenin-2 4'-methyl ether; **4**, neoeriocitrin; **5**, naringin; **6**, rhoifolin; **7**, neohesperidin; **8**, melitidin; **9**, brutieridin; **10**, bergapten; **11**, epoxybergamottin; **12**, sinensetin; **13**, tetramethoxyflavone; **14**, nobiletin; **15**, heptamethoxyflavone; **16**, tangeretin.

Figure 2. Structure assignments of identified flavones.

Figure 3. Structure assignments of identified flavanones.

Figure 4. Structure assignments of identified furocoumarins.

Figure 5. Relative distribution (%) of flavonoids in *C. mirtyfolia* beverage processing homogenate and juice.

Table 1. Polyphenolic contents in *Citrus x myrtifolia* beverage processing homogenate and juice.

	Compound	Homogenate Mean Value (mg/100g FW)[a]	Juice Mean Value (mg/100mL FW)[a]
1	lucenin-2 [b]	0.8 (0.1)	0.65 (0.03)
2	vicenin-2 [c]	3.7 (0.2)	2.3 (0.2)
3	lucenin-2 4'-OMe [b]	1.0 (0.1)	1.2 (0.1)
4	neoeriocitrin	31.1 (1.3)	9.2 (0.6)
5	naringin	70.6 (2.7)	14.3 (0.8)
6	rhoifolin [c]	4.8 (0.3)	0.52 (0.03)
7	neohesperidin	66.0 (3.0)	11.8 (0.7)
8	melitidin [d]	9.5 (0.4)	2.1 (0.1)
9	brutieridin [e]	19.1 (0.9)	3.2 (0.2)
10	bergapten [f]	2.3 (0.6)	0.21 (0.02)
11	epoxybergamottin [f]	2.1 (0.3)	0.30 (0.05)
12	sinensetin	0.44 (0.03)	0.20 (0.02)
13	tetramethoxyflavone [g]	0.53 (0.02)	traces
14	nobiletin [g]	2.62 (0.22)	0.08 (0.02)
15	heptamethoxyflavone [g]	0.11 (0.02)	traces
16	tangeretin [g]	0.82 (0.05)	traces
	Total flavonoids (HPLC)	214.8 (4.4)	46.0 (1.3)
	Total flavonoids (according to Davis)	780.3 (13.7)	102.6 (5.1)
	ORAC[h] total antioxidant activity (μmol T.E.[i]/100 g)	5872 (351)	506 (24)

[a] mean value of five determinations; standard deviation in parentheses.
[b] expressed as diosmetin;
[c] expressed as apigenin;
[d] expressed as naringin;
[e] expressed as neohesperidin;
[f] expressed as bergamottin;
[g] expressed as sinensetin.
[h] Oxygen Radical Absorbance Capacity.
[i] Trolox equivalents.

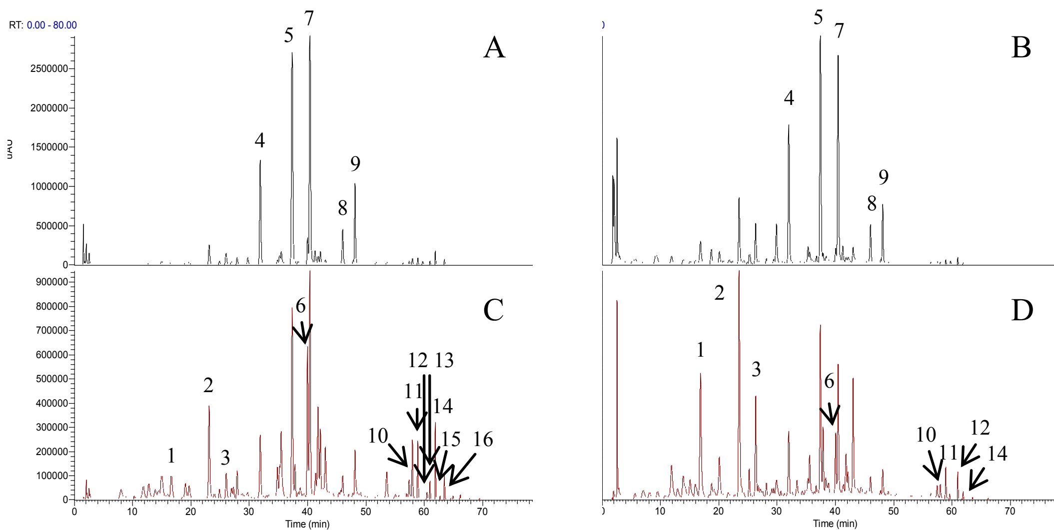

Figure 1.

Peak	Structure assignment	R1	R2	R3	R4	R5	R6	R7	Molecular Weight
1	Luteolin 6,8-di-*C*-glucoside (Lucenin-2)	H	OH	Glu	OH	Glu	OH	OH	610.52
2	Apigenin 6,8-di-*C*-glucoside (Vicenin-2)	H	OH	Glu	OH	Glu	H	OH	594.52
3	Diosmetin 6,8-di-*C*-glucoside (Lucenin-2 4'-Me)	H	OH	Glu	OH	Glu	OH	OCH_3	624.55
6	Apigenin 7-*O*-neohesperidoside (Rhoifolin)	H	OH	H	*O*-Neohesp	H	H	OH	578.53
12	3',4',5,6,7-Pentamethoxyflavone (Sinensetin)	H	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	372.37
13	3',4',5,6,7,8-Hexamethoxyflavone (Nobiletin)	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	402.39
14	5,6,7,4'-tetramethoxyflavone (Scutellarein tetramethylether)	H	OCH_3	OCH_3	OCH_3	H	H	OCH_3	342.35
15	3,5,6,7,8,3',4'-heptamethoxylflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	432.42
16	4',5,6,7,8-Pentamethoxyflavone (Tangeretin)	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	372.37

Figure 2.

Peak	Structure assignment	R1	R2	R3	Molecular Weight
4	Eriodictyol 7-*O*-neohesperidoside (Neoeriocitrin)	*O*-Neohesp	OH	OH	596.54
5	Naringenin 7-*O*-neohesperidoside (Naringin)	*O*-Neohesp	H	OH	580.53
7	Hesperetin 7-*O*-neohesperidoside (Neohesperidin)	*O*-Neohesp	OH	OCH_3	610.56
8	Naringenin 7-(2″-α-rhamnosyl-6″-(3″″-hydroxy-3″″-methylglutaryl)-β-glucoside) (Melitidin)	3-Hydroxy-3-Methylglutaryl	OH	OCH_3	724.23
9	Hesperetin 7-(2″-α-rhamnosyl-6″-(3″″-hydroxy-3″″-methylglutaryl)-β-glucoside) (Brutieridin)	3-Hydroxy-3-Methylglutaryl	H	OH	754.24

Figure 3.

Peak	Structure assignment	R	Molecular Weight
10	5-Methoxypsoralen (Bergapten)	OCH_3	216.19
11	5-(6’,7’-Epoxy)geranyloxypsoralen (Epoxybergamottin)		354.40

Figure 4.

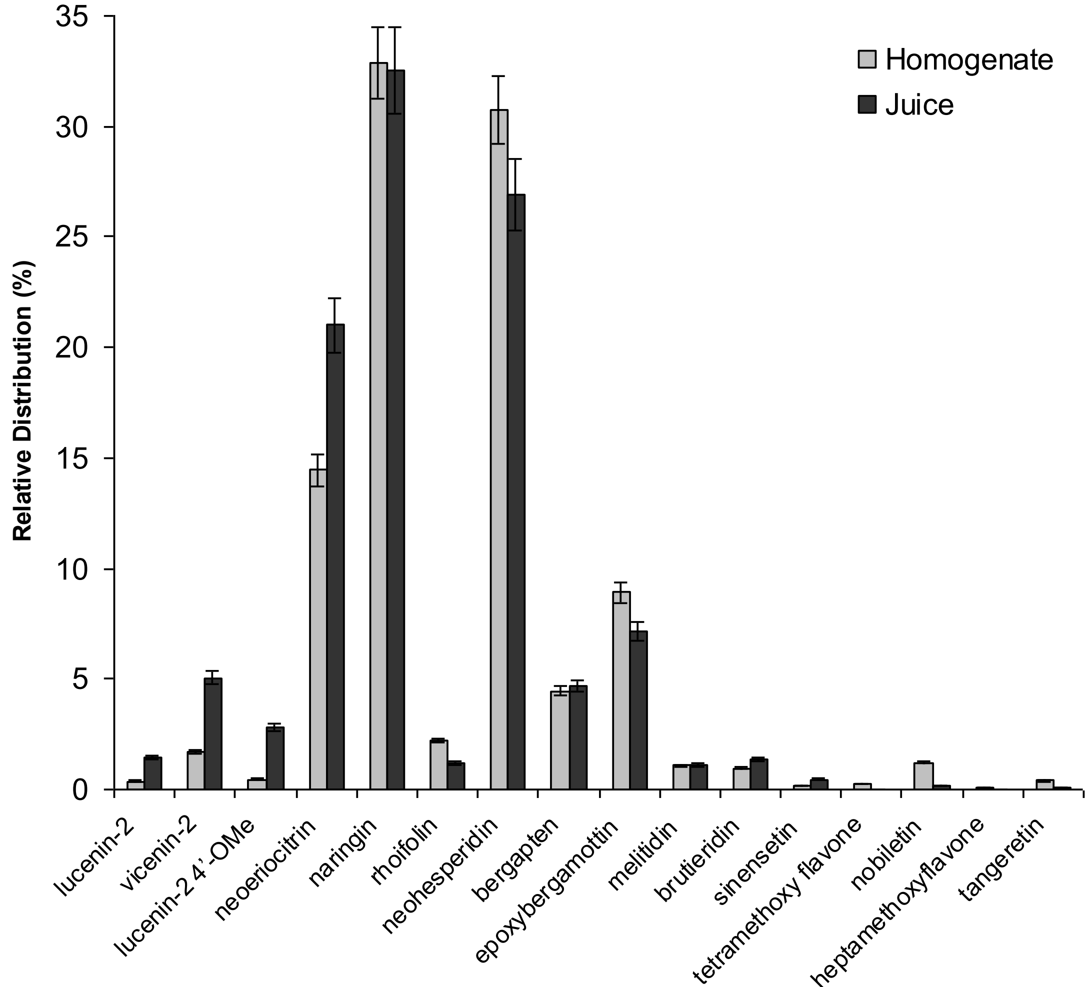

Figure 5.

Paper No 4

Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano.

Characterization of polyphenolic compounds in unripe chinotto (*Citrus myrtifolia*) fruit by HPLC/PDA/ESI/MS-MS.

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CHARACTERIZATION OF POLYPHENOLIC COMPOUNDS IN UNRIPE CHINOTTO (*Citrus myrtifolia*) FRUIT BY HPLC/PDA/ESI/MS-MS.

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BSTRACT:

The flavonoids and the furocoumarins composition of peel and pulp tissues of unripe fruits of *Citrus* × *myrtifolia Rafinesque* was investigates, ingredient of the popular soft drink Chinotto. The compounds separation was made by HPLC-PDA detector coupled to ESI/MS/MS in positive and negative mode. Eighteen compounds (3-hydroxy-3-methylglutaryl-, *C*- and *O*-glycosyl flavonoids, furocoumarins and polymethoxylated flavones) were identified and quantified. Data indicated that the overall amount of flavonoids and furocoumarins compound in peel were higher than in the pulp, even if their relative distribution does not significantly change apart from a lower relative content of naringin in the peel.

Keywords: HPLC/PDA/ESI/MS-MS, chinotto, citrus, flavonoids, furocoumarins.

INTRODUCTION

Over the past few years, increasing attention has been paid by consumers to the nutritional aspects of products containing significant amounts of biologically active components. Nowadays, many studies are carried out on the thousands of natural phytochemicals that may have important physiological effects [1]. Scientific data demonstrated that an increased consumption of fruit and vegetables may protect against degenerative pathologies such as cancer and atherosclerosis [2]. Epidemiological studies have shown an inverse relationship between dietary flavonoids intake and cardiovascular diseases [3]. Many citrus flavonoids have been shown to have antioxidative activity, to inhibit angiogenesis, and to slow down cancer cell migration and proliferation [4, 5]. Among the phytochemicals, flavonoids are widely contained in *Citrus* fruits [3]. Recently our research group has studied the unripe fruits of *Citrus* × *myrtifolia Rafinesque* in terms of bioactive compounds and antioxidant activity; results indicated that it is a good source of phytochemicals, mainly vitamin C and flavonoids [6]. *Citrus* × *myrtifolia* is a species of *Citrus* with foliage similar to that of the common myrtle. It is a compact tree with small leaves and no thorns which grows to a height of three meters and can be found in Malta and in the Liguria, Tuscany, Sicily, and Calabria regions of Italy. The fruit of the tree resemble small oranges. They are sour or bitter and are commonly called by their Italian name, chinotto. They are an essential flavoring agent of most Italian amari, of the popular Campari aperitif, and of several brands of carbonated soft drinks that are generically called "chinotto". Native of southern China, its origin has not been exactly ascertained; probably it is mutation of sour orange that eventually evolved into the species known today [7]. The scientific literature papers about the *C. myrtifolia* [8, 9] are related prevalently to the juice and no information are available about bioactive compounds quantitation and distribution in the whole fruit. The have of interest in the Italian citrus industry for chinotto production could be increased by studies on its composition.
The aim of the present work was to elucidate by HPLC/PDA/ESI/MS-MS technique the identity and the relative distribution of flavonoids and furocoumarins in pulp and peel tissues of unripe *C. myrtifolia* used for beverage industrial purposes.

MATERIALS AND METHODS

Reagents and Standards. HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system. Apigenin, bergamottin, neodiosmin, neoeriocitrin,

naringin, neohesperidin, poncirin and sinensetin were obtained from Extrasynthèse (Genay, France).

Plant materials. Fruits used in this study were grown in Castiglione di Sicilia (Catania, Italy). Immature *Citrus x myrtifolia Rafinesque* (chinotto) fruits were picked up on 2010 October with the degree of fruit maturity determined from the Hunter color parameters, mean diameter, total soluble solids (TSS), titratable acidity (TA) and pH (**Table 1**). Samples for analysis were prepared from 25 fruits; pulp and peel tissues were separated and analyzed individually. The samples were stored at -20 °C until needed for the study.

Extraction and analysis of flavonoids and furocoumarins. Five grams of sample was extracted for 2 h with 10 mL of 50% aqueous methanol containing 0.3% formic acid at room temperature on an orbital shaker. The mixture was centrifuged at 1000rpm for 15 min and the supernatant decanted. The pellets were re-extracted under identical conditions. Supernatants were combined and used for the LC analyses, after filtration through 0.45µm PTFE filters (LabService Analytica, Bologna, Italy). The juice was analyzed before centrifugation and filtration through 0.45µm PTFE filters. The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Luna C18 250 x 4.6mm, 5 µm i.d. (Phenomenex), the flow rate was 1 mL/min, the column temperature 30°C and the injection volume 20 µL. Flow rate was split 1/10 before MS interface. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43 % B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 190–700 nm, and for quantitative determinations the chromatograms were recorded at 285 nm (flavanones), 310 nm (furocoumarins), 335 nm (polymethoxylated flavones) and 340 nm (flavones). Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative and positive ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 4.0 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 12 (arbitrary units). The MS-MS spectra were obtained using collision energy of 25% of instrument maximum, operating in selected reaction monitoring (SRM). Preliminary positive and negative tunings were carried out with continuous introduction of dilute solutions of sinensetin and naringin respectively, at the flow rate of 5 µL/min and the voltages on the lenses were optimized in TunePlus (Excalibur software).

The chinotto flavonoids were characterized in terms of retention times, lambda max, MS and MS/MS data operating in positive and negative mode as follows:

Compound 1: Rt, 16.9 min; UV, 270, 350 nm. MS, 611 $[M + H]^+$ (100); MS, 609 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 611 $[M + H]^+$ (20), 593 $[M + H\text{-}18]^+$ (100), 575 $[M + H - 36]^+$ (10), 545 $[M + H - 66]^+$ (5), 491 $[M + H - 120]^+$ (15), 473 $[M + H - 138]^+$ (15).

Compound 2: Rt, 23.5 min; UV, 270, 335 nm. MS, 595 $[M + H]^+$ (100); MS, 593 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 595 $[M + H]^+$ (20), 577 $[M + H\text{-}18]^+$ (100), 559 $[M + H - 36]^+$ (10), 529 $[M + H - 66]^+$ (5), 475 $[M + H - 120]^+$ (15), 457 $[M + H - 138]^+$ (10).

Compound 3: Rt, 26.3 min; UV, 270, 350 nm. MS, 625 $[M + H]^+$ (100); MS, 623 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 625 $[M + H]^+$ (70), 607 $[M + H -18]^+$ (100), 559 $[M + H - 66]^+$ (5), 505 $[M + H - 120]^+$ (8), 463 $[M + H -162]^+$ (70), 445 $[M + H -180]^+$ (8).

Compound 4: Rt, 29.9 min; UV, 285, 330 nm. MS, 597 $[M + H]^+$ (100); MS, 595 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 597 $[M + H]^+$ (100), 451 $[M + H - 146]^+$ (40), 289 $[M + H - 308]^+$ (80).

Compound 5: Rt, 31.8 min; UV, 285, 330 nm. MS, 597 $[M + H]^+$ (100); MS, 595 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 597 $[M + H]^+$ (100), 451 $[M + H - 146]^+$ (65), 289 $[M + H - 308]^+$ (90).

Compound 6: Rt, 37.4 min; UV, 285, 330 nm. MS, 581 $[M + H]^+$ (100); MS, 579 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 581 $[M + H]^+$ (10), 563 $[M + H\text{-}18]^+$ (30), 545 $[M + H - 36]^+$ (25), 435 $[M + H - 146]^+$ (65), 419 $[M + H - 162]^+$ (100), 315 $[M + H - 266]^+$ (25), 271 $[M + H - 308]^+$ (10).

Compound 7: Rt, 40.0 min; UV, 265, 335 nm. MS, 579 $[M + H]^+$ (100); MS, 577 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 579 $[M + H]^+$ (50), 432 $[M + H\text{-}146]^+$ (50), 273 $[M + H - 308]^+$ (100).

Compound 8: Rt, 40.4 min; UV, 285, 330 nm. MS, 611 $[M + H]^+$ (100); MS, 609 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 611 $[M + H]^+$ (50), 593 $[M + H\text{-}18]^+$ (30), 575 $[M + H - 36]^+$ (30), 490 $[M + H - 120]^+$ (15), 449 $[M + H - 162]^+$ (80), 345 $[M + H - 266]^+$ (20), 303 $[M + H - 308]^+$ (70).

Compound 9: Rt, 42.2 min; UV, 255, 340 nm. MS, 609 $[M + H]^+$ (100); MS, 607 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 609 $[M + H]^+$ (50), 463 $[M + H -146]^+$ (40), 302 $[M + H\text{-}\ 308]^+$ (100). MS-MS focused on $[M - H]^-$, 607 $[M - H]^-$ (40), 299 $[M – H - 308]^-$ (100).

Compound 10: Rt, 46.2 min; UV, 285, 330 nm. MS, 725 $[M + H]^+$ (100); MS, 723 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 725 $[M + H]^+$ (5), 707 $[M + H -18]^+$ (100), 461 $[M + H\text{-}\ 264]^+$ (90), 273 $[M + H - 452]^+$ (10). MS-MS focused on $[M - H]^-$, 723 $[M - H]^-$ (100), 661 $[M – H - 62]^-$ (10), 621 $[M – H - 102]^-$ (20), 579 $[M – H - 144]^-$ (80).

Compound 11: Rt, 48.2 min; UV, 285, 325 nm. MS, 755 $[M + H]^+$ (100); MS-MS focused on $[M + H]^+$, 755 $[M + H]^+$ (100), 609 $[M + H\text{-}\ 146]^+$ (30),

301 $[M + H- 452]^+$ (50); MS-MS focused on $[M - H]^-$, 753 $[M - H]^-$ (100); 691 $[M + H - 62]^-$ (10); 651 $[M + H - 102]^-$ (15); 609 $[M + H - 144]^-$ (40).
Compound 12: Rt, 51.7 min; UV, 285, 330 nm. MS, 595 $[M + H]^+$ (100); MS, 593 $[M - H]^-$ (100). MS-MS focused on $[M + H]^+$, 595 $[M + H]^+$ (20), 577 $[M + H-18]^+$ (30), 559 $[M + H-36]^+$ (40), 449 $[M + H-146]^+$ (70), 433 $[M + H-162]^+$ (100), 287 $[M + H-308]^+$ (25). MS-MS focused on $[M - H]^-$, 593 $[M - H]^-$ (10), 473 $[M – H-120]^-$ (30), 431 $[M – H-162]^-$ (15), 327 $[M – H-266]^-$ (30), 285 $[M – H-308]^-$ (100).
Compound 13: Rt, 57.4 min; UV, 260, 315 nm. MS, 217 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 217 $[M + H]^+$ (10), 202 $[M + H - 30]^+$ (100).
Compound 14: Rt, 59.3 min; UV, 260 (sh), 310 nm. MS, 355 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 355 $[M + H]^+$ (30), 337 $[M + H - 18]^+$ (100).
Compound 15: Rt, 61.3 min; UV, 335 nm. MS, 373 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 373 $[M + H]^+$ (100), 358 $[M + H - 15]^+$ (50), 312 $[M + H - 61]^+$ (30).
Compound 16: Rt, 62.0 min; UV, 335 nm. MS, 403 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 403 $[M + H]^+$ (60), 388 $[M + H - 15]^+$ (100), 373 $[M + H - 30]^+$ (50).
Compound 17: Rt, 62.8 min; UV, 335 nm. MS, 433 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 434 $[M + H]^+$ (60), 419 $[M + H - 15]^+$ (100), 403 $[M + H - 30]^+$ (50).
Compound 18: Rt, 63.6 min; UV, 335 nm. MS, 373 $[M + H]^+$ (100). MS-MS focused on $[M + H]^+$, 373 $[M + H]^+$ (40), 358 $[M + H - 15]^+$ (100), 343 $[M + H - 30]^+$ (40).
The external calibrations were obtained using methanolic standard solutions of known concentration (1-100 mg/L). Lucenin-2, lucenin-2 4'-methyl ether were expressed as neodiosmin; vicenin-2 and rhoifolin were expressed as apigenin; meltidin and brutieridin were expressed as naringin and neohesperidin, respectively; bergapten and epoxybergamottin were expressed in terms of bergamottin relative amount; nobiletin, heptamethoxylflavone and tangeretin have been determined in terms of sinensetin relative amount.
The total flavonoids content was also determined in all of the samples according to the colorimetric Davis method [10] and the values were expressed as mg naringin/100 g FW (fresh weight).
Statistical analysis. To verify the statistical significance of all parameters the values of means and standard deviation (SD) were calculated. Where it was appropriate, the data by two-way ANOVA were tested. The $P_{value}<0.05$ were adopted as statistically significant. All data are means of five measurements.

RESULTS and DISCUSSION

Figure 1 shows the PDA chromatograms of unripe *C. myrtifolia* pulp and peel at different λ allowing for the discrimination of flavanone, furocoumarin and flavone derivatives. All peaks identified were numbered from 1 to 18. The UV spectra, recorded in correspondence with peaks 1, 2, 3, 7 and 9 showed absorptions at 270 and 350 nm (peaks 1, 3 and 9) and 270 and 335 nm (peaks 2 and 7) which can be attributed to bands II (due to the A ring benzoyl system) and I (associated with absorption due to the B ring cinnamoyl system) of a flavone structure, respectively. The UV spectra of compounds 4, 5, 6, 8, 10, 11 and 12 indicated the flavanone nature of the aglycone, showing absorptions centered at 285 and 330 nm. Compounds 13 and 14 showed UV absorption maxima at 260 and 310, indicating a possible coumarin mojety. The lag-times and UV maxima (335 nm) of peaks from 15 to 18 suggested the presence of less polar polimetoxylated flavones.
The MS/MS spectra recorded in positive and negative mode in correspondence with the studied peaks and the comparison with analytical standards and literature data, when available, permitted to obtain an unambiguous structural assignment for all compounds (**Figures 2, 3 and 4**) which were quantified separately in pulp and peel (**Table 2**).
Peaks 1-3 were identified as the flavone-*C*-glucosides lucenin-2, vicenin-2 and lucenin-2 4'-methyl ether. The precursor ions (m/z 611 ($[M+H]^+$) and 609 ($[M-H]^-$); m/z 595 ($[M+H]^+$) and 593 ($[M-H]^-$); 625 ($[M+H]^+$) and 623 ($[M-H]^-$) for 1, 2 and 3, respectively) were in agreement with their nominal masses. The product ions showed the characteristic loss of 120 amu, ascribable to the fragment mass of *C*-glucosyl flavonoids. These flavones were recently detected in chinotto juice [9, 11] and in taxonomically closed Citrus juices such as bergamot and sour orange [12-14]. The peak 7 was assigned to the flavone-*O*-glycoside rhoifolin as confirmed by the molecular fragmentation pattern, which showed the precursor ion at 579 m/z and the loss of *O*-diglycoside fragment mass at 273 m/z. Compound 9 was identified as a diosmetin aglycone *O*-linked with saccharide substituents; MS/MS fragmentation pattern indicated the loss of a rhamnose unit ($[M+H-146]^+$) and a further loss of a glucose unit ($[M+H-308]^+$), revealing that the substituent is a rhamnose–glucose disaccharide. Coelution with a standard allowed us to assign to this peak the structure of neodiosmin (7-*O*-neohesperidosil diosmetin). Neodiosmin presence in *Citrus* was earlier reported by Gattuso *et al*. in *Citrus bergamia Risso* juice [13].
Peaks 4 was ascribed to the flavanone *O*-rutinoside eriocitrin, which have a rutinose (ramnosyl-α-1,6 glucose) residue. Peaks 5, 6, 8 and 12 were recognized as the neohesperidosides flavanones neoeriocitrin, naringin, neohesperidin and poncirin respectively; they consist of a flavanone with neohesperidose (rhamnosyl-α-1,2 glucose) and are responsible of the characteristic fruit bitter taste. All flavanones compounds showed the

characteristic loss of 308 amu, corresponding to the *O*-diglycoside breakage. Moreover, two 3-hydroxy-3-methylglutaric acid conjugates of neohesperidin and naringin, namely, brutieridin and melitidin were identified as peaks 10 and 11 respectively. The positive and negative ESI/MS showed a $[M + H]^+$ ion at m/z 725 and 755 and a $[M - H]^-$ ion at m/z 723 and 753, which corresponded to the elemental composition of melitidin and brutieridin, respectively. A detailed analysis by tandem mass spectrometry showed highly diagnostic fragment ions: the loss of 3-hydroxy-3-methylglutaryl moiety was confirmed by the positive daughter ion at m/z 273 for melitidin and at m/z 303 for brutieridin. These data are in agreement in *C. myrtifolia* by Barreca *et al.* [9] and Scordino *et al.* [11] for chinotto juice.
Finally, the present paper reported the occurrence of polymethoxylated flavones (PMFs) in *C. myrtifolia* (peaks 15-18). PMFs are a class of minor components usually found in the essential oils fraction of citrus peels [15]. Mass spectra generated in positive mode showed $[M+H]^+$ ions at m/z 403, 373 and 433, well-matched in fragmentation, in UV-vis spectra and retention times with what previously reported by Scordino *et al.* [16]. The parent ions $[M+H]^+$ were further fragmented and the breakage of methyl and methoxy groups were in agreement with the loss of 15 and 30 amu. Compounds from 16 to 19 were identified as sinensetin, nobiletin, heptametoxyflavone and tangeretin respectively.
Quantitative evaluation. Naringin and neohesperidin are the flavanone-*O*-glycosides found in the highest amounts in chinotto fruit (**Table 2**), ranging from about 200 to 175 mg/100g in the pulp and from about 347 to 472 mg/100g in the peel respectively. Eriocitrin (6.9 mg/100g) and poncirin (5.6 mg/100g) were detected only in pulp, while neoericitrin was found in both analyzed matrixes in significant quantity (58.9 and 131.5 mg/100g in pulp and peel, respectively). The two 3-hydroxy-3-methylglutaryl flavanone glycosides melitidin and brutieridin were found to be present in great amounts (14.2 and 28.6 mg/100g in the pulp respectively and 30.3 and 95.4 mg/100g in the peel). The flavones *O*-glycosides rhoifolin and neodiosmin were the most abundant flavones of chinotto tissues, ranging from about 7 mg/100g in the pulp to about 29.5 and 57.4 mg/100g in the peel respectively. The peel was also characterized of about 30 mg/100g of the flavone-*C*-glucosides lucenin-2, vicenin-2, lucenin-2 4'-methyl ether; on the contrary, only traces of these compounds were detected in the pulp. The total amount of the two furocoumarins bergapten and epoxybergamottin in the peel (24.3 mg/100g) was two times of the the pulp quantity (12.0 mg/100g). A small but distinctive amount of polymethoxylated flavones (16.4 mg/100g), among which nobiletin predominated was detected in the peel; the same components are detectable in traces in the peel. It's well known that flavonoids contents were usually higher in peels [3], therefore it is not surprising that the total amount of flavonoids and furocoumarins in the peel (1235 mg/100g) was more than two times higher than those of the

pulp (519 mg/100g). The flavonoid contents were also determined in all samples as indicated by Association of the Industry of the Juices and Nectars (AIJN) according to the colorimetric Davis method [10]; data obtained were about 423.7 mg/100g and 736.4 mg/100g for the pulp and the peel, respectively. Taking into account the relative distribution of the different classes above discussed in the two tissues (**Figure 5**), no considerable changes could be observed between the pulp and the peel contribution to the total flavonoids apart from some differences in the relative distribution of the flavones and in the naringin amounts which predominated in the pulp.
In conclusion, our results elucidate the distribution of flavonoids and furocoumarins in *C. myrtifolia* peel and pulp. The HPLC-PDA-ESI/MS^2 technique allowed the identification and relative quantification of 18 compounds, among which many compounds have been detected for the first time in *C. myrtifolia*. Nowadays these aspects are considered to be highly valuable for the commercial valorization of chinotto as a citrus with high potential as nutraceutical source.

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Figure Captions

Figure 1. Typical PDA chromatograms of *C. myrtifolia* pulp and peel from immature fruits recorded at 280 (**A**, **B**) and 330 nm (**C**, **D**). Compounds **1-18** were identified as follows: **1**, lucenin-2; **2**, vicenin-2; **3**, lucenin-2 4'-methyl ether; **4**, neoeriocitrin; **5**, naringin; **6**, naringin; **7** rhoifolin; **8**, neohesperidin; **9**, neodiosmin; **10** melitidin; **11**, brutieridin; **12**, poncirin; **13,** bergapten; **14**, epoxybergamottin; **15**, sinensetin; **16**, nobiletin; **17**, heptamethoxyflavone; **18**, tangeretin.

Figure 2. Structure assignments of identified flavones.

Figure 3. Structure assignments of identified flavanones.

Figure 4. Structure assignments of identified furocoumarins.

Figure 5. Relative distribution (%) of flavonoids and furocoumarins in *C. mirtyfolia* pulp and peel.

Table 1. Polyphenolic contents in *Citrus x myrtifolia Raf*. (Chinotto) pulp and peel.

	Compound	Pulp Mean Value (mg/100g FW)[a]	Peel Mean Value (mg/100g FW)[a]
1	lucenin-2 [b]	traces	4.0 (0.2)
2	vicenin-2 [c]	0.03 (0.01)	21.3 (1.3)
3	lucenin-2 4'-OMe [b]	1.9 (0.2)	4.6 (0.2)
4	eriocitrin	6.9 (0.3)	traces
5	neoeriocitrin	58.9 (3.3)	131.5 (6.7)
6	naringin	200.3 (10.7)	347.0 (17.6)
7	rhoifolin [c]	6.6 (0.4)	29.5 (1.6)
8	neohesperidin	175.7 (7.3)	472.4 (21.9)
9	neodiosmin	7.4 (0.3)	57.4 (1.8)
10	melitidin [d]	14.2 (0.8)	30.3 (1.5)
11	brutieridin [e]	28.6 (0.9)	95.4 (5.2)
12	poncirin	5.6 (0.3)	traces
13	bergapten [f]	7.0 (0.6)	12.0 (0.7)
14	epoxybergamottin [f]	5.0 (0.1)	13.1 (0.6)
15	sinensetin	0.24 (0.02)	1.8 (0.2)
16	nobiletin [g]	0.91 (0.12)	11.2 (0.5)
17	heptamethoxyflavone [g]	traces	0.39 (0.03)
18	tangeretin [g]	traces	3.0 (0.1)
	Total flavonoids (HPLC)	519.1 (13.5)	1235.0 (29.5)
	Total flavonoids (according to Davis)	423.7 (22.7)	736.4 (29.7)

[a] mean value of five determinations; standard deviation in parentheses.
[b] expressed as neodiosmin;
[c] expressed as apigenin;
[d] expressed as naringin;
[e] expressed as neohesperidin;
[f] expressed as bergamottin;
[g] expressed as sinensetin.

Table 2. Parameters of the studied unripe *Citrus x myrtifolia Raf*. (Chinotto).

Parameter	Mean Value[a]	
Hunter colour parameters	Peel	Pulp
a	36.3 (6.2)	5.4 (1.5)
b	-34.4 (4.2)	-14.3 (2.7)
L	66.3 (2.2)	78.8 (2.4)
Mean diameter (cm)	2.5 (0.3)	
Mean weight (g)	27 (2)	
Total soluble solids (Brix)[b]	8.4 (0.1)	
Tritable acidity (% citric acid) [b]	0.83 (0.05)	
pH [b]	3.87 (0.21)	
Ratio (Brix/acidity) [b]	10.1	

[a] mean value of five determinations; standard deviation in parentheses.
[b] referred to the juice.

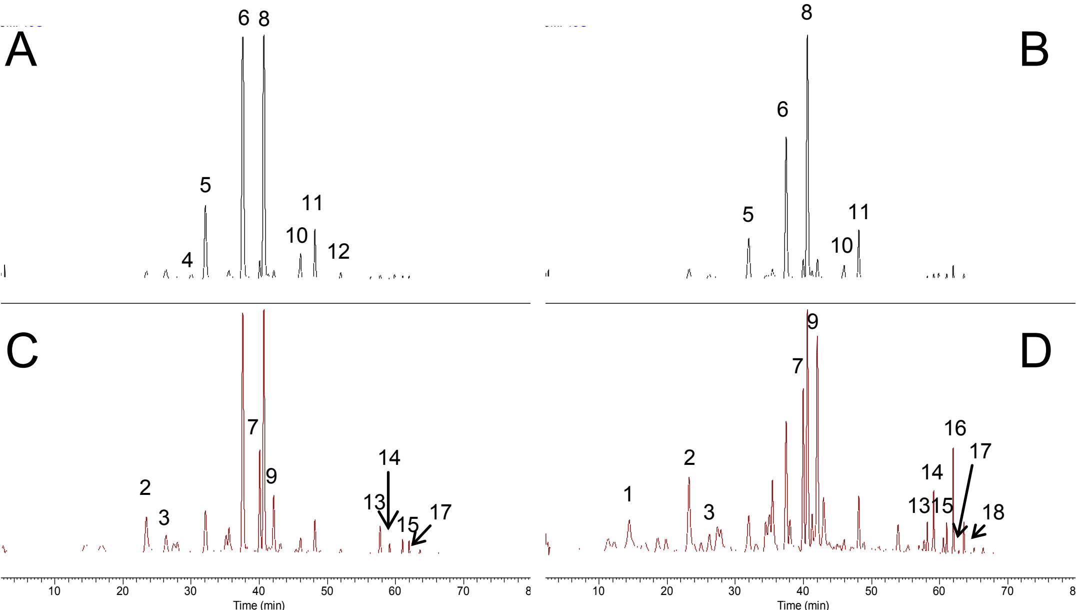

Figure 1.

Peak	Structure assignment	R1	R2	R3	R4	R5	R6	R7
1	Luteolin 6,8-di-*C*-glucoside (Lucenin-2)	H	OH	Glu	OH	Glu	OH	OH
2	Apigenin 6,8-di-*C*-glucoside (Vicenin 2)	H	OH	Glu	OH	Glu	H	OH
3	Diosmetin 6,8-di-*C*-glucoside (Lucenin-2 4'-Me)	H	OH	Glu	OH	Glu	OH	OCH_3
7	Apigenin 7-*O*-neohesperidoside (Rhoifolin)	H	OH	H	*O*-Nh*	H	H	OH
9	Diosmetin 7-*O*-neohesperidoside (Neodiosmin)	H	OH	H	*O*-Nh*	H	OH	OCH_3
15	Sinensetin	H	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3
16	Nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
17	3,5,6,7,8,3',4'-heptamethoxyl flavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
18	Tangeretin	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3

R6
R7
R5
R4
O
R3
R1
R2
O

Figure 2

Peak	Structure assignment	R1	R2	R3
4	Eriodictyol 7-*O*-rutinoside (Eriocitrin)	O-Rutinoside	OH	OH
5	Eriodictyol 7-*O*-neohesperidoside (Neoeriocitrin)	*O*-Neohesperidoside	OH	OH
6	Naringenin 7-*O*-neohesperidoside (Naringin)	*O*-Neohesperidoside	H	OH
8	Hesperetin 7-*O*-neohesperidoside (Neoesperidin)	*O*-Neohesperidoside	OH	OCH_3
10	Naringenin 7-(2″-α-rhamnosyl-6″-(3‴-hydroxy-3‴-methylglutaryl)-β-glucoside (Melitidin)	3-Hydroxy-3-Methylglutaryl	OH	OCH_3
11	Hesperetin 7-(2″-α-rhamnosyl-6″-(3‴-hydroxy-3‴-methylglutaryl)-β-glucoside (Brutieridin)	3-Hydroxy-3-Methylglutaryl	H	OH
12	Isosakuranetin 7-*O*-neohesperidoside (Poncirin)	*O*-Neohesperidoside	H	OCH_3

Figure 3.

Peak	Structure assignment	R
13	5-Methoxypsoralen (Bergapten)	OCH_3
14	5-(6',7'-Epoxy)geranyloxypsoralen (Epoxybergamottin)	

Figure 4.

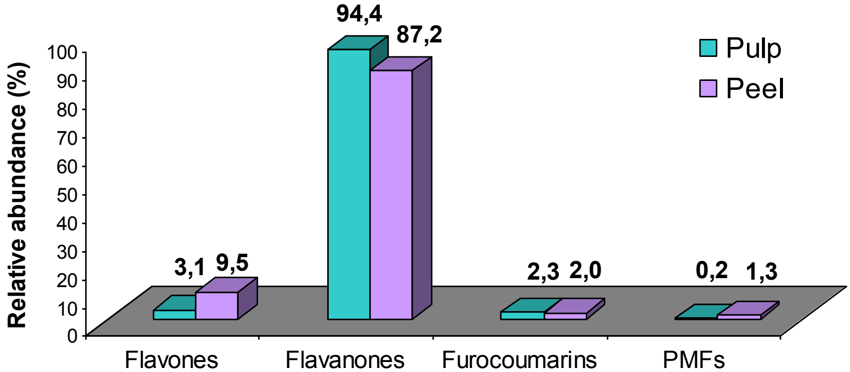

Figure 5.

Paper No 5

Leonardo Sabatino, Monica Scordino, Rosario Caruso, Elena Chiappara, Pasqualino Traulo, Adalgisa Belligno, Giacomo Gagliano.

LC/MS^n detection of short-chain aliphatic amines in glazing agents for fruit coating.

Under review.

LC/MS^{N} DETECTION OF SHORT-CHAIN ALIPHATIC AMINES IN GLAZING AGENTS FOR FRUIT COATING

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ABSTRACT
An analytical protocol was developed for investigating short-chain aliphatic amines occurrence in waxes for fruit coating with the aim of controlling their commercialization in countries where the use of these carriers are forbidden. Seven short-chain aliphatic amines (morpholine, ethanolamine, diethanolamine, triethylamine, 2-Dimethylaminoethanol, triethanolamine and 3-Metoxypropylamine) were detected by LC/MS^{n} operating in positive ion mode in fortified waxes after a two step clean-up procedure. The analytical method was statistically validated. The matrix matched regression lines showed $r^2 > 0.97$. Recoveries ranging from 74 to 115% were obtained for the fortification level of 3.5% m/m and the relative standard deviations ranged from 2 to 8% ($n = 6$). The limits of detection were below 0.2% m/m, while the limits of quantification did not exceed 0.5 % m/m. The method is currently applied in ICQRF Laboratory of Catania on samples of coating waxes collected in the Italian market in the frame of MIPAAF institutional quality control activity.

Keywords: wax, morpholine, food safety, ammonia, carrier

INTRODUCTION

The protection of foods until consumption is becoming one of the most important topic in food preservation. Waxes are usually employed in food manufacturing in order to reduce peels air permeability, avoiding rapid oxidation in fresh fruits and vegetables (*1*). Waxes use became essential when fresh food shelf life has to be extended allowing long-ranges transportation and making products consumers desirable. In order to disperse the wax and facilitate its application free ammonia, primary amines, secondary and tertiary ones are frequently added to coating waxes (*2*). Due to their water solubility and basic properties, amines can be taken advantage of the same target as carrier for glazing agents such as waxes. As defined in ANNEX 1 of REGULATION (EC) No 1333/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on food additives (*3*), 'carriers' are substances used to dissolve, dilute, disperse or otherwise physically modify a food additive or a flavoring, food enzyme, nutrient and/or other substance added for nutritional or physiological purposes to a food without altering its function in order to facilitate its handling, application or use. While ammonium hydroxide is usually admitted by safety food regulations (*3-5*), the amines use is more limited. These law's decisions are often based on peel amines decay time; free ammonia removal from wax coatings is a rapid evaporation process that occurs on fruit surface in a short time. Due to their higher boiling point, amines are hardly removable by evaporation compared to free ammonia (*6*). On the other hand, a higher boiling point does not allow waxes to crumble away, ensuring wax-self stability and holding unharmed coating (*7*).
The occurrence of aliphatic amines in fruits and uncooked vegetables is large health concern, as these substances are potentially dangerous. Not all ingredients acceptable for food coating formulations in the United States are in fact acceptable to all foreign market countries. Among secondary amines morpholine is widely employed. Several countries outside of the European Community such as South Africa, Canada, Chile, and USA have been admitted morpholine as glazing agents applied to fruit [21 CRF 172.235] (*5*). Indeed, no evidence has been made in the European Community to approve this additive. Therefore the use of these carriers is referable on fruit and vegetable coating, leading to food adulteration.
The possibility of detection and determination of amines as carriers of glazing agents in fresh fruit and vegetables can be taking into consideration about food safety reasons.

Unless full scientific studies will carry out on waxes, several analytical approaches have been developed for detection and quantification of aliphatic amines. Dabsyl (*8*) and dansyl (*9*) derivatization for primary and secondary amines determination are the most applied, allowing both spectroscopic and mass spectrometry detection. A new fluorescent probe, the 6-Oxy-(N-succinimidyl acetate)-9-(2'-methoxycarbonyl)fluorescein, was synthesized and used as a pre-column derivatizing reagent for the determination of aliphatic amines in environmental and food samples by high-performance liquid chromatography with fluorescent detection (*10*). A literature method based on thin-layer chromatography combined with ion exchange chromatography and reverse phase high-performance liquid chromatography was shown to be efficient in determining morpholine and its microbiological degradation products (*11*). Gas-chromatography with thermoionic detection was developed for determination of aliphatic amines C7–C20 with prederivatization by N-methyl-bis(trifluoroacetamide) in surface water and wastewater samples (*12*).

Despite a large number of analytical determinations of aliphatic amines through mass spectrometry detection has been done, the most applications have been performed on environmental matrixes (*13*). However amine studies toward food samples have been carried out, no application of liquid chromatography and mass spectrometry has been made up for amines determination in food waxes.

In the present work a LC/ESI-MSn method was developed for detection of morpholine, ethanolammine, diethanolamine, triethylamine, 2-Dimethylaminoethanol, triethanolamine and 3-metoxypropylamine in fruit coating waxes (Figure 1). In order to demonstrate its suitability for routine regulatory purposes, the method was validated in terms of specificity, linearity, precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ). Finally, the method application to amines analysis in commercial waxes collected in Italy during a Ministry quality control investigation was also reported.

MATHERIALS AND METHODS

Chemicals. Standard morpholine (purity 99%), 2-Dimethylaminoethanol (99.4%) and triethylamine (99%) were from Sigma-Aldrich (Milan, Italy), ethanolamine (99%) was obtained by Fluka (Milan, Italy), diethanolamine (99%) was purchased by Carlo Erba (Milan, Italy), triethanolamine (99%) by Merck (Milan, Italy)

and 3-Metoxypropylamine (99.4%) by Chem Service (Milan,Italy) respectively. HPLC-grade methanol was supplied by Romil (Milan, Italy), analytical grade ammonium formiate and 0.1 M HCl were by Carlo Erba (Milan, Italy). Distilled water was purified at 18.2 MΩ with a Millipore MilliQ ULTRA (Milan, Italy) purification system.

Standard solutions preparation and storage. Working standard solutions of amines were prepared each time by diluting stock solutions (1,000 mg/L) in 0.1 M HCl. Stock solutions stored at 4°C were stable at least 3 months.

LC/MS^n conditions for amines determination. The analysis was performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, a Finnigan Surveyor autosampler and a Finnigan LCQ DECA XP MAX detector (Thermo Scientific, Milan, Italy). The analytical column was a Phenomenex (Bologna, Italy) 4µ Fusion RP 80 Å 150 x 2.00 mm, the column temperature 25 °C and the injection volume 10 µL. The mobile phase for LC-MS/MS analysis was ammonium formiate 15 mM and formic acid in water (pH = 3.3) (A) and methanol (B), at a flow rate of 150 mL/min. The gradient program was: 0 min 30% B, 0–15 min 90% B, 15–20 min 30% B, 20-25 min 30% B. Mass spectral analyses were performed using a LCQ ion-trap mass detector operating in the positive ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 3.50 kV. The capillary voltage was 18 V and the heated capillary held at 200°C. A sheath gas flow rate of 20 (arbitrary units) was used and the auxiliary gas was set to 16 (arbitrary units). The amines were detected in both full scan and MS-MS conditions under selected reaction monitoring (SRM) mode. The MS-MS spectra were obtained using an applied collision energy of 30% of instrument maximum and the scan range was 50-200 m/z. Preliminary tunings were carried out with continuous introduction of a dilute solution of 2-Dimethylaminoethanol with flow rate of 5 µL/min and the voltages on the lenses were optimized in TunePlus (Excalibur software).

LC/MS^n Identification of Compounds. Studied amines were characterized in terms of retention times (± standard deviation, n=20), full scan MS and MS-MS data as follows:

Compound 1: diethanolamine. Retention time (Rt), 2.46±0.01 min; MS, 106 $[M + H]^+$ (100); MS-MS focused on $[M + H]^+$, 106 $[M + H]^+$ (30), 88 $[M – H_2O + H]^+$ (100), 70 $[M – 2 H_2O + H]^+$ (10).

Compound 2: triethanolamine. Rt, 2.47±0.01 min; MS, 150 $[M + H]^+$ (100); MS-MS focused on $[M + H]^+$, 150 $[M + H]^+$ (100), 132 $[M – H_2O + H]^+$ (70).

Compound 3: 2-Dimethylaminoethanol. Rt, 2.48±0.02 min; MS, 90 $[M + H]^+$; MS-MS focused on $[M + H]^+$, 90 $[M + H]^+$ (30), 72 $[M – H_2O + H]^+$ (100).
Compound 4: ethanolamine. Rt, 2.48±0.02 min; MS, 62 $[M + H]^+$ (100), 94 $[M + CH_3OH + H]^+$ (30).
Compound 5: morpholine. Rt, 2.49±0.02 min; MS, 88 $[M + H]^+$ (100), 120 $[M + CH_3OH + H]^+$ (30); MS-MS focused on $[M + H]^+$, 88 $[M + H]^+$ (30), 70 $[M – H_2O + H]^+$ (100).
Compound 6: 3-Metoxypropylamine. Rt, 2.49±0.01 min; MS, 90 $[M + H]^+$ (100); MS-MS focused on $[M + H]^+$, 90 $[M + H]^+$ (10), 73 $[M – NH_3 + H]^+$ (30), 58 $[M – CH_3OH + H]^+$ (100).
Compound 7: triethylamine. Rt, 2.97±0.02 min; MS, 102 $[M + H]^+$ (100); MS-MS focused on $[M + H]^+$, 102 $[M + H]^+$ (100).

Statistical data and method validation
System precision. Instrumental precision was checked by repeated scanning (n = 10) of the same standard solution and the peak areas relative standard deviation (RSD) was calculated.
Specificity. The specificity of the analytical method for amines detection was confirmed by obtaining positive results from waxes containing the analyte, coupled with negative results from waxes which do not contain it (negative controls).
Matrix-matched calibration. The LC/MS^n calibration curves were obtained diluting independent solutions of standard amines into negative matrixes (2600 times diluted waxes in 0.1 M HCl) over the range of linearity making two replicates for each concentration. The peak area integration was performed on the total ion current (TIC) of MS/MS related to the fragmentation of $[M + H]^+$, with the exception of ethanolamine that was detected in full scan selecting the $[M+H]^+$ ion. A linear regression of seven calibration points for each amine was used to determine the relationship with the analyte concentrations. The regression equations with linearity range, slope, y-intercept and coefficient of correlation (r^2) were evaluated for all amines studied.
Limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined by signal-to-noise approach. The noise magnitude was taken as an estimate of the blank standard deviation (14). The noise and signal are measured experimentally on the chromatogram printout. LOD corresponds to the analyte amount in the wax for which the signal-to-noise ratio is equal to 3, and LOQ

corresponds to the analyte amount for which the signal-to-noise ratio is equal to 10.

Method accuracy (recovery) and precision (repeatability). The accuracy and the precision of the method were ascertained by spiking negative waxes with a known amount of standard solution of each amine. The spikes were done to obtain a final detectable concentration of 13 mg/L (six replicates each), corresponding to about a content of 3.5% m/m of amine in the wax. The average percentage of recovery, the standard deviation (SD) and the relative standard deviation (RSD) were evaluated.

Amine determination on commercial waxes. Samples of commercial waxes for food application were collected in Italy within the time frame of MIPAAF institutional quality control activity. The pick up was made in four aliquots by MIPAAF officers from many industries. An official report was written up for every sample. The samples were labeled with a description of the pick-up location, pick-up date, sample typology and origin, and sent to ICQRF Catania Laboratory for the analyses. Following the analyses our laboratory submitted a regular or irregular certificate of analysis.

Before LC/MS^n analyses 0.2 g of sample were added of 10 mL of 0.1 M HCl and centrifuged at room temperature at 3000 rpm with an ALC 4236 centrifuge (Italy). After filtration with 0.45 µm PTFE filter (VWR, Italy), 20 µl were added to 1 ml of 0.1 M HCl and analyzed. The analyses were conducted in triplicate. The $P_{value}<0.05$ was adopted as statistically significant.

RESULTS AND DISCUSSION

Method development

In order to develop a sensitive MS-MS method to identify the major species formed in collisional experimental fragmentation of MS^n analysis, a mass characterization study was firstly studied for direct infusion (flow rate 30 µL min^{-1}) of solutions of each investigated aliphatic amine (1 mg L^{-1} in 0.1 M HCl). Mass scans in positive ions mode were performed with ESI source ionization. For each analyte a typical mass spectrum profile was identified and hypotheses about a structure of the most meaningful fragments proposed. All the amines give a m/z signal corresponds to protonated molecule $[M+H]^+$ while in MS^2 analysis the generally loss of a neutral fragment of water leads to the formation of a fragment with a m/z signal corresponding to the species $[M-H_2O+H]^+$; in the case of 3-Metoxypropylamine the ammonia loss generates the ion $[M-NH_3+H]^+$. The tertiary ammine

triethylamine doesn't present further MS^2 fragmentation because of its stable chemical structure, even amplifying the collision energy. Differently ethanolamine spectrum is appreciable only in full scan mode: due to the its low molecular weight (MW 61.18), the possible fragmentation and the following ammonia/water loss will generate a fragment under the detectable range achieved with LCQ DECA XP MAX instrument.

Under the chromatographic conditions described in details in the experimental section, a matrix matched mixture of standard solutions of the seven ammines was injected in the LC-MS system and each component analyzed through both full scan and SRM detection technique. The chromatographic process knocks out the most part of matrix facilitating the detection and quantification. The studied amines were well recognizable on the basis of m/z signals and good sensitivities were obtained. Each analyte show a typical mass spectrum profile previously identified in direct infusion and in some cases methanolic $[M+CH_3OH+H]^+$ abducts is observed as minority components of full scan pattern. Although the chromatographic peaks are partially overlapped as show by the retention times, ion trap resolution allows a good selection of the single compound because the detection is made up on fragments coming out from selected ion species. Experimental evidence of this phenomenon is given by differences <3% in the peak areas between single compound solution and amines mix. Therefore, analytical data indicates that the method is adequate to the aim, supported by the fact that employ of more than one amine in commercial waxes is economically disadvantageous and technologically improbable.

Method validation

Analytical parameters of the proposed method were evaluated according to the criteria given in material and methods section. Results are reported in **Tables 1** and **2**.

Since waxes are complex samples, matrix effect was assessed at method validation. Experiments have been carried out during a 3-month period, in which calibration curves prepared in presence of 2600 times diluted matrix, were prepared and studied. Results demonstrated that matrix-matched calibrations were stable during the period of study. The response was linear in a wide range of concentrations depending on studied molecule, covering from two to three order of magnitude with correlation coefficients better than 0.97. The repeatability of peak area was excellent, ranging from 0.8 to 2.0%. LODs established for a signal to noise ratio of 3 ranged between

0.05 mg L^{-1} for triethanolamine to 0.8 mg L^{-1} for morpholine and 3-Metoxypropylamine; these detection limits corresponded in the waxes to 0.01 and 0.21 % m/m, respectively. LOQs evaluation (signal to noise ratio of 10) showed the lowest value of 0.22 mg L^{-1} for diethanolamine to the higher value of 1.80 mg L^{-1} for 3-Metoxypropylamine, corresponding to and 0.06 and 0.5% m/m in the waxes, respectively. Further improvements of the detection limits can be obtained reducing the wax dilution procedure. It should be remarked that LOQs attained in the proposed HPLC-ESI-MS^n method are fully compatible with concentration levels of amines in waxes, typically added in order of magnitude of 2-5% (*6-7*). Hence no further pre-concentration/cleanup pretreatments were required.

Specificity is the degree to which the procedure applies to a single analyte and was checked by comparing the waxes chromatogram with the chromatogram obtained for the waxes spiked with amine standards. For all studied molecules, responses in control samples were < 30% of LOQ according with validation parameters and criteria of document No. SANCO/10684/2009 (*15*).

The accuracy of the present method was evaluated by means of spiking and recovery study on waxes at levels of about 3.5% m/m, according with a typical concentration level of added amines in waxes (*6-7*). Considering dilution operations, the studied level corresponded to a fortification of about 13 mg L^{-1}. Table 2 showed excellent recoveries for the six ammines detected in MS^n, ranging from 91% for morpholine to 109.5% of diethanolamine. The lower recovery found for ethanolamine is probably imputable to the full scan mass detection method. The AOAC's Single Lab Validation document recommends general recovery limits of 80–115% at levels of 10 mg L^{-1} (*16*). Therefore, the method could be considered accurate at all investigated concentration levels. Moreover, the precision of the method data were good ranging from a minimum of 1.7% to a maximum value of 8.4%.

Method application

The above-discussed methodology is currently applied in ICQRF laboratory of Catania to investigate the occurrence of unpermitted ammines on samples of coating waxes collected in Italy as described in "Materials". The samples were processed as previously described for amines detection. As expected, short chain aliphatic amines were detected only in glazing agents for fruits to be exported in extra-European countries. Among the seven studied amines, these waxes evidenced the occurrence of 2-Dimethylaminoethanol; all sample were labeled as morpholine free. A quantitative estimation of 2-

Dimethylaminoethanol showed mean content ranging from 2 to 4%, confirming the literature data. These results evidenced the efficacy of the analytical protocol on real matrixes beyond the lab-prepared and fortified samples. Figure 2 shows the LC/MS^n chromatograms and relative fragmentation patterns of a wax sample containing 3.0% of 2-Dimethylaminoethanol.

In conclusion the most significant improvement of the HPLC/ESI-MS^n proposed method, with respect to common analytical approaches such as gas and ion chromatography with or without derivatization, was the possibility of avoiding tedious and time consuming preparative purification step, facilitating the sample handling and decreasing the analysis time and the solvent consuming. Moreover, the reverse phase separation coupled with MS^n detection allowed the assured identification of aliphatic amines and the minimization of strong matrix effect which occur utilizing complex formulations like coating waxes. The sensitivity, the precision and the accuracy of the method developed make it adequate to cop its quantification in glazing agents for fruit coating. No time consuming extraction procedure and toxic derivatization reagents were used, making the analysis appropriate for routinely application. This screening should be carried out as routinely analysis to assess quality of European waxes and could be integrated with the detection of unpermitted waxes and fungicides, preserving consumers and importers from commercial illicit and from potential toxicological consequences.

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Figure caption

Figure 1. Chemical formula and physical-chemical property of studied molecules.

Figure 2. Wax sample containing 3.0% of 2-Dimethylaminoethanol. Full scan LC/MS chromatogram (A) and relative TIC spectrum (A'); LC/MS^2 chromatogram (B) and relative MS/MS spectrum (B').

Table 1. Method validation parameters for studied aliphatic amines.

Validation parameters[a]	Diethanolamine	Triethanolamine	2-Dimethylaminoethanol	Ethanolamine	Morpholine	3-Metoxypropylamine	Triethylamine
Linearity range, mg/L [b]	2.0-20.1	1.9-18.6	2.0-52.4	0.9-97.8	2.1-22.0	1.6-48.1	2.2-102.2
y-intercept[b]	1.11E+07	1.48E+08	1.13E+07	1.96E+07	1.90E+05	1.32E+06	5.82E+07
Slope[b]	2.29E+06	3.03E+07	8.21E+05	1.26E+07	7.44E+04	3.37E+05	5.14E+06
Correlation coefficient (r^2) [b]	0.993	0.989	0.992	0.999	0.997	0.971	0.982
System precision (% RSD)[c]	0.8	1.8	2.0	1.6	1.7	2.1	1.3
LOD, mg/L[d]	0.07	0.05	0.30	0.65	0.80	0.80	0.50
LOQ, mg/L[d]	0.22	0.30	0.90	0.85	1.50	1.80	1.40
LOD, % m/m[d]	0.02	0.01	0.08	0.17	0.21	0.21	0.13
LOQ, % m/m[d]	0.06	0.08	0.23	0.22	0.39	0.47	0.36

[a]matrix-mached calibration on the basis of parent ions MS^n fragmentation: n=1 for ethanolamine, selecting the $[M+H]^+$ ion; n=2 for the others amines.
[b]*n*=7 (2 replicates each). [c]*n*=10. [d]calculated considering the wax dilution

Table 2. Method precision and amines recovery data.

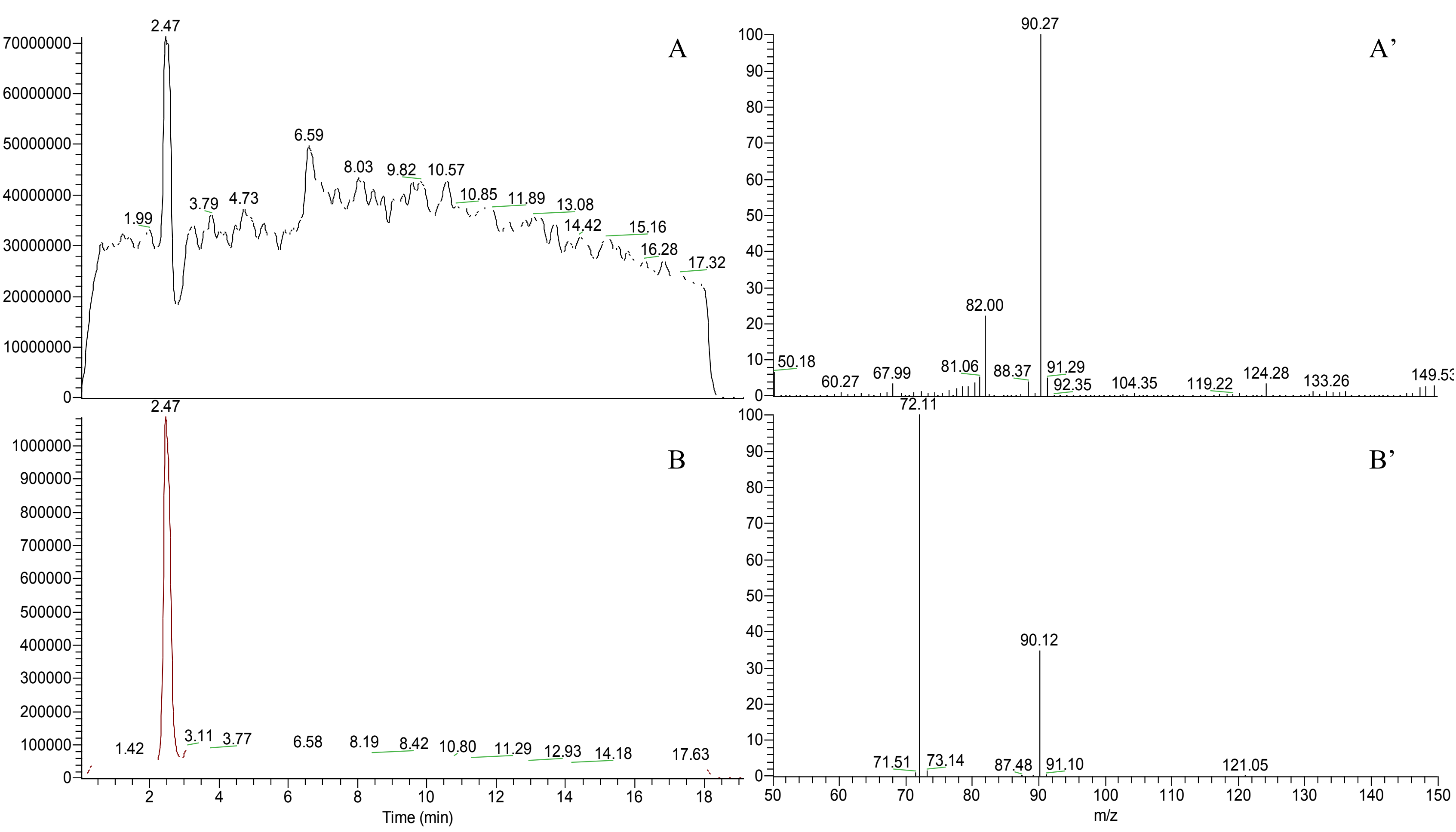
	Diethanolamine	**Triethanolamine**	**2-Dimethylaminoethanol**	**Ethanolamine**	**Morpholine**	**3-Metoxypropylamine**	**Triethylamine**
Theoretical content, mg/L[a]	13.1	12.1	14.1	13.8	14.1	13.0	14.4
Detected content, mg/L[b]	14.4	12.7	16.1	10.2	13.0	13.7	13.6
Precision, SD[c] mg/L[b]	0.2	0.9	1.2	0.9	0.9	0.8	0.4
Precision, RSD[d] %[b]	1.7	7.1	8.4	6.8	6.2	5.9	2.5
Accuracy, %[b]	109.5	104.6	114.9	74.0	91.9	106.0	94.1

[a] concentration corresponding to about 3.5% m/m of amine in the wax. [b] n=6. [c] SD: standard deviation. [d] RSD: relative standard deviation.

Compound name	Common name	Molecular structure	Molecular weight (Dalton)	Dissociation constant (pKb)	Boiling Point (°C)
1-Oxa-4-Azacyclohexane	Morpholine	O N H	87.12	5.6	129
N,N-dimethyl-2-Hydroxyethylamine	2-Dimethylaminoethanol	HO N	89.14	5.1	135
1-Amino-3-Methoxypropane	3-Metoxypropylamine	O NH_2	89.14	3.9	116
2-Aminoethanol	Ethanoloamine	HO NH_2	61.08	4.5	170
2,2'-Iminodiethanol	Diethaoloamine	HO H N OH	105.14	5.1	217
2,2',2''-Nitrilotriethanol	Triethanoloamine	OH HO N OH	149.19	6.2	335
N,N-diethylethanamine	Triethylamine	N	101.19	3.3	89

FIGURE 1.

FIGURE 2.



Paper No 6

Leonardo Sabatino, Monica Scordino, Maria Gargano, Adalgisa Belligno, Pasqualino Traulo, Giacomo Gagliano.

HPLC/PDA/ESI-MS evaluation of saffron (*Crocus sativus*) adulteration.

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LC/PDA/ESI-MS EVALUATION OF SAFFRON (*Crocus sativus*) ADULTERATION

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ABSTRACT

The present study evaluated the reliability of ISO 3632-2 spectrophotometric method for classification of saffron, making determinations on saffron samples added with increasing concentrations of common saffron spice adulterations (safflower, marigold and turmeric). The results showed that the ISO 3632-2 spectrophotometric method is not able to detect addition up to 20% w/w of saffron adulterants. For additions from 20 to 50% w/w of the three adulterants studied, saffron was classified in a wrong category; addition higher of 50% w/w determined variations of the investigated parameters do not allowed the identification of the product as "saffron". In all cases the method did not permit to recognize the adulterant. On the contrary, the specificity of the HPLC/PDA/LC-MS technique allowed the unequivocal identification of adulterants characteristic marker molecules that could be recognized by the values of absorbance/mass. The extraction of characteristic ions of each marker molecule has revealed concentrations of up to 5% w/w for the investigated spice adulterants. Beside, the high dyeing power of turmeric allowed the determination of 5% w/w addition using exclusively the HPLC/PDA technique.

Keywords: spice, saffron, safflower, marigold, turmeric, fraud.

INTRODUCTION

The saffron plant (*Crocus sativus L.*) belonging to the family of *Irridaceae,* is the most valuable spice in the world. It is a perennial meadow grass that reaches 10 to 25 cm and grows from its bulbs. The cultivation of saffron requires Mediterranean continental climate with cold winters, warm and dry summers and a dry Mediterranean humidity regime. The plant is resistant to extreme temperatures in the summer as well as during winter [1]. Saffron is a spice that adds color, taste and aroma to various foods. Saffron odor is related to a colorless terpen essential oil as well as an oxigenous compound of sineole. The bitter taste of saffron is due to picrocrocin and picrocrozioide. The origin of saffron color is crocin and produces glucose and crocetin after hydrolysis [1].

The world production of saffron is about 178 tons in year of which 90% is produced by Iran and the remaining 10% in India, Greece, Morocco, Italy, Spain and other countries. The biggest worldwide exporters are Iranians, followed by the Spanish. In Italy the annual production is around 400 kg [1]. It is cultivated mainly in Sardinia and Abruzzo with about 35 ha and 7 ha respectively, and to a lesser extent in Umbria, Tuscany, Liguria and Sicily. Some productions (Abruzzese di Navelli and San Gimignano) have been awarded the Protected Designation of Origin (PDO). The commercialization costs, including the different process stages, could exceed 1’000 €/kg.

Nowadays saffron production faces a crisis but all nations involved are traditionally related to saffron cultivation and preserve it actively. In the Mediterranean basin the production of saffron has been decreased due to rising standards of living and, inevitably, due to the rise of labor costs. However, the Mediterranean saffron bears the best quality features worldwide, which is attributed to deep cultivation knowledge and careful treatment by all European producers.

Due to its high value, saffron spice has been subjected to many adulterations throughout history [1]. Saffron quality is determined after a series of characteristic parameters for the spice itself (humidity, flower residue or foreign material and ashes content, soluble condensate, coloring power etc) combined with necessary external conditions (absence of parts from other plants, microbiological flora and pesticide residues). Methods applied for quality assurance are widely known and enterprises are able to use the necessary technology in order to guarantee for the product quality to consumers. Since 1980 a standard procedure (ISO/TS 3632) allows the quality saffron

classification [2]. The ISO/TS 3632 was updated in 2003 with the text that currently governs the product quality. This regulation is applicable to saffron strands and saffron ground or dust. The rule divides the saffron in different categories based primarily on physical-chemical criteria (**Table 1**).
European saffron is considered the best in the world due to its chemical, physical and organoleptic features as measured by certain parameters. New high quality verification standards and new evaluation methods should be introduced in order to determine accurately color and to prevent fraud, as many scientific works reported [3-6]. The present study evaluated the reliability of the ISO/TS 3632 spectrophotometric method for saffron classification, making determinations on samples of saffron blended with different concentrations of safflower, calendula and turmeric. The results of the spectrophotometric method were compared and integrated with the HPLC/PDA/ESI-MS technique for the unequivocal identification of adulterants through the identification of specific marker compounds.

MATERIALS AND METHODS

Reagents and Standards. HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system.
Plant material. The strands of saffron from San Gavino (Sardinia, Italy), the turmeric (*Curcuma longa*) powder, the calendula (*Calendula officinalis*) flowers, the safflower (*Carthamus tinctorius*) strands have been taken in the national territory by ICQRF officials. The plant material was treated in accordance with the specifications of ISO/TS 3632-2:2003 sample preparation for the official analysis [2]. Different mixes were prepared with saffron varying percentages from 5 to 70% by weight of each adulterant studied.
UV-Vis spectrophotometry. The method used is based on the technical specification ISO/TS 3632-2:2003 [2] and allowed determining the main characteristics of saffron, in connection with the content picrocrocin, safranal and crocin, moisture and volatile component. The method is based on the spectrophotometric assessment absorption at three wavelengths (λmax): 257 nm (maximum absorption of picrocrocin), 330 nm (maximum absorption of safranal) and 440 nm (maximum absorption of crocin).

HPLC/PDA/ESI-MS Fingerprint. 20±2 mg of sample were extracted with 2 mL of 50% aqueous methanol containing 0.1% formic acid at room temperature in an ultrasonic bath for 30 min. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant decanted and used for the LC analyses, after filtration through 0.45μm PTFE filters (LabService Analytica, Bologna, Italy). The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Gemini C18 150x2.1 mm i.d. 3μm (Phenomenex), the flow rate was 200 μL/min, the column temperature 30°C and the injection volume 10 μL. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43 % B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 200–700 nm and the mass scan range was 100-1600 m/z. Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 3.5 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 14 (arbitrary units).
The main compounds of analyzed plant materials were characterized in terms of retention times, lambda max and MS data operating.

RESULTS and DISCUSSION

In **Table 2** were reported the data related to the different parameters measured according to ISO/TS 3632 (2003) spectrometric method for saffron category assignment performed on unadulterated and on spice spiked San Gavino ISO Category II saffron. Five independent additions at different concentration (10-67%) of saffron spice adulterants (safflower, marigold and turmeric) were made. Results showed that the ISO/TS 3632-2 spectrometric method is not able to detect addition up to 10-20% w/w of saffron adulterants, resulting in a correct classification of the mixes as saffron ISO category II. For additions from 20 to 50% w/w of the three adulterants studied, the mixes were classified as worse ISO category III saffron. Spikes higher of 50% w/w determined variations of the investigated parameters do

not allowed the identification of the mixes as "saffron". In all cases the method did not permit to recognize the kind of adulterant.

Therefore the only use of the spectrometric technique may underestimate the saffron fraud occurrence due to the addition of less valuable spices. The hyphenated techniques using high performance liquid chromatography coupled with UV-Vis spectrophotometers and mass spectrometry may allow a better assessment of the quality of the saffron products. Preliminarily, this study has characterized separately the fingerprint HPLC/PDA/ESI-MS of acidified water-methanolic extracts of saffron, marigold, safflower and turmeric. **Figure 1** showed the UV-Vis chromatograms of the studied spices. In **Table 3** is reported the assignment of the characteristic molecules of each botanical species as a function of retention times, spectral and mass properties.

Crocetin glycosides are responsible of saffron yellow colour; their UV-Vis spectra are characterized by an absorption maximum at about 440-460 nm depending on the molecule. Trans- and cis-crocetin glycoside presented a different spectroscopic behaviour because cis-crocetins presented an additional absorption band around 325 nm in their UV-Vis spectrum respect to their trans-isomers. Six crocetin glycosides (2-7), together with uncolorless picrocrocin (4-(α-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) (1), were identified in the analyzed San Gavino saffron. As confirmed by ESI-MS analysis, in agreement with literature data [5-7], trans-crocetin di-(β-D-gentibiosyl) ester (3), cis-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester (5) and cis-crocetin di-(β-D-glucosyl) ester (7) were the most represented crocetin derivatives, followed by cis-crocetin di-(β-D-gentibiosyl) ester (6), trans-crocetin (β-D-neapolitanosyl)-(β-D-gentibiosyl) ester (2) and trans-crocetin (β-D-glucosyl)-(β-D-neapolitanosyl) ester (4).
Marigold extract was characterized by six main peaks (a-f), which displayed identical UV adsorptions with maximum at about 255 and 350 nm, typical of flavonols. The ESI-MS $[M-H]^-$ parent ions, together with the comparison with scientific references [8], permitted the unequivocal assignation to quercetin 3-*O*-rutinosylrhamnoside (a), quercetin 3-*O*-rutinoside (b), isorhamnetin-3-*O*-rutinosylrhamnoside (c), narcissin (d), isorhamnetin 3-*O*-neohesperidoside (e) and isorhamnetin-3-*O*-glucoside (f). Among them, the isoramnetin derivatives predominated in the analyzed sample.

As reported by many scientific papers [9-10], the main components of safflower red pigments is the carthamin, composed of two chalconoids which conjugated bonds being the cause of the red color; it is derived from the yellow coloured precarthamin by decarboxylation. Both compounds were detected in the analyzed safflower sample as peaks VI and VII. The safflomin A (I), safflor yellow B (IV) and anydrosafflor yellow B (V) were identified as the quinochalcone *C*-glycosides responsible of the yellow colour of the sample. Moreover, two kaempferol derivatives were identified as 6-hydroxykaempferol 3-*O*-β-D-glucoside (II) and kaempferol 3-*O*-β-rutinoside (III). The identification was confirmed by spectroscopic and mass data.

Three molecules (α-γ) were identified as responsible of the yellow distinctive colour of turmeric: all of them clearly possess a maximum absorption wavelength near 420 nm. According with literature data [11], these compounds were identified as dicinnamoylmethane derivatives demethoxycurcumin (α), bisdemethoxycurcumin (β) and curcumin (γ) on the basis of [M-H]- molecular ions generated by the ESI-MS negative soft ionization.

Subsequently, the above-mentioned analytical technique was applied to saffron samples mixed with different concentrations of turmeric, marigold and safflower in the range 2-20% w/w. For each adulterant marker molecules have been choosing for their unambiguous identification in the mixture: their presence is in fact not affected by the saffron matrix effect.

The extraction of the ion with m/z 623, corresponding to isorhamnetin 3-*O*-neohesperidoside (e), is able to detect the presence of marigold. In the case of safflower, the marker molecules for its identification in the mixture were the anydrosafflor yellow B (V) and chartamin (VII) with m/z 1044 and 909 respectively. The marker molecules could be revealed at concentrations of up to 5% w/w of addition for both marigold and safflower.
The characteristic turmeric curcuminoids triplet due to the presence of demethoxycurcumin (α), bisdemethoxycurcumin (β) and curcumin (γ) could easily identify its presence in the mix with saffron also at concentrations of 2%, even using only UV-Vis detector.

The above discussed method is currently applied in ICQRF Laboratory of Catania on samples of saffron collected in the Italian market in the frame of MIPAAF institutional quality control activity.

In conclusion, the results of the present study should be forced the legislative authorities to release new standards for the saffron sector, inviting for maintenance of product purity in order to avoid adulteration and fraud.
The methods applied nowadays are outdated, while newer ones are not positively accepted and are rarely used, even though some of them have proven to be efficient in the field. All national and international quality control standards should be reinforced in order to limit spreading of adulterated saffron in the European market deriving from the third countries that affect considerably the competitiveness of European saffron.

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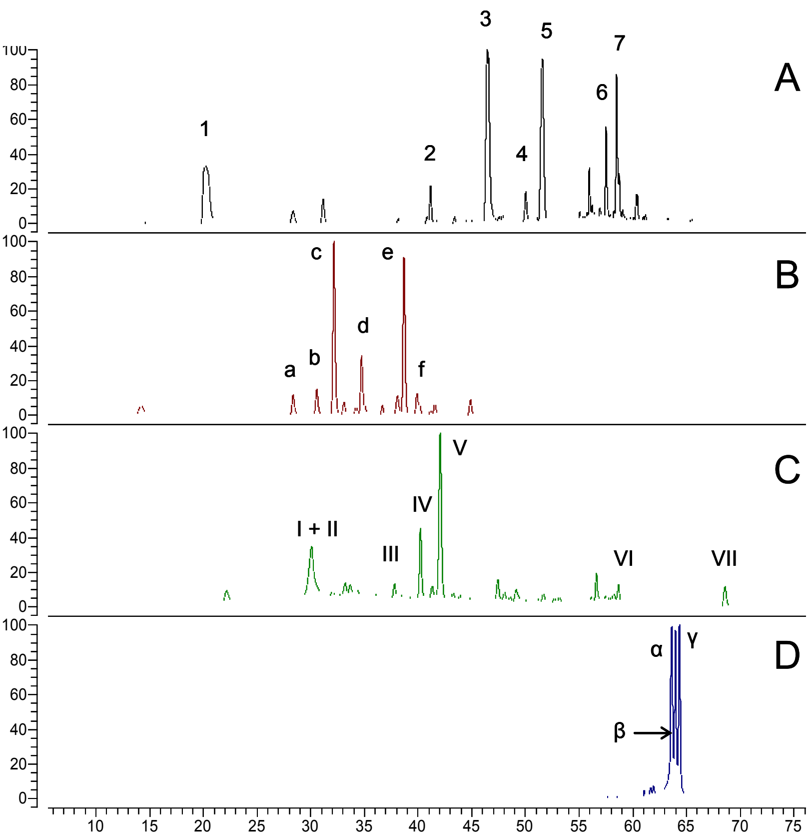

Figure 1: UV-Vis chromatograms of analyzed extracts. **A:** Saffron (λ 250 nm + λ 440 nm); **B:** Marigold (λ 350 nm); **C:** Safflower (λ 410 nm + λ 520 nm); **D:** Turmeric (λ 425 nm). For peak identification see Table 3.

Table 1: Specifications of ISO/TS 3632 "Saffron" [1b].

Characteristics	Specifications			Test method
	Categories			
	I	II	III	
Moisture and volatile matter, % (w/w), max.	12[a] 10[b]	12[a] 10[b]	12[a] 10[b]	ISO/TS 3632-2:2003, Clause 7
Total ash, % (w/w), on dry basis, max.	8	8	8	ISO 928:1997. Clause 8, and ISO/TS 3632-2:2003, Clause 12
Acid-insoluble ash, % (w/w), on dry basis, max.	1	1	1.5	ISO 930:1997. Clause 7, and ISO/TS 3632-2:2003, Clause 13
Bitterness, expressed as direct reading of the absorbance of picrocrocine at about 257 nm, on dry basis, min.	70	55	40	ISO/TS 3632-2:2003, Clause 14
Safranal, expressed as direct reading of the absorbance at about 330 nm, on dry wt basis min. max.	20 50	20 50	20 50	ISO/TS 3632-2:2003, Clause 14
Coloring strength, expressed as direct reading of the absorbance of crocine at about 440 nm, on dry wt basis, min.	190	150	100	ISO/TS 3632-2:2003, Clause 14
Artificial water soluble acid colorants	Absent	Absent	Absent	ISO/TS 3632-2:2003, Clause 16 and/or Clasue17

[a]filaments; [b] powder.

Table 2: Results of ISO/TS 3632 UV-vis spectrophotometry on the analyzed mixes.

Mix composition m/m	$E^{1\%}$ 257 nm	$E^{1\%}$ 330 nm	$E^{1\%}$ 440 nm	ISO Category
Saffron 100%	66	32	170	II
90% Saffron - 10% Turmeric	59	27	150	II
80% Saffron - 20% Turmeric	53	25	132	III
67% Saffron - 33% Turmeric	45	21	111	III
50% Saffron - 50% Turmeric	34	15	81	-
33% Saffron - 67% Turmeric	22	9	49	-
90% Saffron - 10% Safflower	63	31	152	II
80% Saffron - 20% Safflower	59	30	136	III
67% Saffron - 33% Safflower	53	29	113	III
50% Saffron - 50% Safflower	48	27	86	-
33% Saffron - 67% Safflower	42	26	60	-
90% Saffron - 10% Marigold	61	30	151	II
80% Saffron - 20% Marigold	55	29	135	III
67% Saffron - 33% Marigold	49	25	112	III
50% Saffron - 50% Marigold	39	22	83	-
33% Saffron - 67% Marigold	31	18	54	-

Table 3: HPLC/PDA/MS chemical characterization of studied extracts.

Peak	Name	Tr (min)	UV-vis (nm)	$[M-H]^-$ (m/z)
Crocus sativus L. (saffron)				
1	4-(α-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (picrocrocin)	20.2	250	375
2	trans-crocetin (β-D-neapolitanosyl)-(β-D-gentibiosyl) ester	41.2	260, 440	1137
3	trans-crocetin di-(β-D-gentibiosyl) ester	46.5	260, 420, 460	975
4	trans-crocetin (β-D-glucosyl)-(β-D-neapolitanosyl) ester	50.1	260, 440	975
5	cis-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester	51.6	260, 330, 335, 460	813
6	cis-crocetin di-(β-D-gentibiosyl) ester	57.5	260, 320, 435, 460	976
7	cis-crocetin di-(β-D-glucosyl) ester	58.8	260, 325, 440, 465	813
Calendula officinalis (marigold)				
a	Quercetin 3-O-rutinosylrhamnoside	28.4	255, 355	755
b	Quercetin 3-O-rutinoside	30.6	255, 355	609
c	Isorhamnetin-3-O-rutinosylrhamnoside	32.2	255, 350	769
d	Narcissin	34.7	255, 355	623
e	Isorhamnetin 3-O-neohesperidoside	38.7	255, 345	623
f	Isorhamnetin-3-O-glucoside	39.9	255, 355	477
Carthamus tinctorius (safflower)				
I	Hydroxysafflor yellow A (safflomin A)	30.1	225, 410	611
II	6-hydroxykaempferol 3-O-β-D-glucoside	30.6	275, 340	464
III	Kaempferol 3-O-β-rutinoside	37.7	265, 350	593
IV	Safflor yellow B	40.2	225, 410	1060
V	Anydrosafflor yellow B	42.1	225, 410	1044
VI	Prechartamin	58.7	240, 405	955
VII	Chartamin	68.6	370, 520	909
Curcuma longa (turmeric)				
α	Demethoxycurcumin	63.9	250, 425	337
β	Bisdemethoxycurcumin	63.6	250, 420	307
γ	Curcumin	64.3	260, 430	367

Paper No 7

Monica Scordino, Leonardo Sabatino, Adalgisa Belligno, Giacomo Gagliano.

Phenolic characterization of Sicilian yellow-flesh peach (*Prunus persica L.*) cultivars at different ripening stage.

Under review.

PHENOLIC CHARACTERIZATION OF SICILIAN YELLOW-FLESH PEAC (*Prunus persica L.*) CULTIVARS AT DIFFENT RIPENING STAGE

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ABSTRACT
Yellow flesh peaches (Prunus persica L.) from different Sicilian areas (Leonforte, Riesi and Maniace) were investigated for the first time for their polyphenolic composition, with consideration of both peel and flesh tissues. The qualitative and quantitative polyphenolic evaluation was determined at two different ripening stages (market-ripe and fully-ripe) via chromatographic separation by using reverse-phase HPLC-PDA-ESI/MS^2. This technique provides a comprehensive chromatographic evaluation of 10 compounds (hydroxycinnamates and flavonols), differently distributed in the analyzed tissues. Overall, the hydroxycinnamates were present in both pulp and peel, while flavonols resulted exclusively located in the peel. Peels were found to be richest in polyphenolics with respect to the pulps, containing up to 276 mg/kg fresh weight in Riesi market-ripe peaches. Generally, the peach tissues from market-ripe fruits showed higher phenolics contents than fully-ripe fruits.

Keywords: HPLC-PDA-ESI/MS^2, peach, phenolics; hydroxycinnamates, flavonols, pulp, peel.

PRACTICAL APPLICATIONS

It's well documented that the benefits due to the consumption of fruit can be largely attributed to the elevated content of phenolic compounds. High levels of phenolic compounds in fruits are of high importance to maintain the quality of fruits and provide consumers the health benefits of fruit consumption. Thus, the assessment of the distribution of phenolic compounds in various parts of the peach fruit and any variations due at the time of collection are considered interesting from a scientific point of view. The characterization of peach phenolics could represent a useful tool in the area of food authenticity protection since some compounds could represent specific quality markers. Moreover, a study on phenolics quantification at different ripening stages could provide useful information for assessing the fruit harvest timing in order to ensure the best fruit quality.

INTRODUCTION

The typical value of food products involves a niche product diversification through the exploitation of its intrinsic characteristics in types of production and territorial values (Oliveira et al. 2010). The logical step passes through a greater understanding of local products, the protection of their trademarks against imitations and to unfair competition from similar products (Dion et al. 2008). Nowadays a great interest is concentrate on quality productions at risk of extinction, unique regions and ecosystems protection, traditional processing methods recovering, native breeds and local plant varieties safeguard (Lorenzini et al. 2011). The yellow flesh peaches from Leonforte, a Sicilian town close to Enna (Italy), are a Slow Food Presidium derived from the cultivation of local ecotypes of “Bianco” and “Giallona”. This product received recently the Protected Geographical Indication (PGI) under the EC Regulation 622/2010 of 15 July 2010. The PGI is a European official recognition that provides protection for an agrifood product from a place or region having a specific quality, reputation or other characteristic that can be attributed to its geographical origin and which is produced, processed or prepared in the named area (Parrott et al. 2002). The yellow flesh peaches from Leonforte are different from other peach varieties to the late ripening stage in September rather than in June, and to a peculiar agronomic technique that, 120-150 days

before ripening, includes every single fruit in special breathable bags of parchment paper in accordance with its own guidelines for production. The use of this unique cultivation technique fully justifies the lack of chemical treatment, avoiding any presence of toxic residues in fruits which unfortunately are almost unavoidable in conventional peaches. Other typical areas of production of Sicilian yellow flesh peaches are located in Maniace, at the foot of Mount Etna, and in Riesi, located in central Sicily near the valley of the Salso river; in this areas conventional farming techniques are applied.
Epidemiological studies have pointed out that regular consumption of fruits and vegetables imparts health benefits and it seems to be related mainly to the content of antioxidant phenolic metabolites (Cao et al. 1997; Morton et al. 2000; Le Marchand 2002). As a consequence, considerable efforts have been devoted to the quantification of the phenolics of the most common species (Cheng et al. 1995; Gil et al. 2002; Leontowicz et al. 2002; Cevallos-Casals et al. 2006; Di Vaio et al. 2008). Nevertheless, information available about the phenolic content of peach is commonly restricted to the total phenolics evaluation and only few works characterized the complete phenolics pattern (Cheng et al 2000; Tomás-Barberán et al. 2001). Since the polyphenolic composition of yellow flesh peaches from Leonforte has not yet been investigated, the aim of the present work was to elucidate by HPLC-PDA-ESI/MS^2 technique the identity and the relative abundance of these compounds and to compare their relative amount with that of conventional Sicilian yellow flesh peach cultivars from Maniace (Catania) and Riesi (Caltanissetta). The phenolics evaluation was performed at two picking times, comparing peel and flesh tissues.

MATERIALS AND METHODS

Chemicals. HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system. HPLC grade quercetin-3-rutinoside, 5-O-caffeoylquinic acid and 3-O-caffeoylquinic acid were obtained from Sigma Aldrich (Milan, Italy). Quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-rutinoside, isorhamnetin-3-rutinoside and isorhamnetin-3-glucoside were obtained from Extrasynthèse (Genay, France).
Plant material. Yellow flesh peaches from Leonforte (Enna, Italy), Maniace (Catania, Italy) and Riesi (Caltanissetta, Italy) at market-ripe picking time were provided by local manufacturers and were related to

the season 2010. The market-ripe picking time was performed when the fruit is fully developed and almost the full degree of color has been attained but the flesh is firm and the fruit will stand shipping. The sample for the analyses was obtained after collection of 100 fruits (10 per tree). Half of market-ripe sample were processed and analyzed after 7 days at 20°C for the following maturation stage (fully-ripe), when the peach is fully colored, the flesh is soft and the fruit so easily bruised by handling that it will not stand shipment.
Before the phenolics determination, pulp and peel were carefully separated and reduced in small pieces with Turbo Homogenizer HMHF (pbi international). The samples were stored at -20 °C until needed for the study.
Extraction and analysis of phenolics. Two grams of homogenate was extracted for 2 h with 5 mL of 50% aqueous methanol containing 1% formic acid at room temperature on an orbital shaker. The mixture was centrifuged at 1000rpm for 15 min and the supernatant decanted. The pellets were re-extracted under identical conditions. Supernatants were combined and used for the LC analyses, after filtration through 0.45µm PTFE filters (LabService Analytica, Bologna, Italy). The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Luna C18 250 x 4.6mm, 5 µm i.d. (Phenomenex), the flow rate was 1 mL/min, the column temperature 30°C and the injection volume 20 µL. Flow rate was split 1/10 before MS interface. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43 % B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 200–700 nm, and for quantitative determinations the chromatograms were recorded at 325 nm (hydroxycinnamates) and 355 nm (flavonols). Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 4.0 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 15 (arbitrary units). The MS-MS spectra were obtained using collision energy of 25-35% of instrument maximum, operating in selected reaction monitoring (SRM). Preliminary negative tunings were carried out with continuous introduction of dilute

solutions of quercetin-3-rutinoside, at the flow rate of 5 μL/min and the voltages on the lenses were optimized in TunePlus (Excalibur software).
The peach polyphenolics were identified on the basis of retention times, lambda max, MS and MS/MS data operating in negative mode and, wherever possible, by chromatographic comparisons with authentic markers (Table 1). The external calibrations were obtained using methanolic standard solutions of known concentration (5-300 mg/L). The phenolic contents were expressed as mg/kg FW (fresh weight) (Tables 2 and 3).
Statistical analysis. To verify the statistical significance of all parameters the values of means and standard deviation (SD) were calculated. Where it was appropriate, the data by two-way ANOVA were tested. The $P_{value}<0.05$ was adopted as statistically significant. All quantitative data are means of five measurements.

RESULTS and DISCUSSION

Phenolics characterization. Efficient separations of peel and flesh phenolics in the aqueous-methanolic extracts of Sicilian peaches were achieved using the reversed phase C-18 column. Figure 1 reports the HPLC chromatogram from Leonforte cultivar. Peaks in the chromatograms were classified into two groups: hydroxycinnamic acids and flavonols glycosides (Figure 2), by comparison of their UV–Vis spectra and HPLC retention times to those of the available standards. Three peaks (1, 2 and 8) presented spectral characteristics of the hydroxycinnamic acids while other seven major peaks (3-7, 9 and 10) were ascribed to the flavonols family (Table 1). Differently from what reported in literature (Tomás-Barberán et al. 2001; Hong at al. 2004), catechin and epicatechin as well as the dimeric procyanidins were not detected in the analyzed samples. Mass spectra from the negative ionization mode were also used for unequivocal identification of these phenolic compounds. The mass spectra of the conjugated form phenolic compounds showed the de-protonated molecular ion and the de-protonated aglycone ion as a result of the loss of the sugar residue. Table 1 showed the tentative identification of the chromatographic peaks from Sicilian peaches.
The HPLC-ESI/MS analysis showed that hydroxycinnamates ionized with difficulty under the conditions used, or any other condition that was assayed in this study, showing that the specific sensitivity of the ESI/MS detector for this type of compound was smaller than that observed for the other phenolics analyzed such us flavonols. Despite

this, the ESI/MS with negative ion mode of compounds 1 and 2 gave the same [M-H]- ion at m/z 353 in according with a molecular formula $C_{16}H_{18}O_9$. Their molecular ions [M-H]- yielded three peaks at m/z 191, 179 and 135 in MS/MS (Table 1), which suggested that these two compounds were isomers. The comparison with the pure standards confirmed that the structures of components 1 and 2 were neochlorogenic acid (5-*O*-caffeoylquinic acid) and chlorogenic acid (3-*O*-caffeoylquinic acid), respectively. Chlorogenic acids are the esterified form of caffeic acid and quinic acid. The diagnostic fragmentation patterns of chlorogenic acid isomers in ESI/MS with negative ion mode involved cleavage of intact caffeoyl (m/z 179) and quinic acid fragments (m/z 191). A minor compound (peak 8) with the characteristic spectra of hydroxycinnamic derivatives was detected, but its MS spectra did not allow its identification. The occurrence of neochlorogenic and chlorogenic acids in peaches was already reported in literature (Bengoechea et al. 1997; Chang et al. 2000, Tomás-Barberán et al. 2001).

Peaks 3-6, 9 and 10 displayed UV spectra similar to that of quercetin 3-*O*-rutinoside with two absorption maxima at 255 and 355 nm (Table 1). Peak 7 showed a shift of the UV maximum from 355 to 345 nm. The lack of additional hydroxyl/methoxyl groups attached to ring B in kaempferol, compared to quercetin and isorhamnetin, is responsible for this shift. The MS data obtained for the studied flavonols gave the expected molecular ions and diagnostic fragment ions that were characteristic for these compounds, including the loss of 266 amu from the rutinoside flavonoids: the moiety at m/z 301, 285 and 314 were related to the aglycones of quercetin, kaempferol and isorhamnetin respectively. Six peach flavonol peaks were therefore undoubtedly identified as quercetin-3-rutinoside (4), quercetin-3-galactoside (5), quercetin-3-glucoside (6), kaempferol-3-rutinoside (7), isorhamnetin-3-rutinoiside (9) and isorhamnetin-3-glucoside (10). The peak 3 was tentatively identified as a biglycosylated of quercetin. Previous studies have shown the occurrence of quercetin 3-glucoside, 3-galactoside and 3-rutinoside in peaches (Chang et al. 2000, Tomás-Barberán et al. 2001). To our better knowledge, the occurrence of kaempferol and isorhamnetin flavonols in peach has been reported for the first time in the present work. These results suggest that these flavonols may be characteristic of Sicilian cultivars.

Phenolic distribution and quantitative evaluation. The results of this study confirm that chlorogenic is the main hydroxycinamate of peaches, detected in significant amount in all samples. The occurrence

of neochlorogenic acid was related only to the Leonforte and Riesi samples. Traces of the unidentified hydroxycinnamate 9 were found to be localized only in the peach flesh. As reported in Table 2, the Riesi peaches contained the highest amount of hydroxycinnamic acids derivatives (123.3 mg/kg in market-ripe peel samples), while only traces were detected in the Maniace ones (about 1.5 mg/kg). The content variability for hydroxycinnamates in the peach tissues depended on the cultivar: similar amount of hydroxycinnamates were detected in peel and flesh of Leonforte samples (about 40 mg/kg), with no significant variation during the ripening; on the contrary, the Riesi samples showed higher contents in peel with respect to the flesh which significantly decreased with fruit ripening (Table 2).
The seven identified flavonols were exclusively located in the peel in all the three peach studied; similar results where reported in literature for Californian peaches (Tomás-Barberán et al. 2001). Among them, isorhamnetins predominated in Leonforte and Maniace samples, while quercetins were more abundant in the Riesi samples. Differently from the hydroxycinnamates, the content variability for these compounds was not significantly affected by the cultivar, with values ranging from 117.4 mg/kg for Leonforte to 153.2 mg/kg for Riesi market-ripe peel samples (Table 3). A decrement of about 30% during ripening was observed in the Maniace flavonol contents, while Leonforte and Riesi samples showed flavonol amounts reduction of about 50% from market-ripe to fully-ripe maturation stage (Table 3). Analogous results were reported by Wang (2009) in red raspberries: polyphenols such as quercetin and kaempferol derivatives were initially present at high levels but decreased drastically during maturation.
In Figure 3 was reported the phenolics total amount for the studied samples; as a general rule, the peel tissues contained higher amounts of phenolics with respect to the flesh. Differently from what reported in literature for Californian peaches, which evidenced that there was no clear trend in phenolic content with ripening (Tomás-Barberán et al. 2001), our results evidenced a phenolics decrement with maturation stage. The Riesi and the Maniace samples were the richest and the poorer in phenolics respectively, even if the differences among the studied samples tended to level off with the ripening.

CONCLUSIONS

A wide variation of phenolics content in Leonforte, Maniace and Riesi yellow-flesh peaches tissues was observed, that generally decreased with ripening. The peach flesh showed in general lower phenolics

amount than peel. The differences were especially due to the exclusive presence of flavonols in the peel. Kaempferol and isorhamnetin flavonols have been identified for the first time in peel tissues. This result suggests that these flavonols may be characteristic of Sicilian cultivars. The protection of food authenticity represents an area of the food industry where quantification of phenolic compounds has potential as a useful tool for authenticity controls (Fügel et al. 2005). Therefore, research studies on peach phenols would be well advised to examine this aspect more closely. Since there are no literature data on the distribution of phenolics compounds in Italian peaches, further studies will be conducted in the future comparing peach cultivars from different geographic areas.

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Figure Captions

Figure 1. HPLC-UV-vis chromatograms of Leonforte peach phenolics separated from peel (a) and flash (b) and detected at 340 nm. 1: 5-*O*-caffeoylquinic acid; 2: 3-*O*-caffeoylquinic acid; 3: quercetin biglycoside (tentative); 4: quercetin-3-*O*-rutinoside; 5: quercetin-3-*O*-galactoside; 6: quercetin-3-*O*-glucoside; 7: kaempferol-3-*O*-rutinoside; 8: isorhamnetin-3-*O*-rutinoside; 9: hydroxycinnamate (tentative); 10: isorhamnetin-3-*O*-glucoside.
Figure 2. Structures of aglycones and conjugates of phenolics compounds found in Sicilian yellow flesh peaches (peak numbers in parentheses).
Figure 3. Total phenolics (mg/kg fresh weight) in Sicilian yellow flesh peaches. MR: market-ripe; FR: fully-ripe.

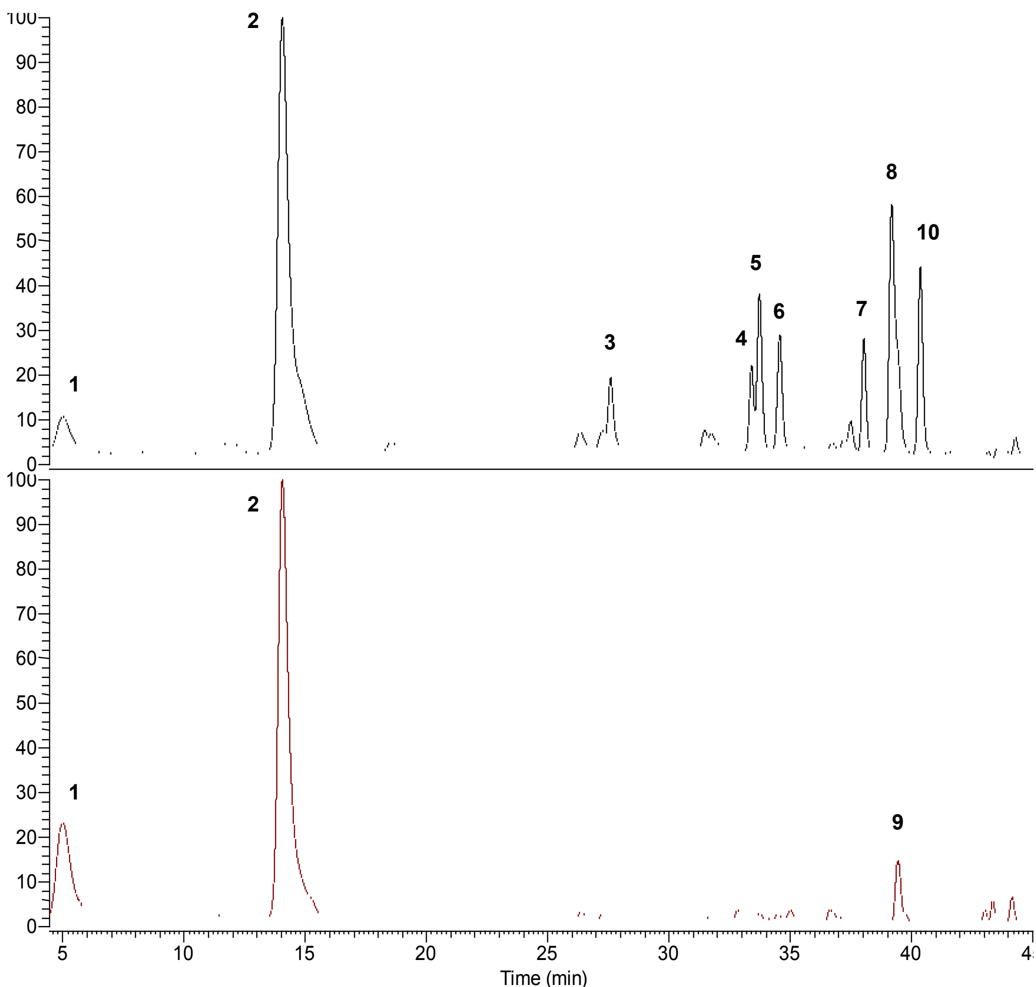

Figure 1.

Quinic acid and Hydroxycinnamics acids	R1	R2	R3
Quinic acid	OH	OH	OH
5-*O*-caffeoylquinic acid (neochlorogenic acid) (**1**)		OH	OH
3-*O*-caffeoylquinic acid (chlorogenic acid) (**2**)	OH	OH	

Flavonol aglyconoes and glycosides	R1	R2	R3
Quercetin	OH	H	H
Quercetin 3-*O*-rutinoside (**4**)	OH	H	rutinose
Quercetin 3-*O*-galactoside (**5**)	OH	H	galactose
Quercetin 3-*O*-glucoside (**6**)	OH	H	glucose
Kaempferol	H	H	H
Kaempferol 3-*O*- rutinoside (**7**)	H	H	rutinoside
Isorhamnetin	OCH_3	H	H
Isorhamnetin 3-*O*- rutinoside (**8**)	OCH_3	H	rutinoside
Isorhamnetin 3-*O*- glucoside (**10**)	OCH_3	H	glucoside

Figure 2.

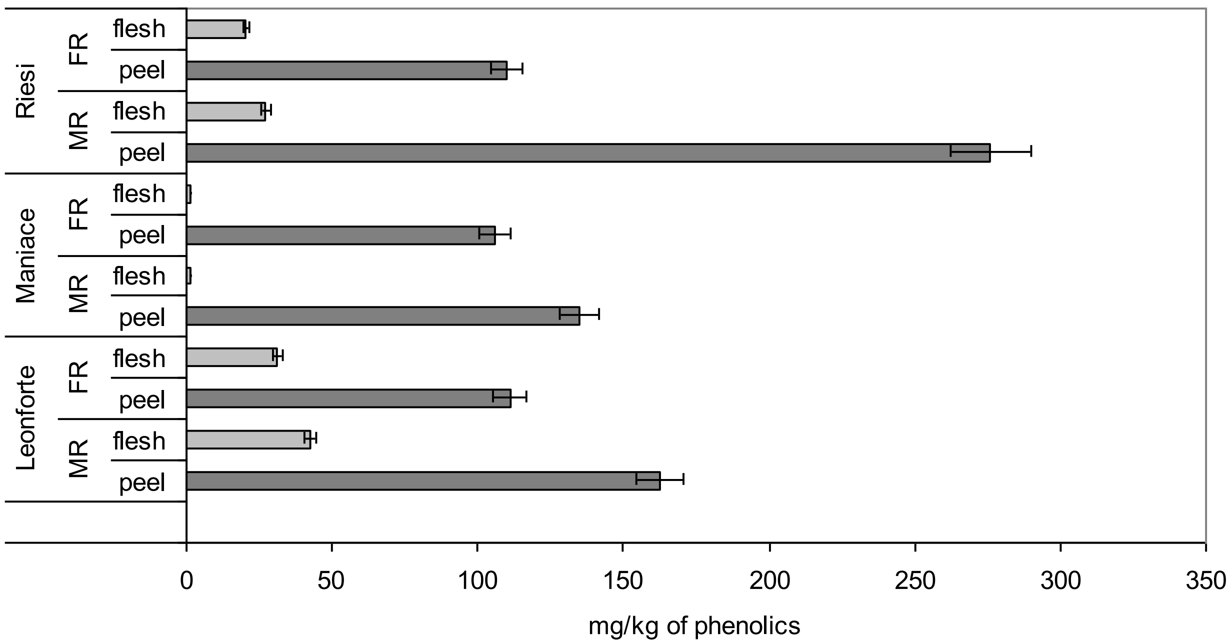

Figure 3.

Table 1. Identification of phenolics in extracts Sicilian peaches by using their spectral characteristics in HPLC-PDA, negative ions in HPLC-MS and MS-MS, respective standards and the literature.

No.	Retention Time (min)	λ_{max} (nm)	Parent Ion $[M-H]^-$ (m/z)	Collision energy (%)	Doughter Ions MS^2 (m/z)	Compound name
1	5.0	250, 300*sh*, 325	353	25	191 (100%), 179 (90%), 135 (20%)	5-*O*-caffeoylquinic acid
2	14.1	250, 300*sh*, 325	353	25	191 (100%), 179 (70%), 135 (25%)	3-*O*-caffeoylquinic acid
3	27.6	255, 265*sh*, 355	625	35	625 (100%), 437 (20%)	Quercetin biglycoside*
4	33.4	260, 270*sh*, 300*sh*, 355	609	35	301 (100%), 609 (30%), 345 (10%)	Quercetin-3-*O*-rutinoside
5	33.7	255, 265*sh*, 355	463	35	301 (100%)	Quercetin-3-*O*-galactoside
6	34.2	255, 265*sh*, 355	463	35	301 (100%), 463 (10%)	Quercetin-3-*O*-glucoside
7	38.1	265, 300*sh*, 345	594	30	285 (100%), 593 (10%)	Kaempferol-3-*O*-rutinoside
8	39.2	255, 265*sh*, 300*sh*, 355	623	35	314 (90%), 623 (45%), 357 (15%)	Isorhamnetin-3-*O*-rutinoside
9	39.4	250, 300*sh*, 325	-	-	-	Hydroxycinnamate*
10	40.4	255, 265*sh*, 300*sh*, 355	477	35	477 (100%), 314 (50%), 357 (15%)	Isorhamnetin-3-*O*-glucoside

*tentatively identified.

Table 2. Hydroxycinnamic Acid Derivatives (mg/kg fresh weight) in Yellow Flesh Sicilian peaches[a].

Peach	Stage	Part	5-*O*-caffeoylquinic acid	3-*O*-caffeoylquinic acid	other[b]	Total
Leonforte	market-ripe	peel	3.4 (0.2)	38.7 (1.9)	-	45.1 (1.9)
		flesh	9.4 (0.5)	31.1 (1.6)	2.0 (0.1)	42.5 (1.6)
	fully-ripe	peel	-	46.3 (2.3)	-	46.3 (2.3)
		flesh	7.9 (0.4)	21.8 (0.9)	1.6 (0.1)	31.3 (1.0)
Maniace	market-ripe	peel	-	2.6 (0.1)	-	2.6 (0.1)
		flesh	-	0.3 (0.02)	0.8 (0.04)	1.1 (0.04)
	fully-ripe	peel	-	0.5 (0.03)	-	0.5 (0.03)
		flesh	-	0.8 (0.04)	0.3 (0.02)	1.1 (0.04)
Riesi	market-ripe	peel	26.4 (1.6)	96.9 (4.9)	-	123.3 (5.1)
		flesh	12.4 (0.6)	13.1 (0.7)	1.8 (0.1)	27.3 (0.9)
	fully-ripe	peel	7.7 (0.4)	28.0 (1.4)	-	35.7 (1.5)
		flesh	10.1 (0.5)	8.6 (0.4)	1.8 (0.1)	20.5 (0.7)

[a] Standard deviations (*n=5*) in parentheses. [b] 3-*O*-caffeoylquinic acid

Table 3. Flavonols (mg/kg fresh weight) in Yellow Flesh Sicilian peaches[a].

Peach	Stage	Part	Querc digly (tentative)[b]	Querc 3-rut	Querc 3-gal	Querc 3-glu	Kaemp 3-rut	Isorham 3-rut	Isorham 3-glu	Total
Leonforte	market-ripe	peel	9.9 (0.5)	9.3 (0.5)	17.3 (0.9)	13.4 (0.7)	10.6 (0.5)	38.0 (1.9)	19.0 (0.8)	117.4 (2.5)
		flesh	-	-	-	-	-	-	-	-
	fully-ripe	peel	6.0 (0.2)	3.9 (0.2)	11.8 (0.6)	8.1 (0.4)	6.4 (0.3)	19.7 (1.0)	8.9 (0.5)	64.8 (1.4)
		flesh	-	-	-	-	-	-	-	-
Maniace	market-ripe	peel	7.8 (0.5)	6.9 (0.4)	26.4 (1.3)	21.5 (1.1)	6.8 (0.3)	40.8 (2.5)	22.1 (1.1)	132.3 (3.3)
		flesh	-	-	-	-	-	-	-	-
	fully-ripe	peel	5.5 (0.3)	7.7 (0.4)	9.6 (0.5)	11.3 (0.6)	12.7 (0.8)	42.8 (2.1)	15.1 (0.9)	104.7 (2.6)
		flesh	-	-	-	-	-	-	-	-
Riesi	market-ripe	peel	8.6 (0.4)	9.6 (0.6)	30.9 (1.6)	50.5 (2.5)	8.3 (0.4)	28.0 (1.4)	17.3 (0.9)	153.2 (3.5)
		flesh	-	-	-	-	-	-	-	-
	fully-ripe	peel	4.9 (0.3)	4.8 (0.2)	14.8 (0.7)	30.0 (1.5)	3.7 (0.2)	16.9 (0.9)	8.3 (0.4)	83.4 (2.0)
		flesh	-	-	-	-	-	-	-	-

[a] Standard deviations (*n=5*) in parentheses. [b] as quercetin 3-rutinoside.

Paper No 8

Muratore Antonio, Mazzaglia Agata, Monica Scordino, Leonardo Sabatino, Lanza Carmela Maria, Adalgisa Belligno, Giacomo Gagliano.

Caratterizzazione fisico-chimica e valutazione sensoriale di pesche di Leonforte (*Persica Vulgaris cv tardiva di Leonforte*) a maturazione commerciale e fisiologica.

Ingredienti alimentari, Chiriotti ed., *in press*.

CARATTERIZZAZIONE FISICO-CHIMICA E VALUTAZIONE SENSORIALE DELLA PESCA DI LEONFORTE (*Persica vulgaris cv Tardiva di Leonforte*) A MATURAZIONE COMMERCIALE E FISIOLOGICA

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RIASSUNTO
La Pesca di Leonforte, unica pesca siciliana ad aver ottenuto l'Indicazione geografica protetta (IGP), è fatta crescere e maturare all'interno di un sacchetto di carta pergamenata, per proteggerla da insetti e intemperie ed evitare trattamenti fitosanitari. Nel presente lavoro per confermare le caratteristiche peculiari della pesca di Leonforte inserita tra i Presìdi Slow Food sono stati determinati alcuni parametri fisico-chimici nonché il profilo sensoriale. Dai risultati, in particolare, emerge che i frutti sono contraddistinti da una buona consistenza della polpa che favorisce la commercializzazione su mercati anche esteri, da un buon contenuto zuccherino e da un profilo sensoriale caratterizzato da un'elevata intensità dei descrittori aroma e flavour di pesca, aroma floreale, compattezza, dolce, succoso e valutazione complessiva.

INTRODUZIONE
La Pesca di Leonforte (Persica vulgaris cv Tardiva di Leonforte), Presidio Slow Food, ottenuta dalla coltivazione degli ecotipi locali di

pesca Bianco e Giallone di Leonforte, comune dell'entroterra siciliano, ha ottenuto per l'Italia l'Indicazione geografica protetta (IGP) con il Regolamento CE n. 622/2010 del 15 luglio 2010. I peschicoltori leonfortesi, per difendere la produzione e impedire l'ovodeposizione della mosca della frutta, ricorrono all'insacchettamento dei frutti, pratica che consiste nell'applicare a ogni frutto un sacchetto di carta pergamenata 120-150 giorni prima della maturazione, proteggendo in tal modo le pesche anche da tutti gli agenti esterni fino alla maturazione completa.

Il ricorso a tale tecnica colturale, unica nel suo genere, giustifica pienamente l'assenza dei trattamenti chimici, evitando l'eventuale presenza di residui tossici nei frutti, che nella peschicoltura convenzionale, purtroppo, sono quasi del tutto ineliminabili. Uno dei limiti della commercializzazione delle pesche è rappresentato dal fatto che spesso sono immesse sul mercato in uno stadio di maturazione inadeguato, con caratteristiche chimico-fisiche e sensoriali non soddisfacenti il consumatore, inducendolo a disaffezionarsi dal prodotto. Pertanto è importante definire il periodo di raccolta ottimale dei frutti, in base all'optimum degli standard qualitativi per la loro commercializzazione.

Dopo un periodo di monitoraggio di due anni la maturità commerciale della pesca di Leonforte è stata definita in un periodo di circa 5 giorni, in cui le caratteristiche dell'epicarpo del frutto permettono la raccolta e il confezionamento per la vendita nel circuito della grande distribuzione nazionale (GDO) ed estera, mentre la maturità fisiologica sopraggiunge nel periodo successivo, in contemporanea alla caduta del frutto nel sacchetto, ed ha una durata di circa 3 giorni permettendo soltanto la vendita locale.

Il presente lavoro ha voluto dare un contributo alla valorizzazione della pesca di Leonforte caratterizzandola mediante parametri quali: colore e consistenza sul frutto intero, quest'ultima a maturità commerciale (C) e fisiologica (F); polifenoli totali, zuccheri e acidi organici su polpa (P) e buccia (B) in frutti a maturità commerciale e fisiologica, allo scopo di valutare la differente distribuzione di tali composti nelle diverse parti del frutto in funzione dell'epoca di maturazione. Inoltre, sui campioni a maturità commerciale è stato definito il profilo sensoriale.

MATERIALI E METODI

I frutti sono stati raccolti nella seconda metà di settembre. La determinazione del colore sui campioni, mediante i parametri di Hunter L*a*b*, è stata eseguita utilizzando un colorimetro portatile (NR-3000,

Nippon Denshoku Ind. Co. Ltd), con illuminante C/2°. La misura dei parametri è stata eseguita su cinque differenti punti della superficie del frutto. La consistenza è stata determinata utilizzando un dinamometro (penetrometro) che misura la forza impressa dall'operatore per penetrare la polpa del frutto per mezzo di un cilindretto di 8 mm di diametro. Il contenuto in polifenoli totali è stato determinato utilizzando il metodo di Folin-Ciocalteau. Il contenuto in zuccheri e acidi organici è stato quantificato mediante un metodo gas cromatografico secondo quanto riportato da Sweeley et al. (1963).

Per definire il profilo sensoriale è stato applicato il metodo UNI 10957 (2003). Le valutazioni sono state condotte nel laboratorio di analisi sensoriale, costruito a norma ISO 8589 (2007), presso il DISPA della facoltà di Agraria di Catania, dotato di uno specifico software per l'acquisizione dei dati sensoriali (FIZZ Biosystemes, Couternon, France). Dodici giudici sono stati selezionati tra gli studenti e addestrati per un periodo di circa un mese con frequenza di due o tre sedute settimanali. In questa fase i giudici hanno familiarizzato con il prodotto e sono stati istruiti sul metodo e sulla scala di valutazione, al fine di definire un vocabolario comune comprendente tutti i descrittori (scelti con una frequenza di citazione del 70%) emersi nel corso delle sedute. I sedici descrittori utilizzati per la definizione del profilo sensoriale dei frutti sono stati: visivi (uniformità colore esterno e intensità del colore della polpa), tattili (compattezza e facilità distacco polpa), olfattivi (aroma di pesca, erbaceo e floreale), gustativi (dolce, acido e amaro), tattili in bocca (pastosità e succosità), flavour (flavour di pesca, erbaceo e floreale) e valutazione complessiva. Per la valutazione dei campioni ai giudici è stato chiesto di quantificare l'intensità dei singoli descrittori su una scala discontinua da 1 (assenza del descrittore) a 9 (massima intensità del descrittore). A ogni giudice è stato presentato un frutto intero in piatti di plastica siglati con un codice a tre cifre.

RISULTATI E DISCUSSIONE

Dai risultati delle analisi chimico-fisiche (Tabella 1) emerge che il parametro L* non subisce variazioni in base all'epoca di raccolta e dai valori si evince che nelle bucce si ha la predominanza del colore bianco. L'indice del rosso-verde a* mostra valori maggiori nei frutti a maturità commerciale, mentre l'indice del colore giallo-blu b* mostra un andamento opposto.

La consistenza dei campioni di pesca raggiunge valori ottimali in un range compreso tra 3 e 4,5 kg (0,5 cm2)-1 a ridosso della maturità commerciale e subisce una diminuzione significativa nella fase F

rispetto alla fase C (-17,95). A differenza di altre varietà di pesca, quella di Leonforte ha una consistenza che favorisce periodi di lunga conservazione.
Il contenuto in polifenoli mostra, sia nella polpa sia nella buccia, una diminuzione alla maturità fisiologica rispettivamente del 5,80% nella polpa e del 5% nella buccia. Per quanto riguarda il contenuto in zuccheri, dalla maturità commerciale a quella fisiologica gli zuccheri totali presentano un incremento del 10% nelle polpe e dell'11% nelle bucce. Inoltre, sia nelle polpe sia nelle bucce, il contenuto di glucosio diminuisce dalla maturazione commerciale a quella fisiologica mentre si ha un incremento del contenuto di fruttosio e di saccarosio. Dalla determinazione del contenuto in acidi organici emerge che l'acido malico a maturità commerciale ha un contenuto più elevato nella polpa rispetto all'acido clorogenico e quinico. Quest'ultimo, sempre a maturità commerciale, presenta un contenuto più elevato nella polpa rispetto alla buccia, mentre l'acido clorogenico ha un contenuto più elevato nelle bucce.
Il profilo sensoriale dei frutti a maturità commerciale è caratterizzato da un'elevata intensità dei descrittori aroma e flavour di pesca, aroma floreale, compattezza, dolce, succoso e valutazione complessiva. I valori medi dell'intensità di ogni descrittore rappresentati in forma grafica su una scala non strutturata disposta a raggiera nel piano, hanno consentito di ottenere lo spider plot (Figura 1) che rappresenta il profilo sensoriale del campione.

CONCLUSIONI

L'applicazione della pratica dell'insacchettamento, impiegata per la pesca di Leonforte, consentendo di ridurre l'impiego di prodotti fitosanitari, ha sicuramente comportato un minor impatto ambientale. Inoltre, data la sua elevata consistenza, confermata anche sensorialmente, che poco varia durante la maturazione, si presta facilmente a essere commercializzata su mercati nazionali ed esteri. Le sue peculiari caratteristiche sensoriali, inconfondibili rispetto a quelle di altre varietà, sono state confermate dal profilo sensoriale ottenuto. La consapevolezza del valore di queste produzioni, insieme ad azioni di protezione e valorizzazione, non solo può portare vantaggi economici ai produttori ma anche ai consumatori.

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-ISO 8589 (2007). Sensory analysis – General guidance for the design of test rooms. International Organization for Standardization.

PHYSICAL-CHEMICAL CHARACTERIZATION AND SENSORY EVALUATION OF PEACH OF LEONFORTE (*Persica vulgaris cv Tardiva di Leonforte*) AT COMMERCIAL AND PHYSIOLOGICAL MATURITY

ABSTRACT

The peach of Leonforte, only Sicilian peach to have obtained the protected geographical indication (IGP), is made to grow and mature into a bag of parchment paper to protect it from insects and weather and to avoid pesticide treatments. To confirm its own characteristics, including sensory aspects, the aim of present study was to characterize the peach of Leonforte by physical-chemical and sensory analysis. The results show that the fruits are characterized by a good firmness of the pulp that promotes the commercialization in abroad markets, a good sugar content and a sensory profile characterized by high intensity of aroma and flavor descriptors fishing, floral aroma, compact, sweet, juicy and overall assessment.

Tabella 1. Valori medi dei parametri chimico-fisici determinati.

Parametri		Prelievi	Leonforte	Parametri		Prelievi	Leonforte
Colore	L*	C	82,95	Consistenza kg (0,5 cm2)-1		C	4,00
		F	85,14			F	3,10
	a*	C	19,29	Polifenoli mg/100 g	Polpa	C	25,24
		F	14,93			F	23,91
	b*	C	-11,14		Buccia	C	45,03
		F	-8,64			F	42,79
Ac. malico mg/100 g	Polpa	C	0,14	Glucosio mg/100 g	Polpa	C	1,02
		F	0,05			F	0,55
	Buccia	C	0,10		Buccia	C	0,94
		F	0,08			F	0,56
Ac. clorogenico mg/100 g	Polpa	C	0,012	Fruttosio mg/100 g	Polpa	C	1,25
		F	0,012			F	1,58
	Buccia	C	0,024		Buccia	C	1,22
		F	0,001			F	1,39
Ac. quinico mg/100 g	Polpa	C	0,13	Saccarosio mg/100 g	Polpa	C	9,84
		F	0,04			F	10,38
	Buccia	C	0,16		Buccia	C	6,62
		F	0,13			F	7,06
Acidi totali mg/100 g	Polpa	C	0,28	Zuccheri totali mg/100 g	Polpa	C	12,11
		F	0,10			F	12,51
	Buccia	C	0,28		Buccia	C	8,78
		F	0,21			F	9,01

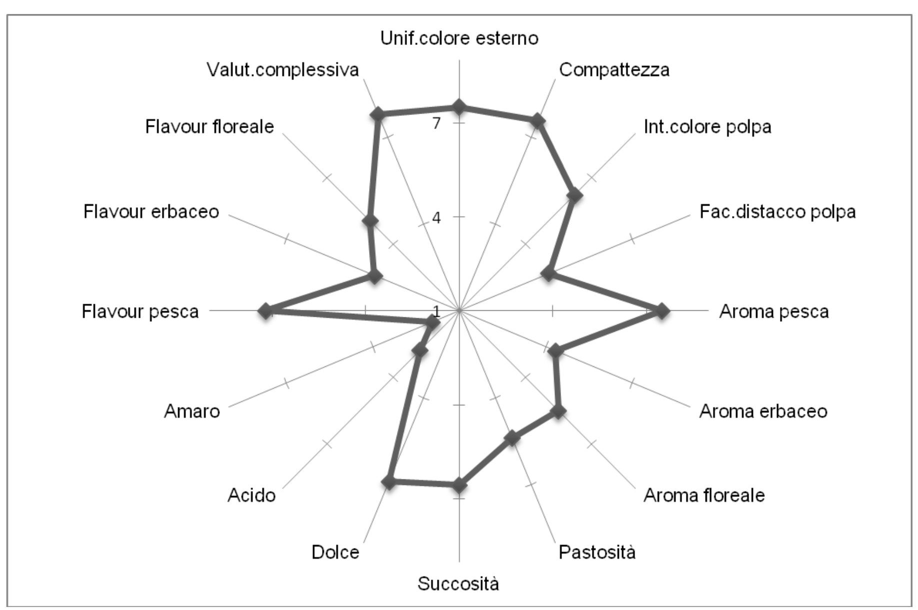

Figura 1. Profilo sensoriale della pesca di Leonforte IGP

Paper No 9

Monica Scordino, Leonardo Sabatino, Rosario Caruso, Adalgisa Belligno, Giacomo Gagliano.

Caratterizzazione di ammine biogene dansil derivate mediante HPLC-UV-ESI/MS.

In progress to be submitted.

CARATTERIZZAZIONE DI AMMINE BIOGENE DANSIL-DERIVATE TRAMITE HPLC-UV-ESI/MS

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ABSTRACT
Il presente lavoro descrive per la prima volta l'applicazione della spettrometria di massa elettrospray per l'analisi di sette ammine biogene dansil-derivate (tripatmina, putrescina, cadaverina, istamina, tiramina, spermidina e stermina), tramite cromatografia liquida ad alte prestazioni utilizzando 1,7 diaminopetano come standard interno. L'addizione di uno o più gruppi dansili alle molecole di interesse determina un incremento sostanziale del peso molecolare, agevolando da una parte la separazione cromatografica e dall'altra la possibilità di rilevare e quantificare inequivocabilmente i relativi derivati mediante spettrometria di massa. Data la notevole riduzione dell'effetto matrice, il metodo risulta adatto per la separazione delle poliammine da fonti animali, vegetali e fungine.

Keywords:
ammine biogene, derivatizzazione pre-colonna, fermentazione, spettrometria di massa.

INTRODUZIONE
Le ammine biogene sono basi azotate a basso peso molecolare dotate di attività biologica. Dal punto di vista biochimico provengono per la maggior parte dalla decarbossilazione degli aminoacidi ad opera di lieviti e batteri. Le ammine biogene sono presenti in molti alimenti, ne

risultano naturalmente ricchi il succo d'arancia, i pomodori, le banane, gli spinaci; si trovano facilmente in bevande ed alimenti fermentati come formaggi, salumi, birra e vino [1,2,3]. Le più importanti sindromi di origine alimentare causate dall'ingestione di ammine biogene sono l'avvelenamento da istamina (sindrome sgombroide) e l'intossicazione da tiramina (sindrome del formaggio), documentate da numerosi studi epidemiologici. Generalmente tali intossicazioni avvengono a seguito di consumo di alimenti in qualche modo alterati: pesce poco fresco, formaggi e vini con fermentazioni anomale.
La difficoltà di quantificare basse concentrazione di ammine biogene attraverso tecniche cromatografiche classiche è determinata dall'assenza di gruppi cromofori e dal notevole effetto matrice. Le moderne tecniche di spettrometria di massa accoppiata all'HPLC permettono di limitare notevolmente l'effetto matrice anche se, nel caso in oggetto, il basso peso molecolare e la struttura chimica delle molecole di interesse ne limita l'applicazione a fini quantitativi; le molecole di interesse vengono infatti tutte eluite nei primi minuti di corsa cromatografica, e risultano spesso sovrapposte tra loro e coeluite con il fronte del solvente. Di contro, la derivatizzazione dei gruppi amminici liberi con reattivi appropriati ne permette una buona separazione cromatografica e la rivelazione come derivati fluorescenti e/o Uv-visibili, benché sia fortemente caratterizzata da un notevole effetto matrice, enfatizzato dall'ampia tipologia di prodotti da sottoporre ad analisi che spaziano dai formaggi, ai pesci e alle bevande alcoliche.
Nell'ottica del controllo qualità delle produzioni agroalimentari, è stato sviluppato un metodo cromatografico che accoppia la spettroscopia UV-vis alla spettrometria di massa elettrospray (ESI) per la separazione e relativa caratterizzazione di 7 ammine biogene mediante derivatizzazione pre-colonna con cloruro di dansile.

MATERIALI E METODI

Preparazione degli standard. Soluzioni madre di circa 1000 ppm di triptamina, putrescina, cadaverina, istamina, tiramina, spermidina, spermina e 1,7-diamminoeptano sono state preparate disciogliendo opportunamente standard ad elevata purezza (Sigma-Aldrich) in HCl 0,1 M. Tali soluzioni sono stabili a -18°C per 2 mesi.
Estrazione dai campioni. 5 g di campione omogeneizzato sono stati addizionati di 20 mL di HCl 0,1 M e 100 μL di soluzione madre di 1,7-diamminoeptano (Standard Interno); l'estratto è stato centrifugato a 3000 rpm per 10 min a 20°C ed il residuo riestratto con ulteriori 20 mL di HCl 0,1 M. I due estratti sono stati riuniti e portati a 100 mL in pallone tarato.
Derivatizzazione pre-colonna. 1 mL di estratto (o 1 mL di soluzioni standard opportunamente diluite) sono state addizionate di 0,5 mL di

bicarbonato di sodio saturo ed 1 ml di cloruro di dansile (5 mg/mL in acetone), agitato per 1 min in vortex e posto al buio per 60 min. Quindi sono stati aggiunti 200 µL di prolina (100 mg/ml in HCl 0,1 M), agitato per 1 min in vortex e lasciato reagire per 15 min. La soluzione è stata estratta con due successive addizioni di etere dietilico (1 mL + 1 mL), le parti organiche sono state riunite e portate a secco in rotavapor. L'estratto è stato ripreso con 2 mL di acetonitrile ed analizzato.
Condizioni cromatografiche HPLC/PDA/MS. I dansil derivati sono stati separati tramite colonna Phenomenex Gemini C18 (150 x 2,1 mm, I.D. 3µm) termostatata a 30°C, utilizzando un gradiente binario di (A) metanolo e (B) acqua (1mM HCOONH4, 0.5% HCOOH), con un flusso di 0.2 mL/min. Gradiente: 0 min, 10% A; 9 min, 50% A; 24,5 min, 90% A; 28 min, 40% A; 30 min, 10% A; 32 min, 10% A. Scan range PDA 190-700 nm; scan range m/z 50-2000, sheath gas 18 units, auxiliary gas 16 units, capillary temperature 220°C, spray voltage 4,00 KV. L'identità dei dansil derivati è stata confermata in spettrometria di massa ESI positivo.

RISULTATI E DISCUSSIONE

Nel presente lavoro la tecnica ifenata HPLC-UV-ESI/MS è stata applicata per la prima volta all'analisi di ammine biogene dansil-derivate secondo la seguente reazione di acetilazione del gruppo amminico con cloruro di dansile:

L'addizione di uno o più gruppi dansili alle molecole di interesse determina un incremento sostanziale del peso molecolare (Tabella 1), agevolando da una parte la separazione cromatografica in fase inversa e dall'altra la possibilità di rilevare e quantificare i relativi derivati mediante spettrometria di massa. In Figura 1 è riportato il cromatogramma ESI/MS delle sette ammine dansil derivate oggetto di studio. La separazione cromatografica mostra una distribuzione dei picchi di interesse a tempi di ritenzione compresi tra i 22 ed i 28 minuti. L'identificazione dei derivati dansilati è stata effettuata tramite spettrometria di massa in modalità ESI positivo, basandosi sul loro spettro di massa. L'analisi LC/MS ha mostrato che i dansil derivati di ciascuna ammina danno luogo alla produzione di uno ione molecolare $[M+H]^+$ molto stabile ed intenso, che non mostra frammentazioni nelle condizioni di analisi. I risultati mostrano che la reazione di dansilazione

produce i derivati stechiometricamente attesi (Tabella 1); nel caso di poliammine tutti i gruppi chimicamente reattivi vengono sostituiti (gruppi amminici e, nel caso della tiramina, anche il gruppo ossidrilico), dando luogo a derivati anche tre/quattro volte più pesanti delle molecole di partenza e quindi facilmente rilevabili tramite spettrometria di massa. L'ausilio della spettrometria di massa in questa tipologia di analisi presenta il notevole vantaggio di limitare l'effetto matrice, grazie all'estrazione del singolo ione di interesse. Sono attualmente in corso applicazioni del metodo suddetto in matrice: risultati preliminari (dati non mostrati) mostrano che il metodo combina una buona capacità estrattiva ed una efficiente separazione cromatografica che ne permette l'applicazione in matrici complesse con recuperi che variano dal 70 al 110%, una buona linearità ($r^2>0{,}998$) e alti livelli di precisione (RSD<5%).

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CHARACTERIZATION OF DANSYL-BIOGENIC AMINES BY HPLC-UV-ESI/MS

ABSTRACT
The present work describes for the first time the application of electrospray mass spectrometry for the analysis of seven dansyl-derived biogenic amines (triptamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine), using high performance liquid chromatography using 1.7 diaminoheptane as internal standard. The addition of one or more dansyl-groups to the studied molecules causes a substantial increase in molecular weight, facilitating the chromatographic separation and the ability to unambiguously detect and quantify its derivatives by mass spectrometry. Given the significant reduction of the matrix, the method is suitable for the separation of polyamines from animal sources, plants and fungi.

Keywords: biogenic amines, pre-column derivatization, fermentation, mass spectrometry.

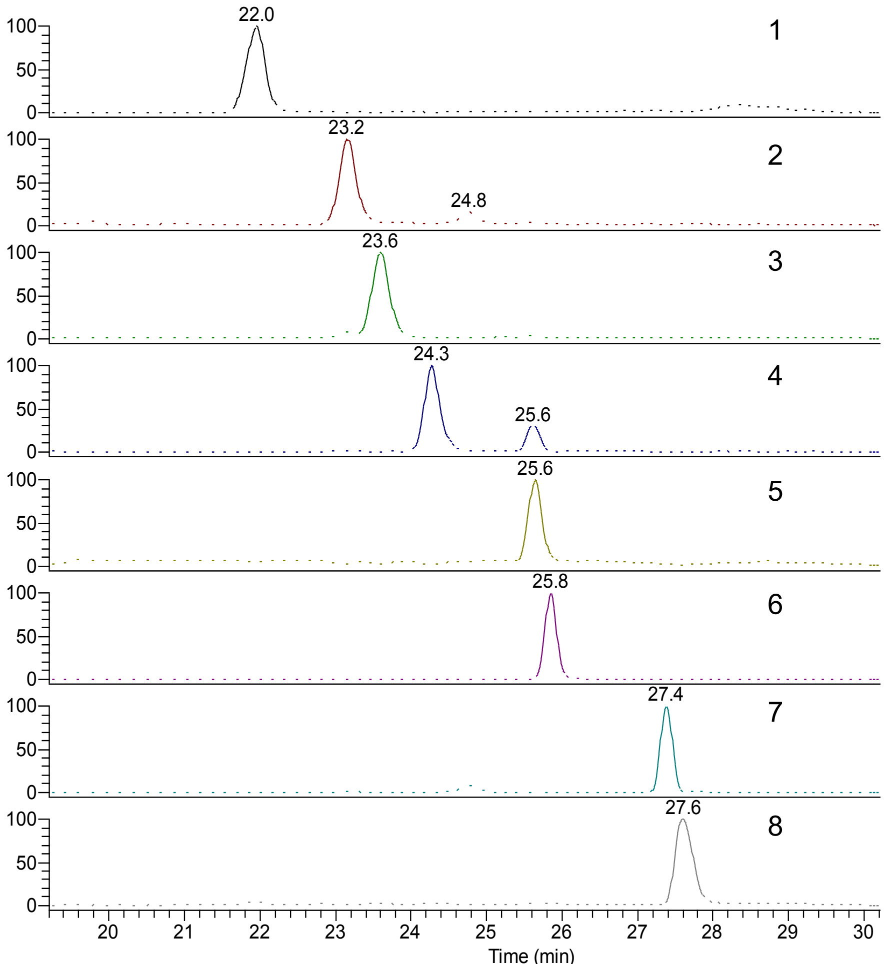

Figura 1. Cromatogrammi ESI/MS dei composti studiati. TIC (Total Ion Current) con estrazione dello ione molecolare $[M+H]^{+}$. Identificazione composti in **Tabella 1**.

N.	Composto	Formula chimica	Peso Molecolare	Dansilammide	Tr (min)	$[M+H]^+$ (m/z)
1	Triptamina		160,22	Dns-Triptamina	22,0	394
2	Putrescina		88,15	$(Dns)_2$-Putrescina	23,2	556
3	Cadaverina		102,18	$(Dns)_2$-Cadaverina	23,6	570
4	1,7-Diaminoeptano		130,23	$(Dns)_2$-1,7-Diaminoeptano	24,3	598
5	Istamina		111,15	$(Dns)_2$-Istamina	25,6	579
6	Tiramina		137,18	$(Dns)_2$-Tiramina	25,8	605
7	Spermidina		145,25	$(Dns)_3$-Spermidina	27,4	846
8	Spermina		202,34	$(Dns)_4$-Spermina	27,6	1136

Tabella 1. Caratterizzazione delle dansilammidi oggetto di studio.

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Hoping to continue my experience in scientific research, I hope that the results of these years can give benefit in the field of food quality control.