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Targeted metabolomics as an advanced tool for the detection of pomegranate juice adulteration

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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Στοχευμένη ανάλυση με τη χρήση μεταβολομικής ως ένα προηγμένο εργαλείο για την ανίχνευση νοθείας στο χυμό ροδιού

ΣΟΦΙΑ ΔΡΑΚΟΠΟΥΛΟΥ

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ABSTRACT

Economically motivated adulteration (EMA) of food, also known as food fraud, is the intentional adulteration of food for financial advantage. A common form of EMA is the undeclared substitution with alternative ingredients and could also pose a health risk to consumers due to potential allergic reactions. Fruit juices have been in the top-7 foods reported from 1980 to 2010 as the most common targets for adulteration.

Several fruit juices, and especially pomegranate juice, have become popular with regard to high levels of antioxidants, presumed to be associated with positive health effects. Similarly to other highly prized food commodities, the economic value and large-scale production of these valuable fruit juices have made them a likely target for adulteration and fraud. One of the most frequent profit-driven fraudulent procedures is the extension of authentic juice with cheaper alternatives (typically juices obtained from apples, grapes and others). Consequently, there is a substantial need for effective food control systems to protect consumers from adulterated food products.

In the present study, the main objective was to explore the feasibility of using targeted analysis under a metabolomics approach. For this reason, a reversed-phase liquid-chromatography coupled to quadruple-time-of-flight mass spectrometry (RPLC-QToF-MS) was used and the data were acquired through broad-band Collision Induced Dissociation (bbCID), which provided information on parent and fragment ions without pre-selection of analytes in one run, in order to discriminate authentic and adulterated fruit juices. Data corresponding to the phenolic composition of fruit juices and their LC-HRMS metabolic fingerprint were considered as a source of potential descriptors for the classification of juices and detection of adulteration.

The data set was treated using advanced chemometric techniques in order to identify possible markers. Finally, pomerganate-fruit extracts were adulterated with different amounts (1–20%) of grape and apple juice and the phenolic profile and mass spectrometric fingerprinting was evaluated for authentication purposes.

SUBJECT AREA: Analytical Food Chemistry

KEYWORDS: fruit juices, adulteration, authenticity, LC-QToF-MS, target screening, chemometrics, metabolomics

ΠΕΡΙΛΗΨΗ

Η οικονομικά παρακινούμενη νοθεία (Economically Motivated Adulteration, EMA) των τροφίμων, γνωστή και ως απάτη τροφίμων, είναι η σκόπιμη νοθεία των τροφίμων με στόχο το κέρδος. Μια κοινή μορφή EMA είναι η αδήλωτη υποκατάσταση με εναλλακτικά συστατικά, γεγονός που μπορεί επίσης να αποτελέσει κίνδυνο για την υγεία των καταναλωτών λόγω πιθανών αλλεργικών αντιδράσεων. Οι χυμοί φρούτων βρίσκονται στις κορυφαίες 7 τροφές που αναφέρθηκαν από το 1980 έως το 2010 ως οι πιο συνηθισμένοι στόχοι για νοθεία.

Αρκετοί χυμοί φρούτων, και ιδιαίτερα χυμοί ροδιού, έχουν γίνει δημοφιλείς στην αγορά όσον αφορά τα υψηλά επίπεδα αντιοξειδωτικών, που θεωρείται ότι σχετίζονται με θετικές επιδράσεις στην υγεία. Όπως και άλλα τρόφιμα υψηλής διατροφικής αξίας που χαρακτηρίζονται από αυξημένη τιμή αγοράς, οι χυμού ροδιού κατατάσσονται στους πιο πιθανούς στόχους για νοθεία και απάτη. Μια από τις πιο συχνές απάτες που βασίζονται στο κέρδος είναι η μερική υποκατάσταση του αυθεντικού χυμού με φθηνότερες εναλλακτικές λύσεις (συνήθως νοθεύονται με χυμούς που λαμβάνονται από μήλα, σταφύλια και άλλα). Κατά συνέπεια, υπάρχει ουσιαστική ανάγκη για αποτελεσματικά συστήματα ελέγχου των τροφίμων για την προστασία των καταναλωτών από νοθευμένα προϊόντα διατροφής.

Στην παρούσα μελέτη, ο κύριος στόχος ήταν να διερευνηθεί η σκοπιμότητα της χρήσης στοχευμένης ανάλυσης με μια μεταβολομική προσέγγιση. Για το σκοπό αυτό χρησιμοποιήθηκε υγροχρωματογραφία αντίστροφης φάσης συζευγμένη με φασματομετρία μαζών με υβριδικό τετραπολικό-αναλυτή χρόνου πτήσης (RPLC-QToF-MS) χρησιμοποιώντας τη λειτουργία bbCID, η οποία παρείχε πληροφορίες για τα πρόδρομα ιόντα και τα θραύσματα, χωρίς προεπιλογή των αναλυτών και με μία ανάλυση, για τη διάκριση αυθεντικών αλλά και νοθευμένων χυμών φρούτων. Τα δεδομένα που αντιστοιχούν στη φαινολική σύνθεση των χυμών φρούτων και στο LC-HRMS μεταβολικό τους αποτύπωμα θεωρήθηκαν ως πηγή δυνητικών βιοδεικτών για την ταξινόμηση των χυμών και την ανίχνευση της νοθείας.

Το σύνολο δεδομένων υποβλήθηκε σε επεξεργασία χρησιμοποιώντας προηγμένες τεχνικές χημειομετρίας, προκειμένου να εντοπιστούν πιθανοί δείκτες. Τέλος, χυμοί ροδιού νοθεύτηκαν επί τούτου με διαφορετικές ποσότητες (1-20%) χυμού σταφυλιών και μήλων

και το φαινολικό προφίλ και τα δεδομένα αξιολογήθηκαν με σκοπό την ανάδειξη της αυθεντικότητας.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική Χημεία Τροφίμων

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: χυμοί φρούτων, νοθεία, αυθεντικότητα, LC-QToF-MS, στοχευμένη σάρωση, χημειομετρία, μεταβολομική

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PREFACE

This master thesis was performed at the laboratory of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens under the supervision of Professor Nikolaos S. Thomaidis.

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CHAPTER 1

Introduction

1.1 Food adulteration

Adulteration of food and beverages is a growing problem in today's global market. Common food are subjected to adulteration either on purpose, more commonly in order to improve the characteristics of the food (flavour, texture, appearance) and increase the shelf-life, or unwillingly as it may be produced on the same production line with other products. The term adulteration has been used, in most cases, in its negative meaning, to indicate the intended degradation of the product.

On its legal term, the meaning of the word adulteration declares that a food product fails to meet legal standards. More specifically, according to the US Code: Title 21 that concerns Food and Drugs [1], adulterated food is declared as:

- Food that contains any poisonous or deleterious (injurious to health, harmful) substance
- Food that contains a pesticide chemical residue that is unsafe
- Food that contains any food additive that is unsafe
- Food that contains a "new animal drug" that is unsafe
- Food that consists in whole or in part of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food
- Food that has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health
- Food that is, in whole or in part, the product of a diseased animal or of an animal which has died other than by slaughter
- Food if its container is composed of any poisonous or deleterious substance which may render the contents injurious to health

- Food that has been intentionally subjected to radiation, unless the use of the radiation was in conformity with a regulation or exemption under the law
- Food wherein any valuable constituent has been in whole or in part omitted, substituted, damage concealed, or substance added to increase bulk or weight
- Food that contains an unsafe colour additive, the regulations then specify what colour additives can safely be used in food
- Confectionery containing alcohol or non-nutritive substance
- Dietary supplement that presents a significant or unreasonable risk of illness or injury
- Dietary supplement prepared, packed, or held under conditions that do not meet current good manufacturing practice regulations
- Additives, microbes, and conditions that could lead to contamination
- Food offered for import that has previously been refused admission, unless the person reoffering the food affirmatively establishes that the food complies with applicable requirements

There are various ways and different motivations for adulterating the food that are presented in **Figure 1**.

Figure 1: Types of intentional contamination [2]

Among them, the most frequent and common food fraud is the Economically Motivated Adulteration (EMA). Economically Motivated Adulteration is the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of production (i.e., for economic gain) [2].

Fruit juices, on which this particular thesis is focused, have been in the top-7 foods reported from 1980 to 2010 as the most common targets of adulteration [3], mainly due to their multiple health effects in combination with their wide-consumption.

1.2 Legislative framework

Food safety and quality are have always been an important issue. In recent years, efforts have been made to deal with these issues. The European Union (EU) has established 4 operational criteria for Food Fraud: violation of EU Food Law, intention, economic gain, deception of customers [4]. In order to limit and control food fraud EU has considered imperative to enhance collaboration /trust between different services within the EU countries and at a European level, namely: food experts (inspectors), Police/Customs (with investigative powers) and Justice.

More specifically, before the horse meat crisis, EU networks were already in place to coordinate and exchange information with Police/Customs (Europol/ European Anti-Fraud Office (OLAF) and with Eurojust, but not with Food Fraud experts. This situation changed in July 2013, with the creation of the EU Food Fraud Network (FFN).

In previous years, back in 2006, the EU had proceeded in another important action to limit Food Fraud, by financing the program, named ‘‘Metabolomics for Plant, Health and OutReach (META-PHOR)’’ in order to establish an international consortium of multi-disciplinary experts to develop common strategies and standards in food research. The major effort of META-PHOR is ‘‘to generate knowledge on these metabolites in our food which determine key

characteristics such as nutritional value, quality and health by developing the advanced tools required for their detection'' [5].

Figure 2: The reorganised network of EU against Food Fraud based on mutual trust [4]

Since the Horse meat crisis in 2013, the main initiatives intended to enhance the EU control system as a whole for detecting and countering frauds in the food chain have been as follows:

- Creation of an EU FFN composed by representatives from the European Commission and all EU countries and Switzerland, Norway and Iceland, for a more efficient cross-border administrative assistance and cooperation
- Development of a dedicated IT tool, the Administrative Assistance and Cooperation System (AAC), to enable the members of the network to rapidly exchange information on potential cases of cross-border fraud. The system has been operational since November 2015
- Organisation of specialised training (in the framework of Better Training for Safer Food initiative) for food inspectors, police and customs officers and judicial authorities of the EU countries, concerning new investigation/control techniques related to food fraud (including eCommerce). Five trainings are held each year
- Coordinated Control Plans at EU level
- The new Official Controls Regulation (OCR)

Figure 3: Current initiatives and dedicated activities of EU against Food Fraud [4]

In conclusion, some basic strategies have been published to protect against adulterated food, such as:

- assess safety of ingredients and additives
- determine that the ingredients and additives can be used in the food, always according to federal regulations
- assure sanitary processing, packaging, storage, transportation, handling
- conduct inspections. Inspections are ongoing, periodic, or voluntary
- devise and implement a food safety/HACCP plan

In EU level, those principals are included in Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety [6].

For the USA and Canada, are described in detail by the Food and Drugs Administration (FDA) (RSC, 1985, Chapter F-27) [7].

In terms of juices, there is not a strict legislative framework as their possible adulteration does not cause any serious problem in human health (excepted potential allergic reactions), as opposed to other foods.

Nevertheless, food industry, and juices in specific, remain a priority for the governments, as juices have an enormous impact on global economy, mainly due to their value, both commercial and nutritional. Hence, a legislative framework has been created which concerns the manufacturing and labelling characteristics of fruit juices that are subject to specific Community rules under Council Directive 2001/112/EC [8].

The products covered by this Directive are: fruit juice, fruit juice from concentrate, dehydrated/powdered fruit juice, water extracted fruit juice, and fruit nectar. These products are defined on the basis of their composition and preparation processes so as to ensure that the terms are used correctly in trade, and not in a manner which may mislead consumers.

Fruit juices are labelled in accordance with the general rules laid down in Directive 2000/13/EC [9] relating to foodstuffs. However, specific provisions are adopted in this Directive in order to improve consumer information. These provisions require to make it clear in the product name if a product is a mixture of different fruits and if a product has been obtained entirely or partly from a concentrate. Moreover, under the same Directive the addition of sugars is no longer authorised in fruit juices [10].

However, food restrictions in juices differ from country to country and are formed according to the organisation that is responsible for their publication. For example, in the case of orange juice, according to European legislation, it consists of juice obtained exclusively from mature oranges (Citrus sinensis) [11].

In contrast, the Codex Alimentarius define as orange juice and concentrated orange juice, the juice obtained from Citrus sinensis and may contain up to 10% citrus juice (Citrus reticulata) [12]. The US Food and Drug Administration (FDA) also allows the addition of mandarin juice of up to 10% and up to 5% juice from sour orange juice (Citrus aurantium) to frozen concentrated orange [13].

1.3 Fruit juices and health benefits

In recent years, there has been a great deal of attention toward the healthy living, part of which is the fruit juices, a high source of antioxidants that seem capable of facing free radicals. Free radicals reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous systems, exposure to different physiochemical conditions or pathological states. A balance between free radicals and antioxidants is necessary for proper physiological function. If free radicals overwhelm the body's ability to regulate them, a condition known as oxidative stress ensues.

A role of oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory condition, certain cancers, and the process of aging [14]. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematous, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism, smoking-related diseases, and many others [15]. An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions [16].

Hence application of external source of antioxidants can assist in coping this oxidative stress [17]. Thus, juices have gained popularity over the past decades, being advertised as an easy-going, accessible superfood which combines both flavour and healthy nutrition at an affordable price.

Fruit juices are generally considered as one of the healthiest foods due to their high content of vitamins, flavonoids and antioxidants, making them effective for the prevention of chronic diseases and the treatment of inflammation in the body [18].

Especially, pomegranates have many health-related beneficial effects, especially in the prevention and treatment of several illnesses. They decelerate the progress of chronic diseases as cancer, diabetes, arthritis [19] due to their

strong antioxidant [20, 21, 22], antitumoral [23], antimicrobial [24], anti-inflammatory [25] and antidiabetic properties [26]. Pomegranate was also shown to reduce the risk of cardiovascular diseases [27], as lower systolic blood pressure [19].

1.4 Antioxidants

Human nutrition science has greatly developed in the past decades, turning from the consideration of foods as simply energy sources to the recognition of their role in maintaining health and in reducing the risk of diseases.

The importance of food for human health is not a new concept, considering Hippocrates's sentence "Let food be thy medicine and medicine be the food", the recent progresses in analytical methods allowed scientists to demonstrate the role of food in human health, and not to simply hypothesise it.

Antioxidants have been declared as such an effective tool for the prevention and the treatment of several illnesses.

1.4.1 Antioxidants' interaction with free radicals

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants [28].

On the other hand, antioxidants are molecules stable enough to donate an electron to a rampaging free radical and neutralise it, thus reducing its capacity to damage. The antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property [29] as they can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body [30]. Other lighter antioxidants

could be found in a diet enriched with fruit and vegetables. Although there are several enzymes system within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and B-carotene [31]. The body cannot manufacture these micronutrients, so they must be supplied in the diet.

1.4.2 Antioxidants' mechanism of action

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify Reactive Oxygen Species (ROS) [32]. The antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property [29]. They can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged.

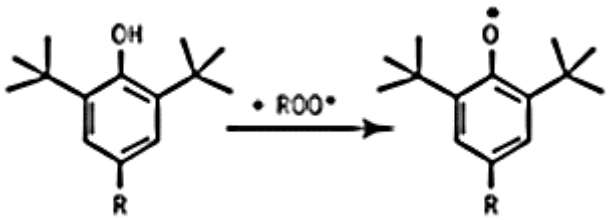
Two principle mechanisms of action have been proposed for antioxidants [33].

Primary or free radical scavenging antioxidants inhibit oxidation via chain terminating reactions. They have reactive OH or NH groups (hindered phenols and secondary aromatic amines) and the inhibition occurs via transfer of a proton to the free radical species. The resulting radical is stable and does not abstract a proton from the polymer chain [34].

ROO* radicals are deactivated by hindered phenol via the reaction presented in **Figure 4(a)** and the phenoxy radical generated is very stable due to their ability to build numerous mesomeric forms **Figure 4(b)**.

The antioxidants found in fruit and fruit juices, are mainly phenolic compounds, the mechanic of which is described in detail follow.

a)



b)

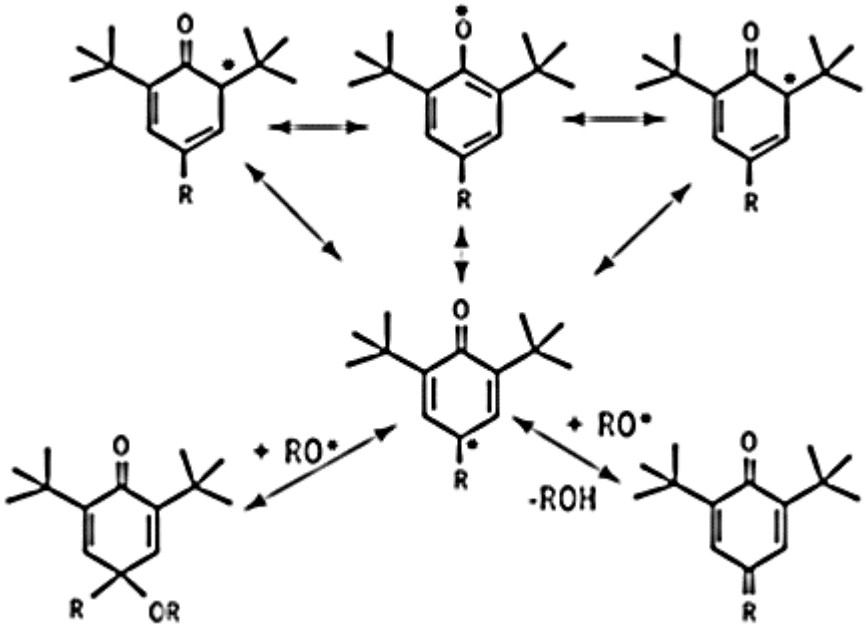


Figure 4: a) The deactivation of free radical ROO* and b) the mesomeric forms of the phenoxy radical [34]

Secondary aromatic amines act as primary antioxidants and are excellent hydrogen donors.

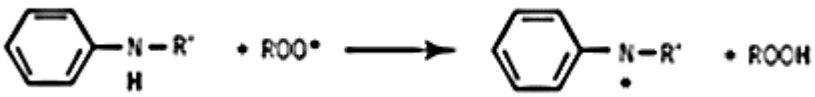


Figure 5: Mechanism of secondary aromatic amines [34]

Secondary antioxidants, frequently referred to as hydroperoxide decomposers, decompose hydroperoxides into non-radical, non-reactive, and thermally stable products. They are often used in combination with primary antioxidants to yield

synergistic stabilisation effects. Hydroperoxide decomposers prevent the split of hydroperoxides into extremely reactive alkoxy and hydroxy radicals [34].

Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation [35].

CHAPTER 2

Detection of possible adulteration in fruit juices- Literature review

2.1 Introduction

In recent years, with the growing complexity of global food supply chains and trade, food fraud, including adulteration of high value foods, such as fruit juices, with cheaper substitutes, has become an increasingly important issue.

To protect the consumers, there is a requirement for more stringent regulations and more diligent monitoring of foods for regulators, vendors and producers. But, as juices adulteration does not include any serious harm for the consumers, none or little assays have been made in order to control and reduce the problem. Although juices adulteration does not have a strong health effect, it remains a priority for the governments, as juices have an enormous impact on global economy, mainly due to their value, both commercial and nutritional.

The most common forms of fraud occurring in the fruit juice industry usually include dilution with water, addition of sugars or other additives, or mixing with cheaper fruit juices [36]. These processes are applied alone or in combination in order to make the fraud more difficult to detect [37].

Thus, due to the high value of juices, different studies have been undertaken to detect fraud using simple or more sophisticated techniques, such as foodomics, in order to find special markers in each and every juice, if possible, or their ratios, that will suggest the occurrence of some kind of fraud.

2.2 Sample treatment

Juices, due to their simple matrix, they do not have any important treatment and they are generally considered an easy sample to handle. The most common sample treatment is the dilution with water [38], and in some cases with

methanol (1:1) [39] or acetonitrile (1:1) [40]. Filtration is considered necessary to prevent the column from large particles and possible irreversible damage.

In some studies centrifugation also takes place [40, 41, 42], or even more sophisticated techniques are used, such as lyophilising [43] and pre-column derivatization [44].

2.3 Analytical Techniques - Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

In food industry, the application of MS in combination with chromatography has been well recognised as the “gold standard” for both quantification and semi-quantitative screening of particular compounds in food [47]. Especially, LC and MS have resulted in very powerful instrumentation for sensitive and selective determination of other more polar or ionic contaminants, comparing to GC-MS instrumentation, at trace levels in food [12, 13] including veterinary medicines [43, 46], pesticides [44, 47] and toxins [39, 46].

2.3.1 Reversed Phase Ultra High-Performance Liquid Chromatography (RP-UHPLC)

Developments in chromatography are enabling more rapid, highly efficient LC separations [49, 50] and providing opportunities for the analysis of ionic or polar compounds [51, 52 and 53].

UHPLC is a promising technique that uses small-diameter particles in the stationary phase and short columns achieving in this way fast and high-resolution separation that increases LC-MS sensitivity and minimises matrix interference [51]. UHPLC is commonly performed in reversed-phase (RP) mode using C18 columns. The mobile phase consists of an aqueous and an organic solvent.

2.3.2 Mass spectrometry (MS)

In many studies [39, 42 and 46], the developed analytical methods include liquid chromatography coupled to tandem mass spectrometry using low resolution mass analysers, usually triple quadrupole (QqQ), because this technique is reliable for qualitative and quantitative determination [54].

Among the possible ionisation techniques in LC-MS, electrospray ionisation (ESI) [55] remains the most common ionisation technique employed for the determination of chemical compounds in food by LC-MS. The use of atmospheric pressure chemical ionisation (APCI) [56] appears to have been left in the wake of the overwhelming popularity of ESI. This may be related to the increasing number and the wider range of analytes currently sought but may also reflect the improvements in source and probe design for ESI not yet paralleled in APCI [57].

LC-HRMS has an excellent performance providing the ability of full spectral information with the added bonus of high mass-resolving power that boost selectivity and the capability for accurate mass measurement to aid identification with the additional advantage of retrospective analysis [58, 59 and 60].

With full-spectral accurate-mass data, a theoretically unlimited number of analytes that are present in a sample can be identified, because the acquisitions have been made as ‘all ions all the time’ [61]. The simultaneous determination of a broad number of compounds in one injection, with a corresponding reduction of time and cost, and even when reference standards are not available, makes LC-HRMS one of the most widely used in analytical chemistry [62].

Time-of-flight (TOF) is one of the most used HRMS analysers due to its desirable specifications comparing to other instruments **(Table 1**) and it is easily coupled to ultra high-performance liquid chromatography (UHPLC). Mass resolution typically ranges from 20.000 up to 80.000 FWHM and mass accuracy is lower than 2 ppm.

Table 1: Common parameters used to compare performance of mass spectrometers used for LC-MS [63]

Mass analyser type	Resolving Power ($\times 10^3$)	Mass accuracy (ppm)	Upper limit of m/z range ($\times 10^3$)	Acquisition speed (Hz)	Linear dynamic range	Price
Q	3-5	Low	2-3	2-10	10^5-10^6	Low
IT	4-20	Low	4-6	2-10	10^4-10^5	Moderate
ToF	10-60	1-5	10-20	10-100	10^4-10^5	Moderate
Orbitrap	100-240	1-3	4	1-5	5×10^3	High

2.3.3 Tandem mass spectrometry (MS/MS)

The basic principle of MS/MS is the selection of precursor ion, fragmentation of this ion, usually by collision-induced dissociation (CID), and measurement of the m/z ratio of the product ions formed. There are two fundamentally different approaches to MS/MS: tandem in space and tandem in time.

Tandem-in-space instruments have separate independent mass analysers in physically different locations of the instrument. A hybrid mass spectrometer is an instrument which combines analysers of different types. Hybrid configurations, such as Quadrupole-Time-of-flight (QqToF) (**Figure 6**), increase the potential of the analyser for screening purposes and provide relevant structural information by obtaining accurate-mass product-ion spectra after MS/MS experiments [54]. Other examples of tandem mass spectrometers include, but are not limited to, triple/tandem quadrupole (QqQ), and Orbitrap hybrid instruments.

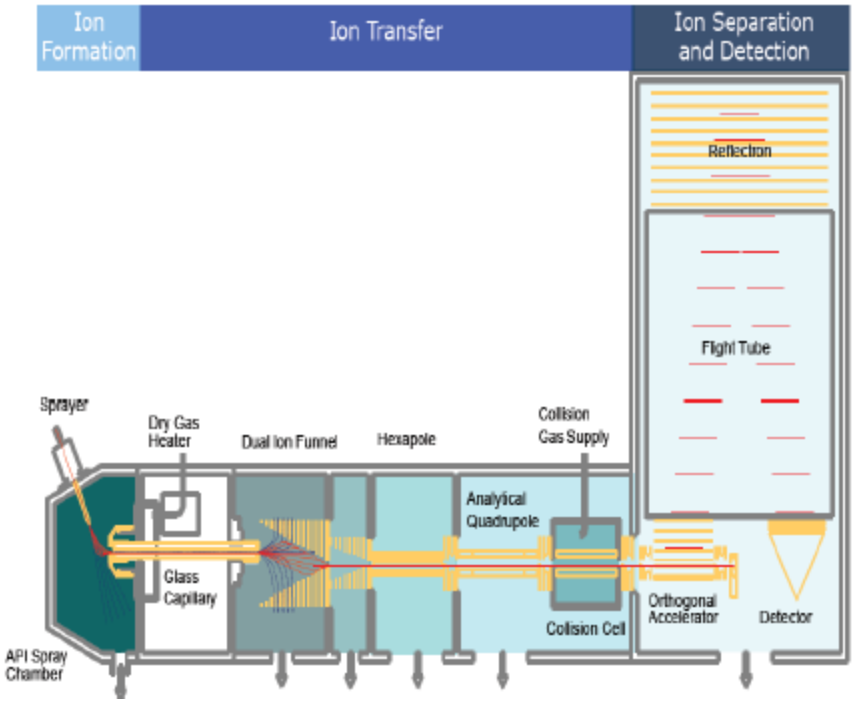


Figure 6: Course of ions in the QTOF sections (maXis Impact, Bruker)

Tandem-in-time instruments are typically ion-trapping mass spectrometers, which comprise 3-D quadrupole ion traps (QIT), linear ion traps (LIT) and Fourier transform ion cyclotron resonance (FTICR) instruments. The various stages of MS are conducted within the same physical trapping volume but at different times during the experiment [57].

2.3.3.1 Data Independent Acquisition (DIA)

In this acquisition that applied in this particular project there is no need to pre-select the precursor ion. Full-scan spectra at different collision energies are obtained in one injection.

This acquisition provides simultaneously accurate mass data of parent compounds and fragment ions in a single run using two scans, one at low and one at high collision energy. By applying low energy (LE) in the collision cell, no fragmentation is performed. A full-scan spectrum is obtained that provides information for the parent ion (the (de)-protonated molecule) and, in some

cases, the adduct ions and the in-source fragments. By applying high energy (HE) in the collision cell, fragmentation is performed and a spectrum similar to MS/MS experiments is obtained. This approach is called all-ions MS/MS, MS^E or bbCID, according to the QTOF manufacturer [54].

2.3.3.2 Data Dependent Acquisition (DDA)

In DDA, there is firstly a full scan which is defined as the survey scan and data are processed “on-the-fly” to determine the candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. If the selection criteria are met, MS/MS analysis is then triggered and MS/MS scans (data-dependent) are performed [54, 60].

2.4 Data treatment

After the sample preparation and the LC-HRMS analysis, raw data can be treated with three different approaches, target, suspect and non-target screening. A systematic workflow for all three approaches is shown in **Figure 7.**

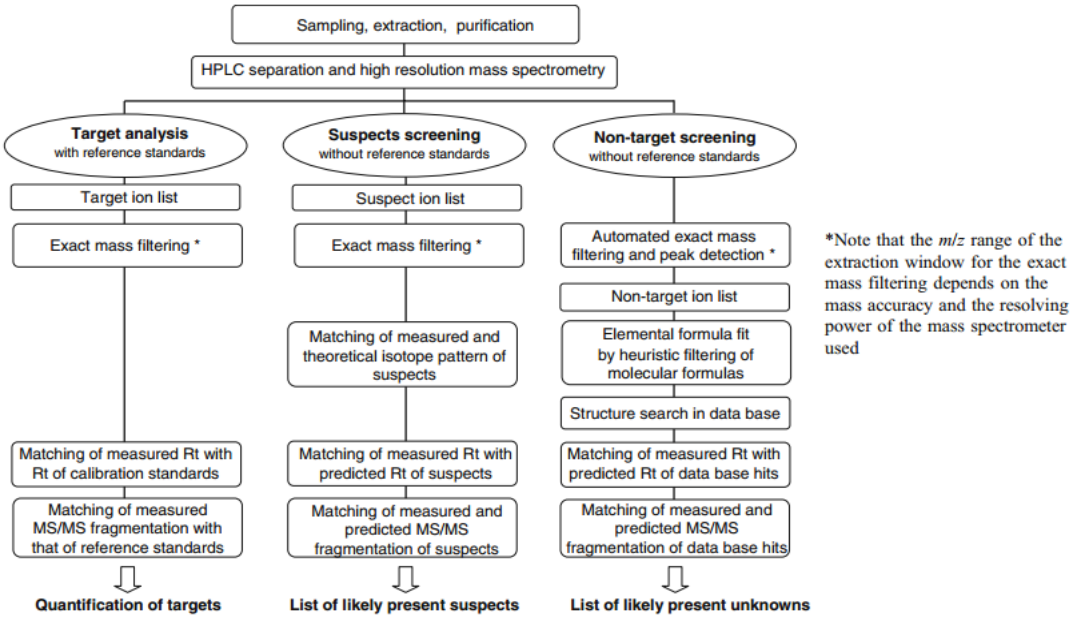


Figure 7: Systematic workflow for target, suspect and non-target screening by LC-HRMS/MS [60]

2.4.1 Target screening

In this approach, an in-house developed database is used for the screening of a large number of compounds. The information included in the database is based on the analysis of the available reference standards [59]. The reference standard is necessary for comparison of the retention time, the MS spectrum profile (precursor ion, adducts, in-source fragments), as well as the MS/MS spectrum (fragment ions and ion ratios) [65].

2.4.2 Suspect screening

In this approach, a list of suspect compounds that are possible to be found in specific samples is built. The screening is based only on the exact m/z of the expected ions, which, in case of the ESI source, are usually the pseudomolecular ions $[M+H]^+$ and $[M-H]^-$, except for some compounds which exclusively show adduct formation. Molecular formula and structure are known, so this information can be efficiently used in the identification and confirmation process [60].

2.4.3 Non-target screening

In non-target methodologies, samples are searched for compounds without any previous information on them. These unknown compounds are actually new, unexpected or not searched ones in specific samples. Identification is a challenge in this approach, as more than one elemental formula and several plausible structures are obtained for a given unknown compound detected in a sample [59]. Except for the elucidation of unknowns, non-target screening is used for the identification of metabolites and transformation products, arising from in vivo and in vitro experiments, in-silico modeling and degradation laboratory studies [59, 62].

2.4.4 Metabolomics

In order to export and correlate the experimental data that present some kind of tendency, the omics technologies seem to have gain popularity over the past decades, as a large, or even exhaustive number of measurements can be taken in a fairly short time period. Starting from the four major types of omics measurements (genomics, transcriptomics, proteomics, and metabolomics), a variety of omics subdisciplines (epigenomics, lipidomics, interactomics, metallomics, etc.) has emerged [66].

Thanks to the omics approach, researchers are now facing the possibility of connecting food components, foods, the diet, the individual, the health, and the diseases, but this broad vision needs not only the application of advanced technologies, but mainly the ability of looking at the problem with a different approach, a “foodomics approach”.

Foodomics has been defined as a discipline that studies the food and nutrition domains through the application of advanced omics technologies to improve consumer’s well-being, health, and knowledge [67, 68, 69].

The main idea behind the use of this term has been not only to use it as a flag of the new times for food analysis, but also to highlight that the investigation into traditional and new problems in food analysis in the postgenomic era can find exciting opportunities and new answers through the use of epigenomics (that studies the mechanisms of gene expression that can be maintained across cell divisions, and thus the life of the organism, without changing the DNA sequence), genomics, transcriptomics, proteomics, and metabolomics tools [70].

Figure 8 presents the multiple tools that are used in foodomics and how they can be used to different appilications.

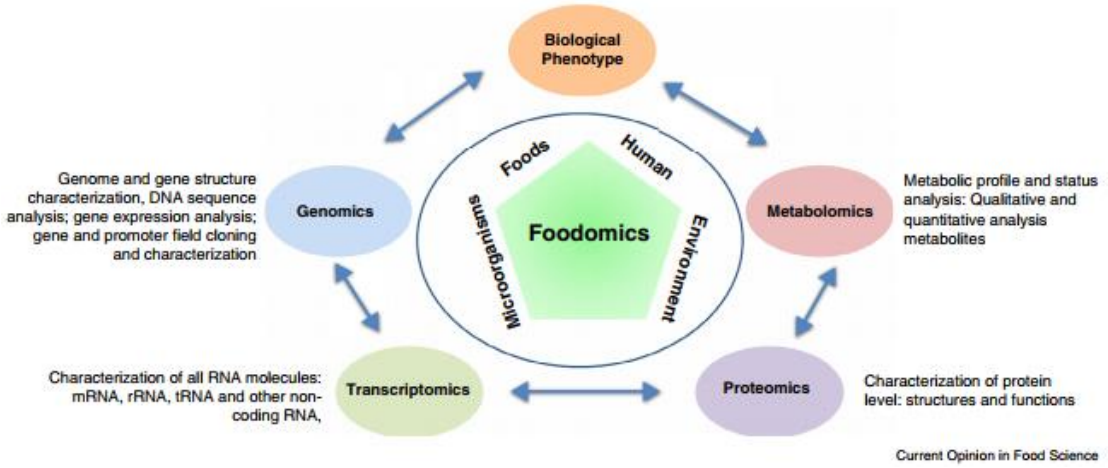


Figure 8: Foodomics involves the use of multiple tools to deal with the different applications [71]

Transcriptomic, proteomic and metabolomic approaches are also valuable tools to distinguish between similar food products and to detect food frauds (adulteration, origin, authenticity, etc.), food-borne pathogens, toxic species, food allergens, and so forth [71].

Specifically, metabolomic approaches that are typically classified as either top-down/non-targeted or bottom-up/targeted have been applied in foodomics. Due to the chemical complexity and concentration diversity in food metabolites, a single analytical technology is insufficient for adequate coverage. Frequently, multi-analytical technologies are required to make coverage of food-related metabolites as complete as possible. The technologies most frequently used in foodomics are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). These techniques either stand alone, or combined with separation techniques (typically LC-NMR, GC-MS, LC-MS, and CE-MS) [70].

Mass spectrometry (MS) has emerged as the foremost technology in metabolomics studies due to its unparalleled sensitivity and specificity, high resolution and wide dynamic range, enabling comprehensive quantitative and qualitative measurement of large-scale small-molecular metabolites in complex biological samples (i.e. cells, body fluids, tissues or organisms) [72].

From the perspective of metabolomics, food safety can be depicted as tolerable, safe contents of adulterants, contaminants, toxins or any other substances that may be harmful to human health in a given food and feed. Obviously, chemically characterising (identifying and quantifying) food constituents by MS-based metabolomics approaches is essential for the assessment of food safety and quality, especially with current developments in MS, because it enables differentiation between food products with molecular features that cannot otherwise be evaluated by external factors of food, such as texture, flavour or colour [72].

In MS-based metabolomics study, procedures, including sampling and sample preparation, instrumental separation analysis, data analysis, identification of potential candidates and biological interpretation, are often performed sequentially to complete the whole metabolomics approach. Of all the procedures, instrumental separation analysis is considered essential, as it directly influences the quality of the raw metabolomics data. However, the importance of other procedures cannot be neglected because they also contribute to the adequacy and the accuracy of the metabolomics approach [72].

2.4.5 Principal Component Analysis (PCA)

Principal component analysis (PCA) is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components [73].

In other words, the idea behind PCA is to find principal components which are linear combinations of the original variables describing each specimen. The principal components are also chosen so that the first principal component (PC1), accounts for most of the variation in the data set, the second (PC2), accounts for the next largest variation and so on. Hence, when significant correlation occurs the number of useful PCs is much less than the number of original variables.

Figure 9 illustrates the method when there are only two variables and hence only two principal components. This figure also shows that PCA is equivalent to a rotation of the original axes in such a way that PC1 is in the direction of maximum variation, but with the angle between the axes unchanged. With more than two variables it is not possible to illustrate the method diagrammatically but again we can think of PCA as a rotation of the axes in such a way that PC1 is in the direction of maximum variation, PC2 is in the direction of next greatest variation, and so on.

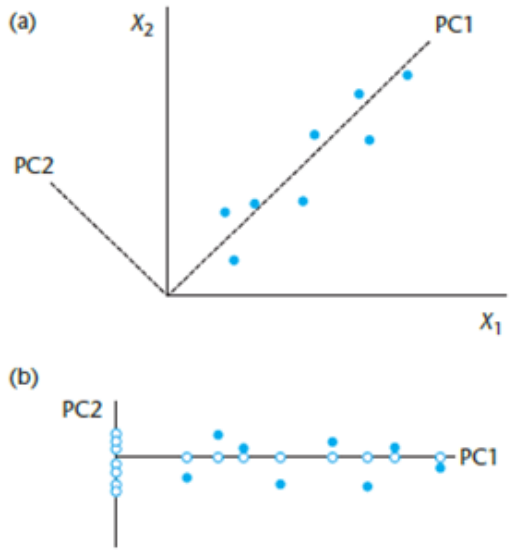


Figure 9: (a) Diagram illustrating the two principal components, PC1 and PC2, for the two variables, (b) Points referred to the principal component axes • indicates data points, ○ their projection on to the axes [74]

It is often found that PC1 and PC2 then account between them for most of the variation in the data set. As a result the data can be represented in only two dimensions instead of the original n [74].

PCA is often used in combination with metabolomics. More specifically, one approach to finding meaning in metabolomics datasets involves multivariate analysis (MVA) methods such as principal component analysis (PCA), where spectral features contributing most to variation or separation are identified for

further analysis. In essence, PCA aim to differentiate between classes in highly complex data sets, despite within class variability [75].

2.5 Research for possible adulteration in fruit juices – Analytical methods performed

So far, there is a satisfying number of studies that have been undertaken in order to detect juice fraud, from simple techniques such as soluble solids, color rating, suspended pulp etc. [46], to more complex ones, based on amino acid analysis profiles [44], sugars [40], flavonoids, antioxidants [38, 39, 40, 43, 76] using techniques such as high performance liquid chromatography (HPLC), in combination with various types of detectors, while new promising techniques such as metabolomics have also been applied.

The studies that have been reviewed are categorised according to the juice sample, the markers proposed, the technique applied and the sample treatment, and are shortly presented below.

The markers proposed are either antioxidants [38, 43, 46, 49], or amino acids [40, 47] and their selection depends from their respective standards available in every lab. The standards are necessary both for the qualification and the quantification of each compound.

The main technique applied is HPLC (and UPLC) in combination with HILIC (for the determination of amino acids) [40], as a supplementary technique. More specifically, a Reversed-Phase chromatography (RP-HPLC) was selected as the antioxidants and the amino acids studied are considered rather polar compounds. Moreover, technique such as capillary electrophoresis has also been reported [43].

A variety of detectors have been used including non-destructive detectors such as diode array detector (DAD or PDA) [40, 41, 43], a UV detector that measures the UV absorption of the effluent continuously at single or multiple wavelengths, fluorescence detector (FLD) [44], that irradiates the effluent with a light of set wavelength and measure the fluorescence of the effluent at a single or multiple

wavelength, as well as destructive detectors such as mass spectrometer (MS) [39, 40, 42, 46, 46].

The sample preparation in juices is quite simple, including dilution and filtration as main procedures. Centrifugation is sometimes needed [40, 41, 42] in order to receive a sample easier to handle.

A literature review of the analytical methods applied for the determination of adulteration in fruit juices is presented in **Table 2**.

Table 2: Detected markers of possible fraud in fruit juices

Juices	Markers	Technique	Sample treatment	LOD (mg/L)	Reference
Orange	citric acid isocitric acid malic acid tartaric acid	Capillary electrophoresis P/ACE (Beckman, Palo Alto, CA), in 200nm Injection time: 5s Capillary column: polyacrylamide (Beckman, Madrid, Spain) 57 cm length and 50 mm i.d. Function in 14 kV	a) dilution 1:1 with Milli-Q water b) filtration	-	[38]
Mandarin Orange Grapefruit	didymin rhoifolin isorhoifolin neohesperidin hesperidin naringin narirutin limonin glucoside vicenin-2	UPLC–QToF MS (Quattro Micro, ESI +, MRM) Στήλη: Atlantis T3 C18 column (100 × 2.1 mm I.D., 3 mm) with a Atlantis T3 guard column (10 mm × 10 mm I.D., 5 mm, 100 Å) MS: QqQ mass spectrometer (Waters, Milford)	a) dilution with methanol (1: 1) LC-MS /MS screening b) infusion directly in UPLC-QToF MS ESI pos.	-	[39]

		Solvent A: aqueous formic acid (0.3%) Solvent B: formic acid (0.3%) in ACN Solvent C: formic acid (0.3%) in 2-propanol Flow: 0.35 ml/min Column temperature: 20 ºC Injection volume: 10 µL			
Apple Grape Orange and other citric fruits	sorbitol fructose, glucose, sucrose sorbitol fructose, glucose sorbitol, fructose, glucose, sucrose hesperidin naringin narirutin diosmin didymin neohesperidin	HILIC-MS/ESI, UV UPLC-PDA Column: Waters BEH Amide UPLC C18 (2.1 × 100 mm, 1.7 µm) MS: Waters Synapt G2 Q-TOF-MS (Waters Corporation, Milford, MA), ESI neg. Capillary voltage: 2.5 kV (neg.) Solvent A: 10 mM ammonium acetate	a) 10 times dilution in 50% ACN b) centrifugation	2.0 - 2.0 - 2.0 - 0.3 - - - - -	[40]

		Solvent B: 10 mM ammonium acetate in 95% ACN Chromatogram time: 14 min Flow: 0.25 ml/min Column temperature: 35ºC			
Apple Pear Strawberries Raspberries	Dihydrochalcones	direct RP-HPLC-DAD Column: Hicarbosphere 3ODS (Hichrom Ltd, Berkshire, U.K.) silica based (15 × 4.6 mm, 5 μm) Capillary voltage: 2.5 kV Solvent A: 10 mM ammonium acetate Solvent B: 10 mM ammonium acetate in 95% ACN Flow: 0.35 ml/min Column temperature: 30ºC Injection volume: 20 μL	a) centrifugation b) filtration through cellulose acetate filter	1.2-1.6	[41]

Orange Apple Grapefruit	Metabolomics	HPLC–QqTOFMS Column: Restek Ultra II Aqueous C18 RP (50 × 2.1 mm i.d., 3 μm) MS: AB SCIEX 4000 QTRAP QqQ/ IT-MS Capillary voltage: -4 kV Solvent A: 5 mM ammonium acetate Solvent B: methanol Chromatogram time: 10 min Flow: 0.25 ml/min Column temperature: 35ºC Injection volume: 10 μL	a) centrifugation b) filtration through 0.22 μm PTFE filter c) 100 times dilution with ultra-pure water	-	[42]
Orange Mandarin Lemon	eriodictyol-7-*O*-rutinoside-4′-*O*-glucoside eriodictyol-7-*O*-rutinoside diosmetin-6,8-di-*C*-glucoside diosmetin-8-*C*-glucoside luteolin-7-*O*-rutinoside	Solvent A: acetic acid-water (0.5:99.5, v/v) Solvent B: methanol Chromatogram time: 136 min Flow: 0.8 mL/min Column temperature: 30ºC Injection volume: 50 μL	RP-HPLC–DAD Column: Phenomenex Luna C18 (150 × 4.6 mm i.d., 3 μm) with a Waters NovaPack guard column C18 (10 × 3.9 mm i.d, 4 μm) Solvent A: acetic acid-water (0.5:99.5, v/v)	Solvent extraction in lyophilized fruit juice samples	[43]

Grapefruit	diosmetin-6-*C*-glucoside diosmetin-6,8-di-*C*-hexosideacylhexoside naringenin-7-*O*-neohesperidoside naringenin-7-*O*-neohesperidoside-4-*O*-glucose naringenin-*O*-hexosylhexoside hesperetin-7-*O*-neohesperidoside naringenin-*O*-rhamnosylmalonylhexoside isosakuranetin-7-*O*-neohesperidoside hesperetin-7-*O*-rutinoside apigenin-6-*C*-hexoside-*O*-hexoside apigenin-7*O*-neohesperidoside scopoletin-*O*-hexoside		Solvent B: methanol Chromatogram time: 136 min. Flow: 0.8 mL/min Column temperature: 30ºC Injection volume: 50 µL		
Apple Orange Cherries Pear Pomegranate	Determination of the amount of amino acids and comparison with standard values	RP-HPLC-FLD Column: ODS (250mm×4 mm) RP, (Knauer) Solvent A: MeOH – Na_3PO_4 (10:90, v/v)	Pre-column derivatisation with ortho-phthalate aldehyde (OPA)	-	[44]

Peach Grape		Solvent B: MeOH – THF (97:3, v/v) Chromatogram time: 32 min Flow: 1.0 ml/min, λex/λem =330/450 nm Injection volume: 20 µL			
Grape	tartaric acid malic acid (in white grape)	LC-MS/MS (Quattro Micro ESI-, MRM) Column: HPLC Organic Acids 250 × 4.6 mm (5 µm), fitted with a 10 × 4.6 mm (5 µm) guard column, and an extra column of ZIC-HILIC Sequant 150 × 2.1 mm (5 µm), fitted with a 20 × 2.1 mm (5 µm) guard column Solvent A: ACN – H_2O (90:10, v/v, containing 0.1% ammonium acetate) Solvent B: water containing 0.1% ammonium acetate	Sample and standards preparation	0.3 0.2	[45]
Apple	quinic acid tartaric acid			- 0.3	
Orange	citric acid isocitric acid			- - 0.3	
Cranberries	tartaric acid citric acid isocitric acid quinic acid tartaric acid			- - - 0.3 -	

		Chromatogram time: 40 min Flow: 0.7 ml/min Column temperature: 30ºC Injection volume: 10 µL			
Pear Orange Grapefruit	Metabolomics	UPLC–QToF MS (ESI- and ESI+) Column: ACQUITY UPLC™ BEH C18 and BEH HILIC (100 × 2.1 mm, 1.7 µm) Capillary voltage: 2,5 kV Solvent A: 10 mM aqueous ammonium acetate Solvent B: ACN (in reverse in HILIC) Chromatogram time: 10 min Flow: 0.4 ml/min Column temperature: 45ºC Injection volume: 3 µL	Fruit samples preparation	-	[46]

CHAPTER 3

Scope

Fruit juices due to their health benefits, have gained an important position in the global market, with a large number of regular consumers that present eager to spent a significant amount of money to get them. Thus, fruits juices are often subjected to food fraud, also known as Economically Motivated Adulteration (EMA). A common form of EMA is the undeclared substitution with alternative ingredients.

More specifically, pomegranates are of particular interest because of their high nutritional value and high content of antioxidants and phenolics. Similarly, to other highly prized food commodities, the economic value and large-scale production of these valuable fruit juices have made them a likely target for adulteration and fraud. One of the most frequent profit-driven fraudulent procedures is extension of authentic juice with cheaper alternatives (typically juices obtained from apples, grapes, grapefruits, and others). Consequently, there is a substantial need for effective food control systems to protect consumers from adulterated food products.

LC-HRMS allows the wide-scope screening of antioxidants present in fruit juices with an acquisition of accurate-mass full spectrum data. These data can be used for target screening in order to detect antioxidants present in particular fruit juices that can be used as markers for the determination of possible fraud.

Recent studies focus on the determination of either antioxidants or amino acids in fruit juices. However, efforts for screening of a wide range of antioxidants in fruit juices by LC-HRMS are very limited.

The scope of this study is to detect and find antioxidants that will be used as markers, as they exist exclusively in a particular juice, in this case in red and white grape and in apple juice as well, in order to detect possible adulteration. For this reason, an in-house database of antioxidants is used for the

qualification and quantification of antioxidants found in pomegranate, apple, red and white grape juice.

CHAPTER 4

Materials and Methods

4.1 Chemicals and Materials

For the sample preparation, syringes of 2 mL volume that were used for juices filtration were obtained from HSW Norm-Ject (Germany). Regenerated cellulose (RC) syringe filters (diameter 15 mm, pore size 0.2 μm) were obtained from Phenomenex (Torrance, CA, USA).

For the dilution of juices, when necessary, bottled water was used. All the solvents for the LC-QTOF-MS analysis were UHPLC-MS grade. Methanol was purchased from Merck (Darmstadt, Germany) and the eluent additives ammonium acetate were purchased from Fluka (Buchs, Switzerland). Ultrapure water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA).

4.2 Sampling and Storage

A variety of fruit juices were provided from “Delta Foods S.A.” including different fruit juices samples, such as apple, white and red grape and pomegranate juice (**Table 3**).

The samples were stored in the freezer at -20 ^{o}C until analysis.

Table 3: Categorisation of juices by brand, content and origin

Juice	Brand	Content	Origin	Sample Code
Apple	Starkin, Granny, Granny Smith	Concentrated apple juice	Greece	N1
	Starkin, Granny, Granny Smith, Delicious, Golden	Non-concentrated apple juice	Greece	N4
White grape	Trebbiano	Concentrated white grapejuice	Italy	I1, I2, I4, I5
Red grape	Sangiovese, Montepulcianoo, Lambrusco, Schiava, Shiraz, Ciliegiolo, Merlot	Concentrated red grape juice	Italy / Puglia	TH1, TH2, TH3, TH4, TH5
Pomegranate	Hicaz	Concentrated pomegranate juice	Turkey	L1

Table 4: Dilution in juices depending on Brix number

Juice	Sample Code	Content	Brix before dilution	Brix after dilution	Dilution
Apple	N1	Concentrated apple juice	69-71	11.2	6.5 times
	N4	Non-concentrated apple juice	11-13	-	Non-dilution
White grape	I1, I2, I4, I5	Concentrated white grapejuice	64.7-65.3	15.9	4 times
Red grape	TH1, TH2, TH3, TH4, TH5	Concentrated red grape juice	65-68	15.9	4 times
Pomegranate	L1	Concentrated pomegranate juice	64.5-65.5	15	4.5 times

4.3 Sample preparation

In the samples, provided by “Delta Foods S.A.”, a Brix number (°Bx), that refer to the sugar content of an aqueous solution (1°Bx=1g sucrose/100g of solution) [77], before and after dilution was provided, according to which the samples were diluted properly, as described in **Table 4**.

Every sample was then filtered directly into a 2 mL vial using a syringe fitted with a 0.2 μm RC membrane filter in order to remove the solid particles that were present and may cause blockage of the column filter. Finally, they were ready for LC-HRMS/MS analysis.

4.4 Instrumentation

An Ultra-High Performance Liquid Chromatography (UHPLC) system (UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a Quadrupole-Time of Flight Mass Spectrometer (QToF-MS) (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for the analysis of the samples. The UHPLC apparatus consists of a solvent rack degasser, a binary pump with solvent selection valve (HPG-3400), an auto-sampler and a column. The QTOF-MS apparatus consists of an Electrospray Ionisation (ESI) source operating in positive and negative mode.

Figure 10: UHPLC-QToF-MS, Maxis Impact, Bruker Daltonics

In our analysis, a reversed-phase chromatographic run was performed in negative ESI mode. An Acclaim RSLC 120 C18 column (2.1 × 100 mm, 2.2 µm) (Dionex Bonded Silica Products, Thermo Scientific, Dreieich, Germany), preceded by an ACQUITY UPLC BEH C18 1.7 µm guard column of the same packaging material (VanGuard Pre-Column, Waters, Dublin, Ireland), and thermostated at 30 ^{o}C, was used.

In the negative ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5 mM CH_3COONH_4 and the organic mobile phase consisted of CH_3OH, 5 mM CH_3COONH_4. The gradient elution program applied changes in mobile phase and in flow rate. It started with 1.0% of organic phase (flow rate 0.200 mL/min) for 1 min, increasing to 39.0% by 3 min (flow rate 0.200 mL/min), and then to 99.9% (flow rate 0.400 mL/min) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.480 mL/min) and then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.200 mL/min for the last minute. The injection volume was set to 5 µL.

The operating parameters of the ESI interface were the following: capillary voltage 3000 V for negative mode, end plate offset 500 V, nebulizer pressure (N_2) 2.0 bar, drying gas (N_2) 8.0 L/min, drying temperature 200 ^{o}C.

Data were acquired through a Data Independent Acquisition (DIA) scan mode, called broad-band Collision Induced Dissociation (bbCID), which provided both MS and MS/MS spectra simultaneously using two different collision energies with a scan rate of 2 Hz and a mass range of 50-1000 Da. Low collision energy (4 eV) provided a full scan spectrum (MS) and high collision energy (25 eV) provided a spectrum where all ions were fragmented (bbCID MS/MS).

An external calibration of the QTOF mass spectrometer was performed with a sodium formate solution before analysis. Also, a calibrant injection was performed automatically at the beginning of each run and the segment of 0.1-0.25 min was used for internal calibration. The calibrant solution of sodium formate consisted of 10 mM sodium formate clusters in a mixture of water: isopropanol 1:1. The theoretical exact masses of calibration ions with formulas $Na(NaCOOH)_{1-14}$ in the range of 50−1000 Da were used for calibration. The

instrument provided a typical resolving power of 36.000-40.000 during calibration.

Bruker's software that was used for raw data analysis was DataAnalysis 4.3, TASQ Client 1.4 and TargetAnalysis 1.3.

4.5 Method validation in pomegranate juice

A validation dataset of 33 available antioxidants was used in order to evaluate linearity, accuracy, precision, matrix effects and detectability of the screening method. The compounds of the validation dataset and some of their fragments in negative ESI mode are shown in **Table 5**.

Linearity was studied for each compound by analyzing standard solutions at 6 different concentrations ranging from 0.25-10 mg/L.

Accuracy was assessed with recovery experiments. Method recovery was calculated by dividing the peak area of the spiked samples by the peak area of the matrix-matched samples at 5 mg/L. The initial samples were analysed for determination of the analytes of the validation dataset and if the sample already contained the analyte, its peak area was subtracted from the peak area of the spiked sample and the peak area of the matrix-matched sample. Precision was expressed as method repeatability in terms of relative standard deviation (%RSD) in 4 spiked samples at 5 mg/L. After the calculation of the matrix factor by dividing the peak area of matrix-matched samples by the peak area of the standard solutions, matrix effect was assessed by the equation: %Matrix Effect = (Matrix Factor - 1) × 100. The method limits of detection (MLOD) and quantification (MLOQ) were calculated by spiking experiments ranging from 0.25-10 mg/L.

Table 5: Validation dataset

Compound name	CAS Number	Molecular formula	Calculated m/z of $[M-H]^-$	Retention time (min)	Fragm 1 m/z	Fragm 2 m/z	Fragm 3 m/z
3,4- dihydroxybenzoic acid (Protocatechuic acid)	(99-50-3)	C7H6O4	153.0193	1.3	109.0294	108.0218	
2,5-dihydroxybenzoic acid (gentistic acid)	(490-79-9)	C7H6O4	153.0193	2.5	108.0215	109.0278	
4-hydroxybenzoic acid	(99-96-7)	C7H6O3	137.0244	1.4	93.0342	65.0398	
Apigenin	(520-36-5)	C15H10O5	269.0455	8.1	151.0031	117.0340	
Cinnamic acid	(140-10-3)	C9H8O2	147.0452	4.5	103.0553	146.8973	
Epicatechin	(490-46-0)	C15H14O6	289.0718	4.3	123.0452	151.0401	137.0244
Ferulic acid	(537-98-4)	C10H10O4	193.0506	3.0	134.0361	178.026	149.061
Hydroxytyrosol	(10597-60-1)	C8H10O3	153.0557	3.5	123.0452		
Luteolin	(207-741-0)	C15H10O6	285.0405	7.4	285.0399	133.0287	
Myricetin	(208-463-2)	C15H10O8	317.0303	6.1	151.0035	178.9986	317.0303
p-coumaric acid	(501-98-4)	C9H8O3	163.0401	2.3	119.0502	93.0344	
Quercetin	(117-39-4)	C15H10O7	301.0354	7.1	151.0036	178.9959	121.0288
Salicylic acid	(200-712-3)	C7H6O3	137.0244	3.6	93.0340	65.0399	

Syringic acid	(530-57-4)	C9H10O5	197.0455	1.4	123.0080	166.9976	
Taxifolin	(480-18-2)	C15H12O7	303.0510	4.8	125.0227	285.0408	153.0193
Tyrosol	(501-94-0)	C8H10O2	137.0608	4.1	119.0495	107.0496	93.034
Vanillin	(121-33-5)	C8H8O3	151.0401	4.7	136.0158		
Vanillic acid	(121-34-6)	C8H8O4	167.0350	1.4	125.0244		
Eriodictyol	(4049-38-1)	C15H12O6	287.0561	6.3	151.0038	135.045	
Genistein	(446-72-0)	C15H10O5	269.0455	7.5	133.0284	225.0546	159.044
Galangin	(548-83-4)	C15H10O5	269.0455	10.0	213.0546	169.0657	197.0597
Hesperitin	(520-33-2)	C16H14O6	301.0718	7.4	151.0025	195.9988	
Rosmarinic acid	(20283-92-5)	C18H16O8	359.0772	4.3	161.0233	197.0444	179.0338
Chrysin	(480-40-0)	C15H10O4	253.0506	9.7	209.0597	143.0491	253.0495
Pinobanksin	(548-82-3)	C15H12O5	271.0612	7.2	253.0495	197.0597	225.0546
Pinocembrin	(480-39-7)	C15H12O4	255.0663	9.2	151.0025	213.0546	
Oleuropein	(32619-42-4)	C25H32O13	539.1770	6.0	275.0919	149.0244	
Caffeic acid	(331-39-5)	C9H8O4	179.0350	1.4	135.0453	134.0346	

Ethyl vanillin	(121-32-4)	C9H10O3	165.0557	5.6	136.0156	137.0222	108.0219
Gallic acid	(149-91-7)	C7H6O5	169.0142	1.3	125.0244	69.0344	97.0295
Syringaldehyde	(134-96-3)	C9H10O4	181.0506	4.7	151.0028	123.0091	166.0265
8-Prenylnaringenin	(53846-50-7)	C20H20O5	339.1238	10.0	219.0660	119.0492	339.1232
2',4'-Dihydroxychalcone	(1776-30-3)	C15H12O3	239.0714	10.1	119.0496		

4.6 Experiments of adulteration

In order to investigate whether it is possible to detect adulteration in pomegranates juices with this particular method and in which level, experiments of adulteration were occured. More specifically, red and white grape juice, as well as apple juice were added as adulterants in levels of 20, 10, 5, 3, 2 and 1% in pomegranate juice matrix.

For the quantification of all compounds mentioned above Internal Standard (IS) was used. The IS was the Oleuropein, a phenolic compound that is found in olive oil. Oleuropein was selected as a phenolic compound (antioxidant), with similar structure of the antioxidants included in the database (**Table 6**) that cannot be found in fruit juices.

Oleuropein was spiked in the ready-to-run samples in a concentration of 5 mg/L. The IS deviation is presented in the Quality Chart (**Figure 11**) in a sequence of 30 injections.

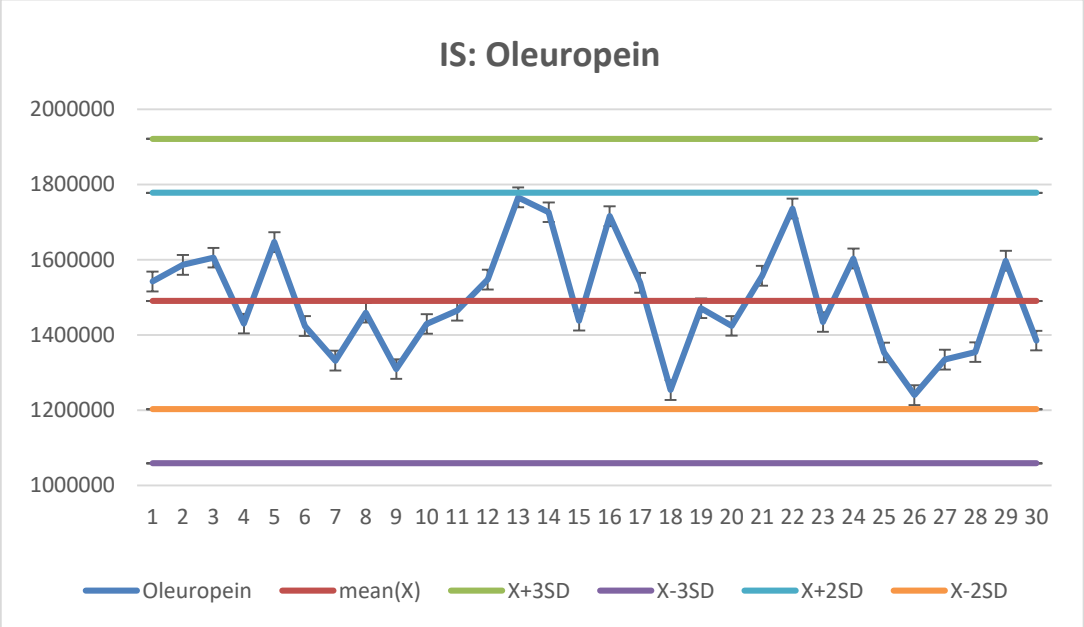
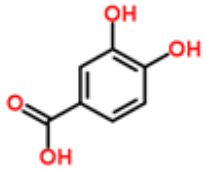
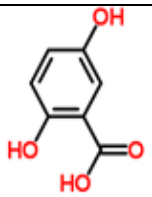
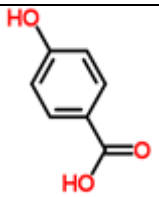
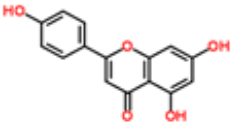
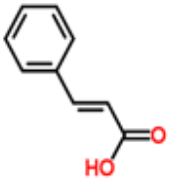
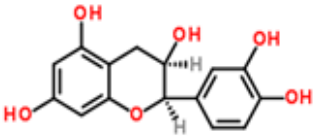
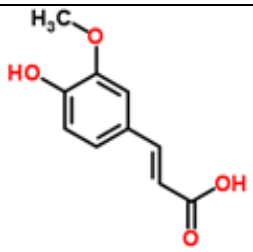
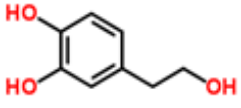
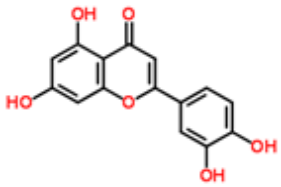
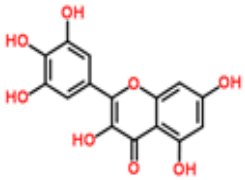
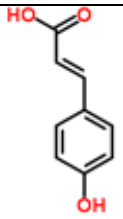
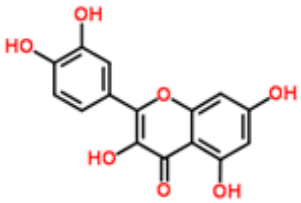
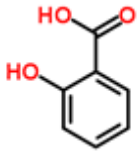
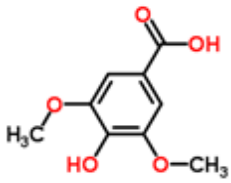
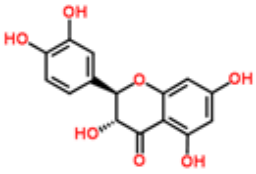
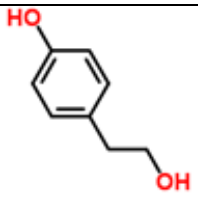
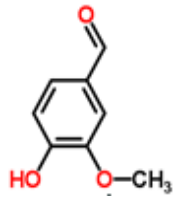
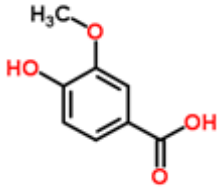
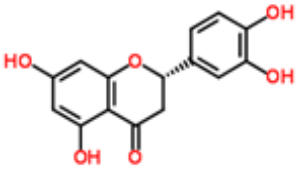
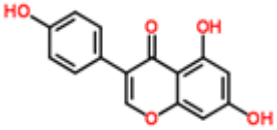
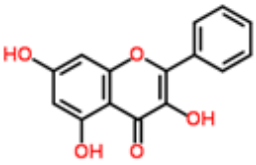
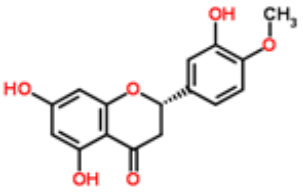
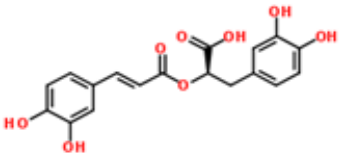
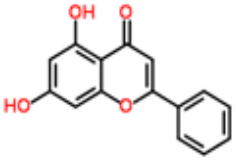
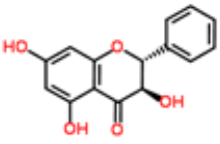


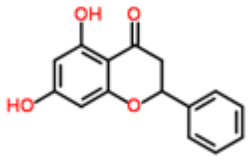
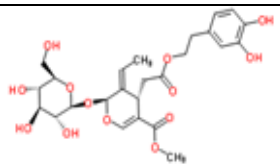
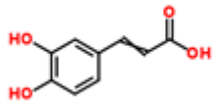
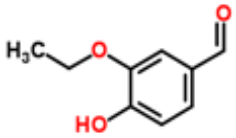
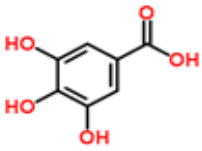
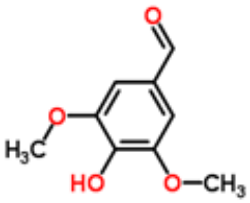
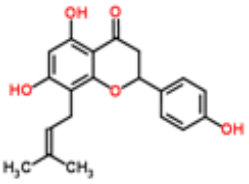
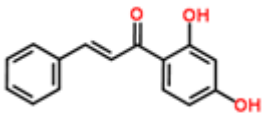
Figure 11: QC chart of Oleuropein used as IS

Table 6: Table of antioxidants and properties: formula, neutral mass and skeletal formula

Compound Name	Formula	Neutral mass	Skeletal formula
3,4- dihydroxybenzoic acid (Protocatechuic acid)	C7H6O4	154.027	
2,5-dihydroxybenzoic acid (Gentistic acid)	C7H6O4	154.027	
4-hydroxybenzoic acid	C7H6O3	138.032	
Apigenin	C15H10O5	270.053	
Cinnamic acid	C9H8O2	180.042	
Epicatechin	C15H14O6	290.079	
Ferulic acid	C10H10O4	194.058	
Hydroxytyrosol	C8H10O3	154.063	

Luteolin	C15H10O6	286.048	
Myricetin	C15H10O8	318.038	
p-coumaric acid	C9H8O3	164.047	
Quercetin	C15H10O7	302.043	
Salicylic acid	C7H6O3	138.032	
Syringic acid	C9H10O5	198.053	
Taxifolin	C15H12O7	304.058	
Tyrosol	C8H10O2	138.068	

Vanillin	C8H8O3	152.047	
Vanillic acid	C8H8O4	168.042	
Eriodictyol	C15H12O6	288.063	
Genistein	C15H10O5	270.053	
Galangin	C15H10O5	270.053	
Hesperetin	C16H14O6	302.079	
Rosmarinic acid	C18H16O8	360.084	
Chrysin	C15H10O4	254.058	
Pinobanksin	C15H12O5	272.068	

Pinocembrin	C15H12O4	256.074	
Oleuropein	C25H32O13	540.518	
Caffeic acid	C9H8O4	180.042	
Ethyl vanillin	C9H10O3	166.063	
Gallic acid	C7H6O5	170.022	
Syringaldehyde	C9H10O4	182.058	
8-Prenylnaringenin	C20H20O5	340.131	
2',4'-Dihydroxychalcone	C15H12O3	240.079	

CHAPTER 5

Results and Discussion

5.1 Validation results

As mentioned in chapter 4.5, for the evaluation of linearity, accuracy, precision, matrix effects and detectability of the screening method, a representative validation dataset of 33 antioxidants was used.

Regarding linearity, the slope, the intercept and the correlation coefficient (R^2) of the standard solution calibration curve for each compound are presented in **Table 7**.

The method limits of detection (MLODs) and the method limits of quantification (MLOQs) that were calculated from the data of the calibration curves are presented in **Table 8**.

Table 7: Validation results - Linearity: Slope, intercept and correlation coefficient (R^2) of the standard solution calibration curve of 6 different concentrations ranging from 0.25-10 mg/L for each compound

Analyte	Slope (b) (mg/L)	Standard error (S_b)	Intercept (a) (mg/L)	Standard error (S_a)	Correlation coefficient (R^2)
3,4- dihydroxybenzoic acid (Protocatechuic acid)	$39.6x10^3$	1.8×10^3	26.8×10^3	8.5×10^3	0.992
2,5-dihydroxybenzoic acid (gentistic acid)	52.4×10^2	9.4×10^2	-78×10^2	44×10^2	0.99
4-hydroxybenzoic acid	$257\times0\cdot10^2$	9.6×10^2	123×10^2	45×10^2	0.994
Apigenin	$101\times5\cdot10^2$	7.2×10^2	138×10^2	33×10^2	0.98
Cinnamic acid	65.1×10^3	1.5×10^3	7.5×10^3	6.9×10^3	0.998
Epicatechin	200.1×10^3	9.9×10^3	119×10^3	46×10^3	0.99
Ferulic acid	88.7×10^3	1.4×10^3	-1.3×10^3	6.8×10^3	0.99

Hydroxytyrosol	12.70×10^4	0.50×10^3	5.6×10^3	2.4×10^3	0.99
Luteolin	725×10^3	51×10^3	10.9×10^3	2.4×10^3	0.98
Myricetin	249.1×10^3	8.7×10^3	48×10^3	40×10^3	0.995
p-coumaric acid	56.7×10^3	1.4×10^3	-2.7×10^3	6.5×10^3	0.998
Quercetin	621×10^3	39×10^3	797.9×10^3	1.8×10^3	0.98
Salicylic acid	369.6×10^3	8.7×10^3	134×10^3	41×10^3	0.998
Syringic acid	24.7×10^3	1.2×10^3	8.8×10^3	5.8×10^3	0.98
Taxifolin	33.6×10^4	2.2×10^4	29.3×10^4	10.2×10^4	0.98
Tyrosol	49.43×10^2	0.65×10^2	20.7×10^2	3.3×10^2	0.9994
Vanillin	49.8×10^3	1.5×10^3	6.7×10^3	6.8×10^3	0.996
Vanillic acid	132.2×10^2	5.8×10^2	73×10^2	27×10^2	0.992
Eriodictyol	51.0×10^3	1.2×10^3	4.0×10^3	5.6×10^3	0.998
Genistein	74.7×10^4	5.1×10^4	141×10^4	24×10^4	0.98
Galangin	147.7×10^4	6.1×10^4	83×10^4	28×10^4	0.993
Hesperitin	33.3×10^4	2.4×10^4	43×10^4	11×10^4	0.98
Rosmarinic acid	137.1×10^4	1.4×10^3	-5.5×10^3	6.6×10^3	0.9996
Chrysin	138.2×10^4	7.3×10^4	250×10^4	34×10^4	0.99
Pinobanksin	141.0×10^4	8.8×10^4	202×10^4	41×10^4	0.98
Pinocembrin	16.5×10^5	1.2×10^5	19.9×10^5	5.5×10^4	0.98
Naringenin	129.0×10^4	9.2×10^4	190×10^4	43×10^4	0.98
Caffeic acid	32.0×10^4	1.8×10^4	12.4×10^4	8.3×10^4	0.99

Ethyl vanillin	98.7×10^3	5.1×10^3	26×10^3	24×10^3	0.99
Gallic acid	19.9×10^4	1.1×10^4	-5.6×10^4	5.1×10^4	0.99
Syringaldehyde	45.7×10^3	3.1×10^3	20×10^3	14×10^3	0.98
8-Prenylnaringenin	50.1×10^4	2.0×10^4	-30.1×10^4	9.4×10^4	0.993
2',4'-Dihydroxychalcone	32.3×10^4	2.2×10^4	-17×10^4	10×10^4	0.98

Table 8: Validation results - MLODs & MLOQs

Analyte	MLOD (mg/L)	MLOQ (mg/L)
3,4- dihydroxybenzoic acid (Protocatechuic acid)	0.23	0.69
2,5-dihydroxybenzoic acid (gentistic acid)	0.10	0.30
4-hydroxybenzoic acid	0.42	1.3
Apigenin	0.060	0.18
Cinnamic acid	0.11	0.33
Epicatechin	0.040	0.12
Ferulic acid	0.18	0.54
Hydroxytyrosol	0.030	0.090
Luteolin	0.12	0.36
Myricetin	0.080	0.24
p-coumaric acid	0.27	0.81
Quercetin	0.040	0.12
Salicylic acid	0.050	0.15

Syringic acid	0.51	1.5
Taxifolin	0.040	0.12
Tyrosol	0.070	0.21
Vanillin	0.070	0.21
Vanillic acid	0.050	0.15
Eriodictyol	0.040	0.12
Genistein	0.060	0.18
Galangin	0.20	0.60
Hesperitin	0.24	0.72
Rosmarinic acid	0.25	0.75
Chrysin	0.080	0.18
Pinobanksin	0.76	2.3
Pinocembrin	0.48	1.4
Naringenin	0.090	0.27
Caffeic acid	0.030	0.090
Ethyl vanillin	0.41	1.2
Gallic acid	0.22	0.66
Syringaldehyde	0.14	0.42
8-Prenylnaringenin	0.26	0.79
2',4'-Dihydroxychalcone	0.29	0.86

Recovery experiments were performed at 5 mg/L. The recoveries measured are satisfying for the majority of the compounds, while method repeatability in terms of %RSD in 4 spiked samples at 500 ng/L was below 6% for all analytes.

The last column of the table (%Matrix Effect, ME) indicates in which cases we have enhancement (ME>0) or repression (ME<0) of the signal.

The results for recoveries, repeatability, matrix effects and matrix factors are presented in total in **Table 9**.

Table 9: Validation results - Recoveries, repeatability, matrix effects and matrix factors

Analyte	% Recovery	%RSD (n=4)	%Matrix Effect
3,4- dihydroxybenzoic acid (Protocatechuic acid)	68	4.1	-32
2,5-dihydroxybenzoic acid (gentistic acid)	75	3.3	37
4-hydroxybenzoic acid	73	3.9	14
Apigenin	77	1.6	-23
Cinnamic acid	84	0.95	-19
Epicatechin	82	1.2	-62
Ferulic acid	71	1.9	-23
Hydroxytyrosol	77	3.7	-23
Luteolin	67	1.1	11
Myricetin	71	1.6	80
p-coumaric acid	88	5.2	-100
Quercetin	71	1.0	10
Salicylic acid	92	1.2	77
Syringic acid	88	1.6	18

Taxifolin	73	1.6	-43
Tyrosol	82	4.6	-19
Vanillin	85	1.7	111
Vanillic acid	67	2.3	37
Eriodictyol	65	4.3	-37
Genistein	76	1.2	-24
Galangin	72	1.7	32
Hesperitin	78	1.8	0
Rosmarinic acid	80	1.1	31
Chrysin	75	0.92	-1.0
Pinobanksin	95	1.6	-16
Pinocembrin	73	1.4	-12
Naringenin	94	1.6	-15
Caffeic acid	80	2.1	-6.0
Ethyl vanillin	72	3.0	12
Gallic acid	61	1.3	-62
Syringaldehyde	94	4.4	4.0
8-Prenylnaringenin	88	4.5	18
2',4'-Dihydroxychalcone	73	2.1	30

5.2 Target screening results

For target screening, different fruit juices were analysed (pomegranate, apple, red and white grape juice). The variation of the antioxidants in samples that have more than one code (i.e. red and white grape as well as apple) is shown in **Table 10**, **11** and **12** respectively.

Table 10: Variation of antioxidants in red grape juice

C (mg/L) / Analyte	Range	Average	TH1	TH2	TH3	TH4	TH5
2,5-dihydroxybenzoic acid (gentistic acid)	3.0-5.3	3.5	3.4	5.3	3.6	3.0	4.2
Caffeic acid	0.28-1.4	0.57	0.39	0.25	0.40	0.28	1.4
Epicatechin	4.4-17	8.6	9.0	17	12	6.0	4.4
Ferulic acid	0.27-0.90	0.45 <LOQ	0.51 <LOQ	0.90		0.45 <LOQ	0.27 <LOQ
Hydroxytyrosol	2.3-4.4	3.1	3.2	3.6	4.4	2.3	2.6
Myricetin	0.20-0.60	0.24			0.28	0.20 <LOQ	0.60
p-coumaric acid	1.3				1.3		
Quercetin	0.050-0.43	0.20	0.050 <LOQ	0.11 <LOQ	0.31	0.15	0.43
Salicylic acid	0.56-2.4	1.3	2.1	1.3	2.4	0.56	0.67
Taxifolin	0.32-0.84	0.62	0.32	0.78	0.43	0.78	0.84
Tyrosol	0.45-0.93	0.65	0.53	0.53	0.59	0.75	0.93
Eriodictyol	0.060-0.19	0.12	0.060 <LOQ	0.19	0.14	0.12	0.15
Naringenin	0.16-0.36	0.25	0.16	0.17	0.18	0.36	0.20

Table 11: Variation of antioxidants in white grape juice

C (mg/L) / Analyte	Range	Average	I1	I2	I4	I5
Hydroxytyrosol	1.5-2.3	1.8	1.5	1.6	1.8	2.3
Salicylic acid	0.22-0.25	0.24	0.22	0.24	0.25	0.25
Tyrosol	0.070-0.15 <LOQ	0.11 <LOQ	0.090 <LOQ	0.070 <LOQ	0.12 <LOQ	0.15 <LOQ
Naringenin	0.10-0.21	0.20	0.21	0.09	0.10	0.18

Table 12: Variation of antioxidants in apple juice

C (mg/L) / Analyte	Range	Average	N1	N4
2,5-dihydroxybenzoic acid (gentistic acid)	0.49-0.61	0.55	0.61	0.49
Epicatechin	2.6	2.6	2.6	2.6
Hydroxytyrosol	0.10-0.11	0.10	0.11	0.10
p-coumaric acid	0.30 <LOQ		0.30 <LOQ	
Quercetin	0.040 <LOQ	0.040 <LOQ	0.040 <LOQ	
Taxifolin	0.060-0.10 <LOQ	0.08 <LOQ	0.10 <LOQ	0.060 <LOQ
Tyrosol	0.17-0.35	0.26	0.17 <LOQ	0.35
Vanillin	0.090		0.090	
Eriodictyol	0.35-0.42	0.39	0.42	0.35
Naringenin			0.14 <LOQ	

5.2.1 Experiments of adulteration

Then, in order to detect possible adulteration in pomegranate juice, juices of red and white grape as well as apple were added in purpose, at a rate of 20, 10, 5, 3, 2 and 1% of adulteration. The results are shown in **Table 13**, **15** and **17** respectively, in the second column of which the content of pomegranate juice (sample code L1) is included.

Table 13: Antioxidants found in red grape and pomegranate juice matrix and in rates of adulteration

C (mg/L) / Analyte	Pomegranate (L1)	Red grape (TH2)	20%	10%	5%	3%	2%	1%
2,5dihydroxybenzoic acid (gentistic acid)	1.2	5.3	1.8	1.2	1.0	0.86	0.68	0.73
Caffeic acid		0.25						
Cinnamic acid	0.44		0.73	0.56	0.61	0.44	0.49	0.37
Epicatechin	**0.060 <LOQ**	**17**	**3.7**	**1.2**	**0.66**	**0.33**	**0.27**	**0.12**
Ferulic acid	0.63	0.90	0.96	0.64	0.70	0.48 <LOQ	0.44 <LOQ	
Hydroxytyrosol	**0.040 <LOQ**	**3.6**	**2.2**	**1.8**	**1.6**	**1.5**	**1.5**	**1.4**
Luteolin	0.24 <LOQ		0.23 <LOQ	0.19 <LOQ	0.22 <LOQ	0.17 <LOQ	0.18 <LOQ	0.16 <LOQ
Myricetin	0.32		0.12 <LOQ	0.090 <LOQ	0.090 <LOQ			
p-coumaric acid	0.35 <LOQ		0.66 <LOQ	0.49 <LOQ	0.85	0.30 <LOQ	0.32 <LOQ	0.31 <LOQ
Quercetin	0.070 <LOQ	0.11 <LOQ	0.21	0.16	0.20	0.14	0.13	0.20
Salicylic acid	**0.07 <LOQ**	**1.3**	**0.37**	**0.17**	**0.090 <LOQ**	**0.060 <LOQ**	**0.080 <LOQ**	
Taxifolin	0.06 <LOQ0	0.78	0.18	0.080 <LOQ	0.070 <LOQ	0.050 <LOQ	0.040 <LOQ	
Tyrosol	0.11 <LOQ	0.53	5.4	4.8	4.8	4.4	4.3	3.6
Eriodictyol	0.12	0.19	0.16	0.13	0.090 <LOQ	0.080 <LOQ	0.11 <LOQ	0.080 <LOQ

Syringaldeyde	0.37 <LOQ		0.71	0.44	0.48	0.44	0.47	0.36
Naringenin	0.20 <LOQ	0.17 <LOQ	0.14 <LOQ	0.11 <LOQ	0.12 <LOQ		0.090 <LOQ	

Possible markers in order to detect adulteration with red grape juice are Epicatechin, Hydroxytyrosol and Salicylic acid. What makes these antioxidants ideal as markers is their low concentrations in pomegranate juice, as shown in **Table 13**. Moreover, these antioxidants follow a satisfying linear regression model (**Table 14**).

Table 14: Linearity: Slope, intercept and correlation coefficient (R^2) of the markers in red grape and pomegranate juice matrix

Analyte	Slope (b) (mg/L)	Standard error (S_b)	Intercept (a) (mg/L)	Standard error (S_a)	Correlation coefficient (R^2)
Epicatechin	18.3×10^{-2}	1.6×10^{-2}	-2.1×10^{-2}	15×10^{-2}	0.97
Hydroxytyrosol	3.77×10^{-2}	0.26×10^{-2}	140.1×10^{-2}	2.0×10^{-2}	0.98
Salicylic acid	1.74×10^{-2}	0.12×10^{-2}	1.5×10^{-2}	1.2×10^{-2}	0.98

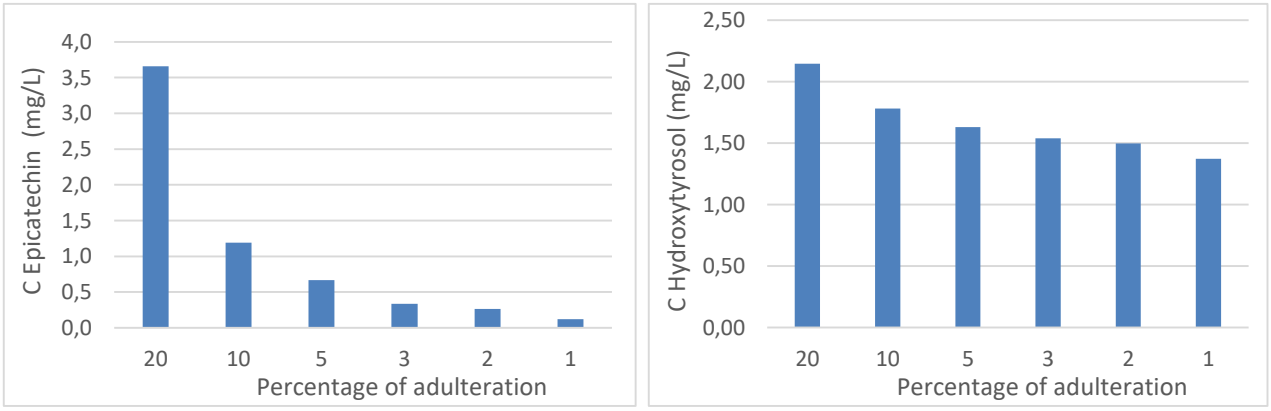


Figure 12: Variance of Epicatechin and Hydroxytyrosol in different rates of red grape juice adulteration in pomegranate juice (Hicaz's variety)

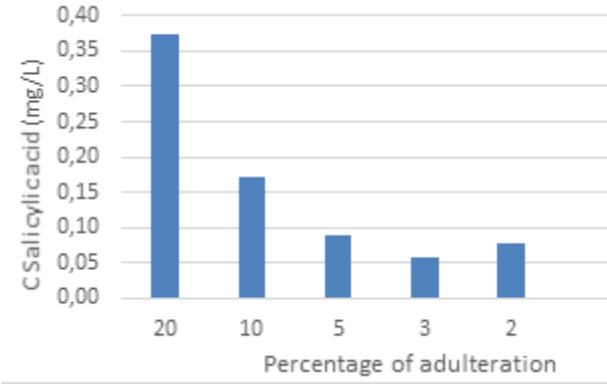


Figure 13: Variance of Salicylic acid in different rates of red grape juice adulteration in pomegranate juice (Hicaz's variety)

In the case of salicylic acid in red grapes juices, possible adulteration can be detected at 20 and 10% percentages under a satisfying level of confidence. The percentages of 5, 3, 2 and 1% give questionable results, as the concentrations calculated are below the LOQ of the method. In **Figure 13** there are included in the chart for better visualisation of the results.

Table 15: Antioxidants found in white grape and pomegranate juice matrix and in rates of adulteration

C (mg/L) / Analyte	Pomegranate (L1)	White grape (I1)	20%	10%	5%	3%	2%	1%
2,5dihydroxybenzoic acid (gentistic acid)	1.2		0.30	0.67	0.82	0.99	0.94	0.99
Cinnamic acid	0.44		0.27 <LOQ	0.28 <LOQ	0.28 <LOQ	0.33	0.34	0.27 <LOQ
Epicatechin	0.060 <LOQ		0.070 <LOQ	0.080 <LOQ	0.11 <LOQ	0.050 <LOQ	0.040 <LOQ	0.0400 <LOQ
Ferulic acid	0.63							
Hydroxytyrosol	**0.040 <LOQ**	**1.5**	**0.66**	**0.36**	**0.14**	**0.050 <LOQ**	**0.050 <LOQ**	**0.030 <LOQ**
Luteolin	0.24 <LOQ							
Myricetin	0.32							
p-coumaric acid	0.35 <LOQ					0.28 <LOQ		

Quercetin	0.070 <LOQ		0.33	0.34	0.060 <LOQ	0.050 <LOQ	0.060 <LOQ	0.050 <LOQ
Salicylic acid	**0.070 <LOQ**	**0.22**	**0.14 <LOQ**	**0.10 <LOQ**	**0.080 <LOQ**	**0.070 <LOQ**	**0.060 <LOQ**	**0.050 <LOQ**
Taxifolin	0.060 <LOQ					0.040 <LOQ	0.040 <LOQ	0.040 <LOQ
Tyrosol	0.11 <LOQ	0.090 <LOQ	0.10 <LOQ	0.21	0.11 <LOQ	0.10 <LOQ	0.11 <LOQ	0.080 <LOQ
Eriodictyol	0.12		0.040 <LOQ	0.080 <LOQ	0.070 <LOQ	0.050 <LOQ	0.080 <LOQ	0.080 <LOQ
Syringaldeyde	0.37 <LOQ		0.28 <LOQ	0.27 <LOQ	0.27 <LOQ	0.28 <LOQ	0.25 <LOQ	0.25 <LOQ
Naringenin	0.20 <LOQ	0.21 <LOQ	0.17 <LOQ	0.17 <LOQ	0.15 <LOQ	0.15 <LOQ	0.14 <LOQ	0.14 <LOQ

Possible markers in order to detect adulteration with white grape juice are Hydroxytyrosol and Salicylic acid, which follow a linear regression model (**Table 16**).

Table 16: Linearity: Slope, intercept and correlation coefficient (R^2) of the markers in white grape and pomegranate juice matrix

Analyte	Slope (b) (mg/L)	Standard error (S_b)	Intercept (a) (mg/L)	Standard error (S_a)	Correlation coefficient (R^2)
Hydroxytyrosol	3.43×10^{-2}	0.16×10^{-2}	-1.8×10^{-2}	1.5×10^{-2}	0.991
Salicylic acid	45.2×10^{-4}	2.9×10^{-4}	552.3×10^{-4}	28.1×10^{-4}	0.98

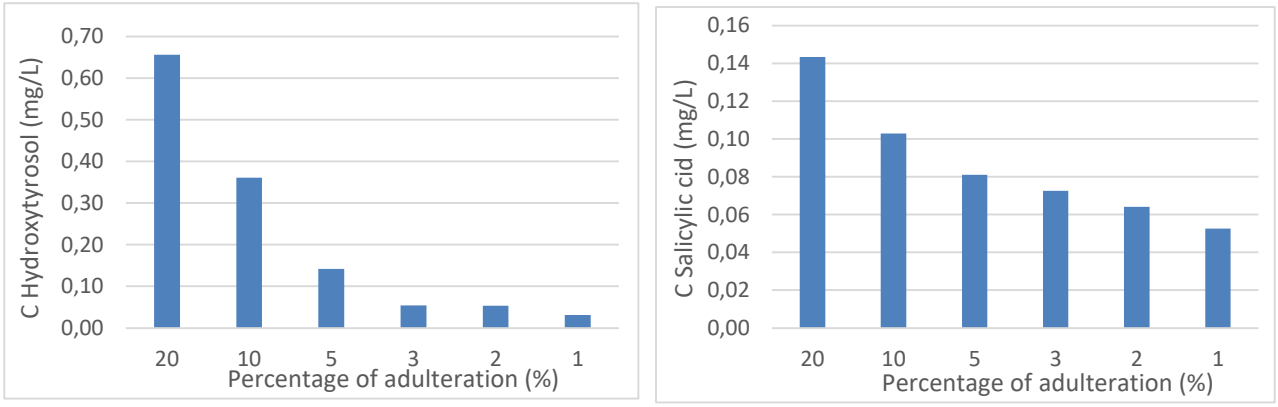


Figure 14: Variance of Hydroxytyrosol and Salicylic acid in different rates of white grape juice adulteration in pomegranate juice (Hicaz's variety)

In white grape juice adulteration two markers are exported. In the case of Hydroxytytrosol, possible adulteration can be detected at 20, 10 and 5% percentages under a satisfying level of confidence. The percentages of 3, 2 and 1% give questionable results, as the concentrations calculated are below the LOQ of the method. In Salicylic acid on the other hand all percentages of adulteration are below the LOQ of the method. In **Figure 14** all results are included in the chart for better visualisation of the results.

Table 17: Antioxidants found in apple and pomegranate juice matrix and in rates of adulteration

C (mg/L) / Analyte	Pomegranate (L1)	Apple (N1)	20%	10%	5%	3%	2%	1%
2,5dihydroxybenzoic acid (gentistic acid)	1.2	0.61	1.0	1.2	1.2	1.3	1.1	1.1
Cinnamic acid	0.44		0.12 <LOQ	0.19 <LOQ	0.20 <LOQ	0.46	0.33	0.38
Epicatechin	**0.060 <LOQ**	**2.6**	**0.31**	**0.21**	**0.13**	**0.090 <LOQ**	**0.070 <LOQ**	**0.070 <LOQ**
Ferulic acid	0.63		0.20 <LOQ	0.21 <LOQ	0.25 <LOQ	0.73	0.49 <LOQ	0.54
Hydroxytyrosol	0.040 <LOQ	0.11	0.060 <LOQ	0.040 <LOQ	0.040 <LOQ	0.040 <LOQ	0.060 <LOQ	0.050 <LOQ
Luteolin	0.24 <LOQ		0.16 <LOQ	0.25 <LOQ	0.22 <LOQ	0.20 <LOQ	0.17 <LOQ	0.21 <LOQ
Myricetin	0.32			0.15 <LOQ	0.16 <LOQ	0.20 <LOQ	0.18 <LOQ	0.22 <LOQ
p-coumaric acid	0.35 <LOQ	0.30 <LOQ	0.50 <LOQ	0.61 <LOQ	0.56 <LOQ	0.40 <LOQ	0.29 <LOQ	0.28 <LOQ
Quercetin	0.070 <LOQ	0.040 <LOQ	0.65	0.60	0.41	0.100 <LOQ	0.070 <LOQ	0.070 <LOQ
Salicylic acid	0.070 <LOQ		0.080 <LOQ	0.12 <LOQ	0.090 <LOQ	0.070 <LOQ		0.090 <LOQ
Taxifolin	0.060 <LOQ	0.10 <LOQ				0.070 <LOQ	0.040 <LOQ	0.060 <LOQ
Tyrosol	0.11 <LOQ	0.17 <LOQ	0.16 <LOQ	0.16 <LOQ	0.11 <LOQ	0.11 <LOQ	0.13 <LOQ	0.08 <LOQ
Vanillin		0.090 <LOQ						
Eriodictyol	0.12	0.42	0.080	0.16	0.11 <LOQ	0.12	0.090 <LOQ	0.090 <LOQ
Syringaldeyde	0.37 <LOQ		0.51	0.62		0.39 <LOQ	0.35 <LOQ	0.30 <LOQ

Naringenin	0.20 <LOQ	0.14 <LOQ	0.11 <LOQ	0.12 <LOQ	0.18 <LOQ	0.22 <LOQ	0.16 <LOQ	0.19 <LOQ

The only possible marker in order to detect adulteration with white grape juice is Epicatechin, which follow a linear regression model (**Table 18**). In 3, 2 and 1% the concentrations are below the LOQ of the method, they are included in the chart for better visualisation of the results (**Figure 15**).

Table 18: Linearity: Slope, intercept and correlation coefficient (R^2) of the marker in apple and pomegranate juice matrix

Analyte	Slope (b) (mg/L)	Standard error (S_b)	Intercept (a) (mg/L)	Standard error (S_a)	Correlation coefficient (R^2)
Epicatechin	13.11×10^{-3}	0.90×10^{-3}	58.5×10^{-3}	8.6×10^{-3}	0.98

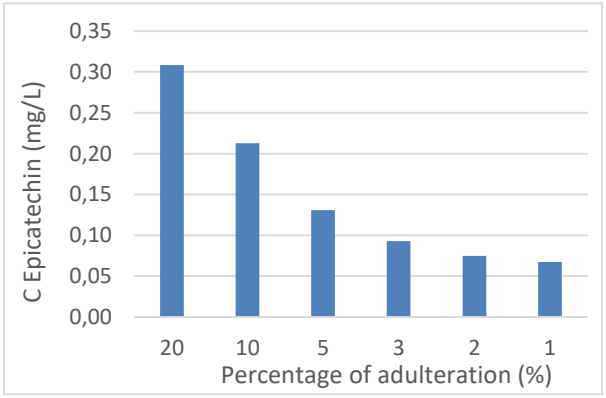


Figure 15: Variance of Epicatechin in different rates of apple juice adulteration in pomegranate juice (Hicaz's variety)

5.2.2 Confirmation of results

Due to the fact that only one variety of pomegranate juice was taken into account for the export of results, it was considered appropriate to repeat the experiment, this time with freshly- squeezed pomegranate juice (code L2). The

pomegranates that were used in the experiment belonged to the Ermioni's variety. The adulteration was conducted in same levels, those of 20, 10, 5, 3, 2, and 1%, in the same matrices: red and white grape juice as well as apple juice.

The experiment's data verified the initial results and are presented in **Table 19**.

Table 19: Antioxidants found in red grape and freshly-squeezed pomegranate juice matrix and in rates of adulteration

C (mg/L) / Analyte	**Pomegranate (L2)**	**Red grape (TH2)**	**20%**	**10%**	**5%**	**3%**	**2%**	**1%**
2,5dihydroxybenzoic acid (gentistic acid)	2.9	5.3	2.4	1.9	2.2	2.3	2.9	2.2
Epicatechin	**2.2**	**17**	**4.0**	**3.1**	**2.7**	**2.3**	**2.3**	**2.0**
Ferulic acid		0.90						
Hydroxytyrosol		**3.6**	**0.55**	**0.27**	**0.17**	**0.11**	**0.10**	**0.040 <LOQ**
Myricetin	0.080 <LOQ							
Quercetin		0.11 <LOQ						
Salicylic acid	**0.060 <LOQ**	**1.3**	**0.36**	**0.25**	**0.090 <LOQ**	**0.070 <LOQ**	**0.060 <LOQ**	**0.050 <LOQ**
Taxifolin	0.050	0.78	0.19	0.10 <LOQ	0.090 <LOQ	0.070 <LOQ	0.060 <LOQ	0.040 <LOQ
Tyrosol		0.53	0.12 <LOQ	0.080 <LOQ				
Eriodictyol		0.19	0.050 <LOQ					
Syringaldeyde	0.59		0.48	0.45	0.47	0.48	0.50	0.56
Naringenin	0.17 <LOQ							

Same markers were found and more specifically Epicatechin, Hydroxytyrosol and Salicylic acid with satisfying linearity (**Table 20**). In Ermioni's variety Epicatechin was found to be higher in content than in Hicaz variety.

Hydroxytyrosol's concetration in 1% percentage of adulteration, was calculated below the LOQ of the method, but is included in the chart for better visualisation of the results (**Figure 16**).

Table 20: Linearity: Slope, intercept and correlation coefficient (R^2) of the markers in red grape and freshly-squeezed pomegranate juice matrix

Analyte	Slope (b) (mg/L)	Standard error (S_b)	Intercept (a) (mg/L)	Standard error (S_a)	Correlation coefficient (R^2)
Epicatechin	9.9×10^{-2}	0.62×10^{-2}	205.7	5.9×10^{-2}	0.98
Hydroxytyrosol	25.5×10^{-3}	9.1×10^{-3}	32×10^{-3}	86×10^{-3}	0.994
Salicylic acid	18×10^{-2}	17×10^{-2}	2.5×10^{-2}	1.6×10^{-2}	0.96

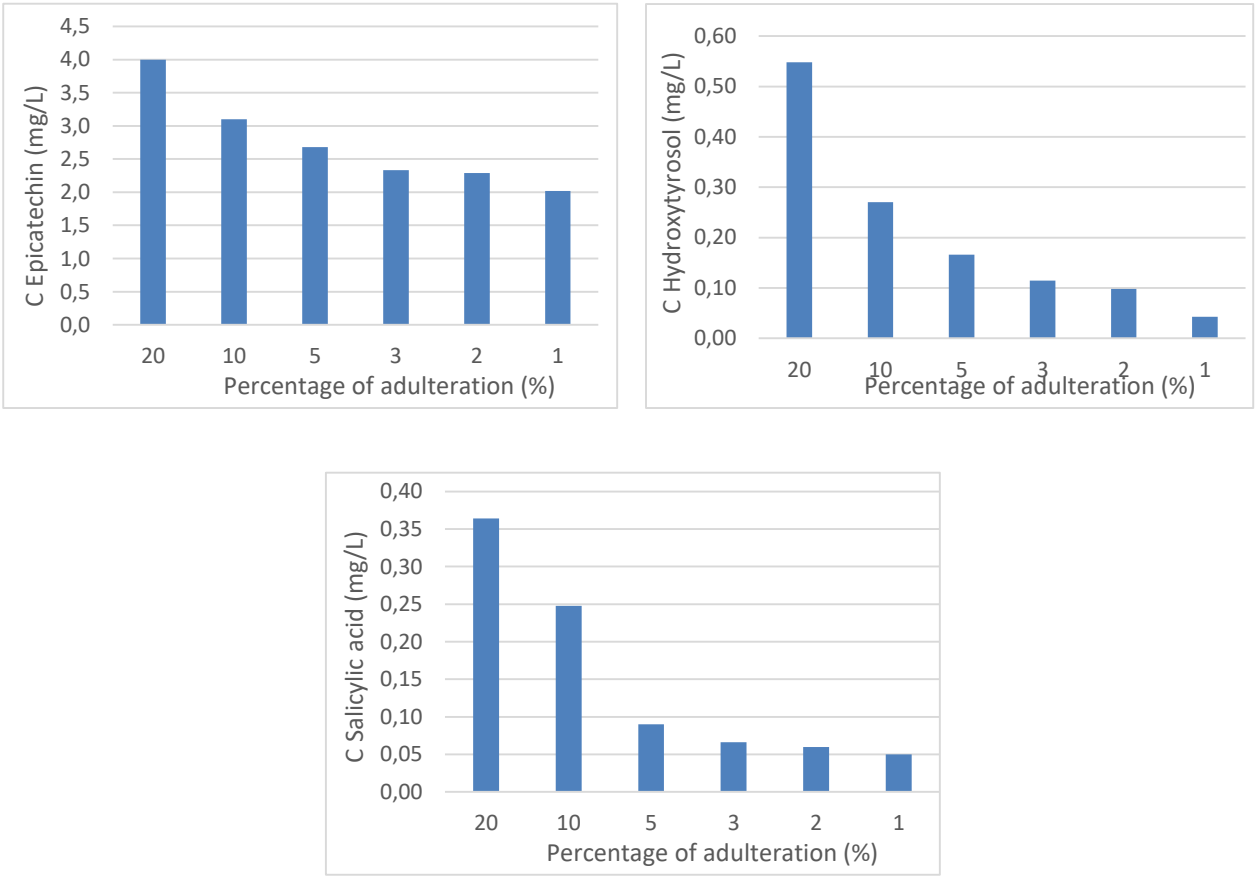


Figure 16: Variance of Epicatechin, Hydroxytyrosol and Salicylic acid in different rates of red grape juice adulteration in freshly-squeezed pomegranate juice (Ermioni's variety)

Table 21: Antioxidants found in white grape and freshly-squeezed pomegranate juice matrix and in rates of adulteration

C (mg/L) / Analyte	Pomegranate (L2)	White grape (I1)	20%	10%	5%	3%	2%	1%
2,5dihydroxybenzoic acid (gentistic acid)	2.9		1.5	1.9	1.9	2.7	2.9	2.0
Epicatechin	2.2			1.9	1.7	2.2	2.4	1.8
Hydroxytyrosol		**1.5**	**0.34**	**0.19**	**0.13**	**0.050 <LOQ**	**0.040 <LOQ**	
Myricetin	0.080 <LOQ							
Salicylic acid	**0.11**	**0.22**	**0.12**	**0.080**	**0.050**	**0.040**	**0.030**	**0.030**
Taxifolin	0.050 <LOQ			0.040 <LOQ	0.040 <LOQ	0.050 <LOQ	0.050 <LOQ	0.040 <LOQ
Tyrosol		0.090 <LOQ						
Syringaldeyde	0.59		0.37 <LOQ	0.50	0.50	0.76	0.78	0.54

Same markers were found and more specifically Hydroxytyrosol and Salicylic acid with satisfying linearity (**Table 22**). Hydroxytyrosol's concetration in 3 and 2% percentage of adulteration, is calculated below the LOQ of the method, when in 1% adulteration cannot be detected at all. However, both 3 and 2% percentages are included in the chart for better visualisation of the results (**Figure 17**).

Table 22: Linearity: Slope, intercept and correlation coefficient (R^2) of the markers in white grape and freshly-squeezed pomegranate juice matrix

Analyte	Slope (b) (mg/L)	Standard error (S_b)	Intercept (a) (mg/L)	Standard error (S_a)	Correlation coefficient (R^2)
Hydroxytyrosol	1.67×10^{-2}	0.12×10^{-2}	1.4×10^{-2}	1.1×10^{-2}	0.98
Salicylic acid	4.9×10^{-3}	0.26×10^{-3}	24.8×10^{-3}	2.4×10^{-3}	0.98

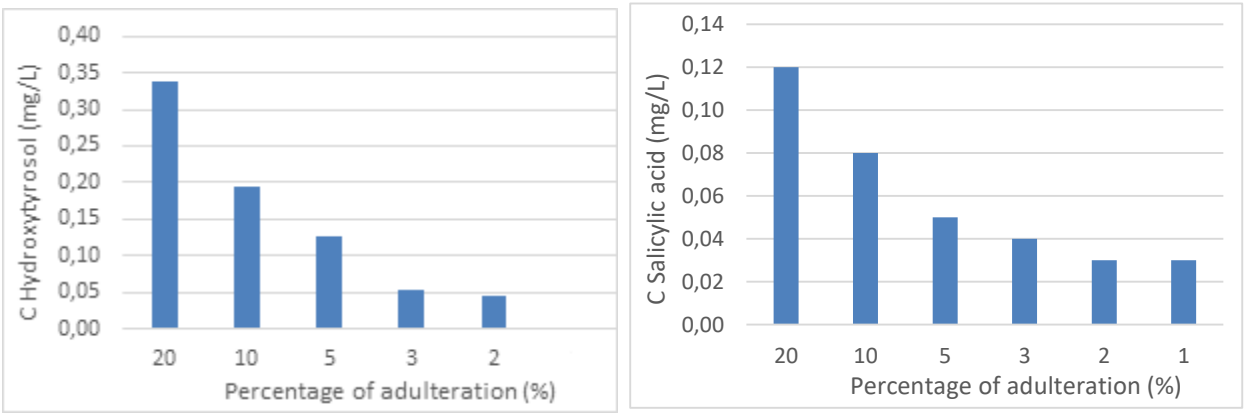


Figure 17: Variance of Hydroxytyrosol and Salicylic acid in different rates of white grape juice adulteration in freshly-squeezed pomegranate juice (Ermioni's variety)

Table 23: Antioxidants found in apple and freshly-squeezed pomegranate juice matrix and in rates of adulteration

C (mg/L) / Analyte	Pomegranate (L2)	Apple (N1)	20%	10%	5%	3%	2%	1%
2,5dihydroxybenzoic acid (gentistic acid)	2.9	0.61	2.0	2.0	2.2	2.8	2.5	2.4
Epicatechin	2.2	2.6	1.8	2.0	2.0	2.1	2.0	2.2
Hydroxytyrosol		0.11	0.030 <LOQ		0.030 <LOQ			
Myricetin	0.080 <LOQ							
p-coumaric acid		0.30 <LOQ						
Quercetin		0.040 <LOQ						
Salicylic acid	0.060 <LOQ	0.020 <LOQ	0.11 <LOQ	0.090 <LOQ	0.060 <LOQ	0.070 <LOQ	0.050 <LOQ	0.060 <LOQ
Taxifolin	0.050 <LOQ	0.10 <LOQ		0.050 <LOQ	0.050 <LOQ	0.050 <LOQ	0.050 <LOQ	0.050 <LOQ
Tyrosol		0.17 <LOQ						
Vanillin		0.090 <LOQ						
Eriodictyol		0.42	0.040 <LOQ					

Syringaldeyde	0.59		0.44	0.48	0.55	0.62	0.49	0.57
Naringenin		0.14 <LOQ						

In this case, due to the fact that this variety of pomegranate (Ermioni's variety) has a high amount of Epicatechin, a discrimination of possible adulteration was not possible, as Epicatechin was not anymore a representative marker of pomegranate juice adulteration with apple.

In order to compare the adulterations in the two different pomegranate juices, relative charts were made in red grape, white grape and apple adulteration presented in **Figures 18, 19** and **20** respectively.

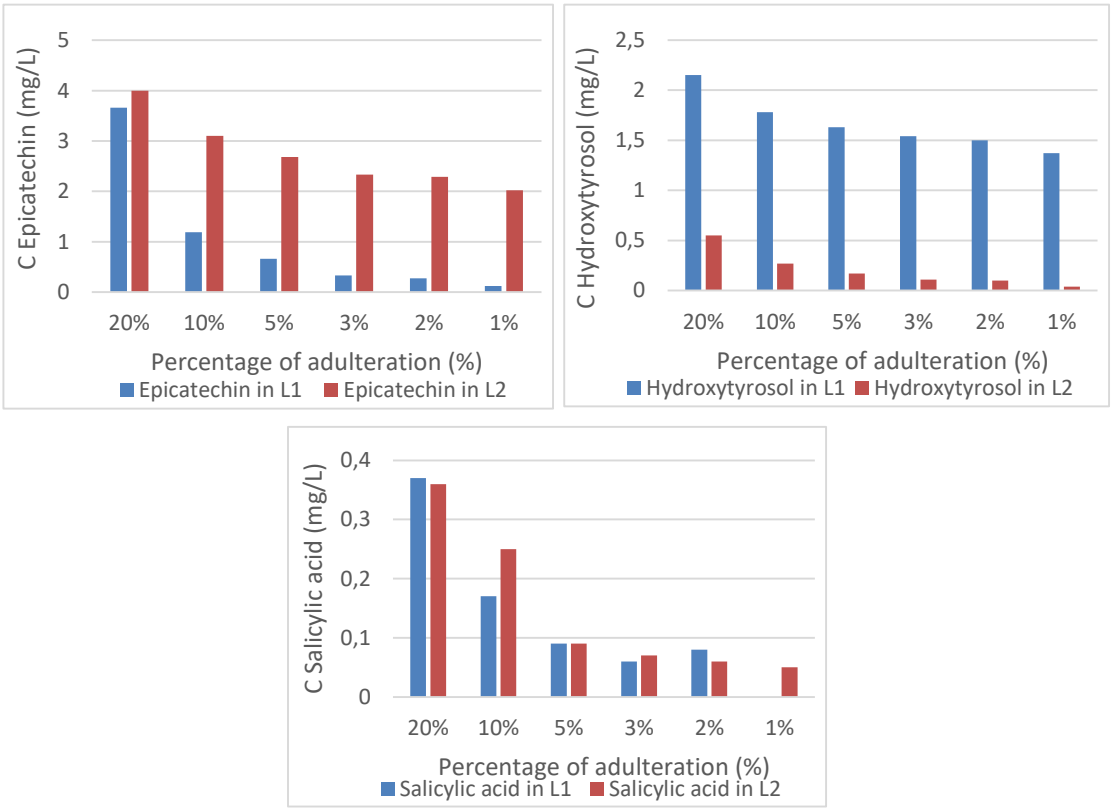


Figure 18: Comparing the variance of Epicatechin, Hydroxytyrosol and Salicylic acid in different rates of red grape juice adulteration in Hicaz's and Ermioni's variety pomegranate juice

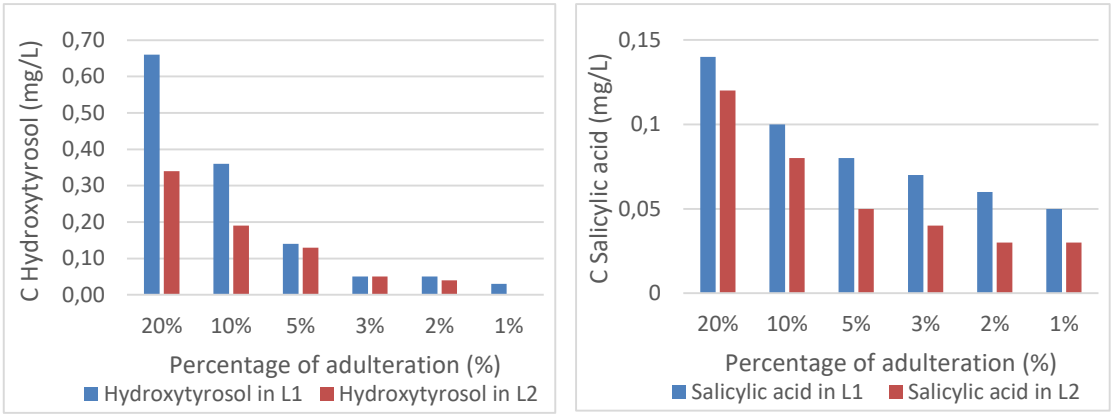


Figure 19: Comparing the variance of Hydroxytyrosol and Salicylic acid in different rates of white grape juice adulteration in Hicaz's and Ermioni's variety pomegranate juice

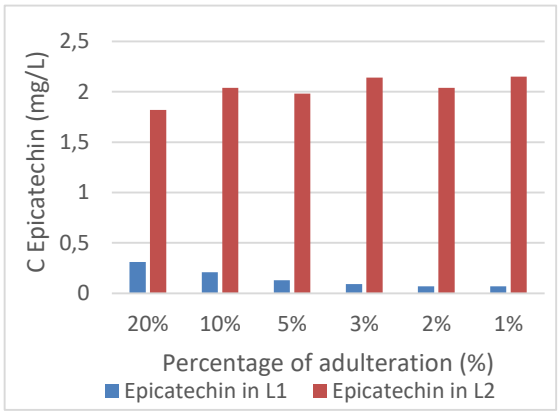


Figure 20: Comparing the variance of Epicatechin in different rates of apple juice adulteration in Hicaz's and Ermioni's variety pomegranate juice

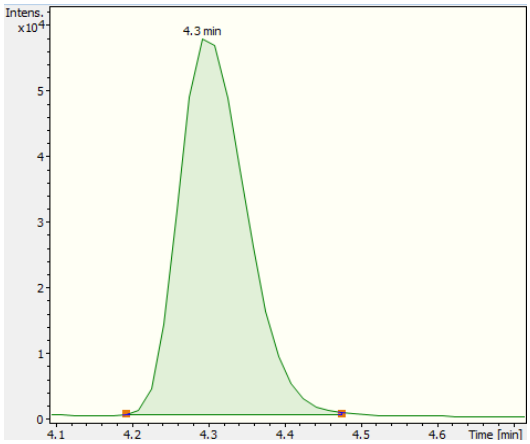
5.3 Identification of compounds

The identification of the compounds was based on the retention time (RT) and the study of the fragments according to the database of antioxidants (see **Table 5**).

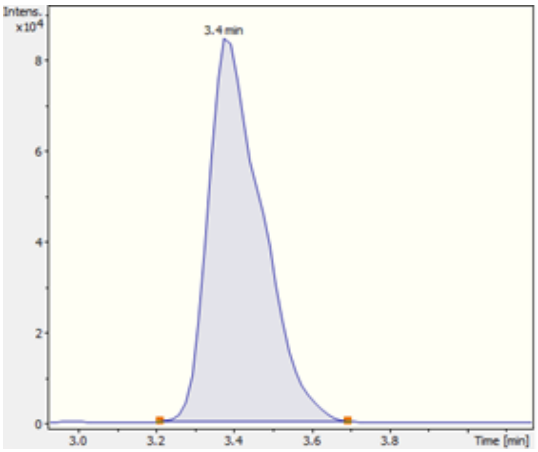
All the identified compounds had the same retention time with the standards in the database (±0.2 min) and fragments were also identified. More specifically, some selected chromatograms and fragments of the markers are presented in **Figure 21**.

Subsequently the MS/MS spectra is presented in which the fragments of the compounds- markers and their chemical structure are presented (**Figures 22, 23, 24**).

(a)



(b)



(c)

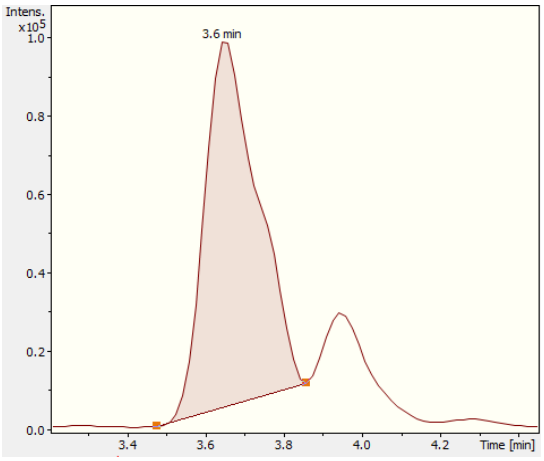


Figure 21: Chromatograms and retention times of (a) Epicatechin, (b) Hydroxytyrosol and (c) Salicylic acid

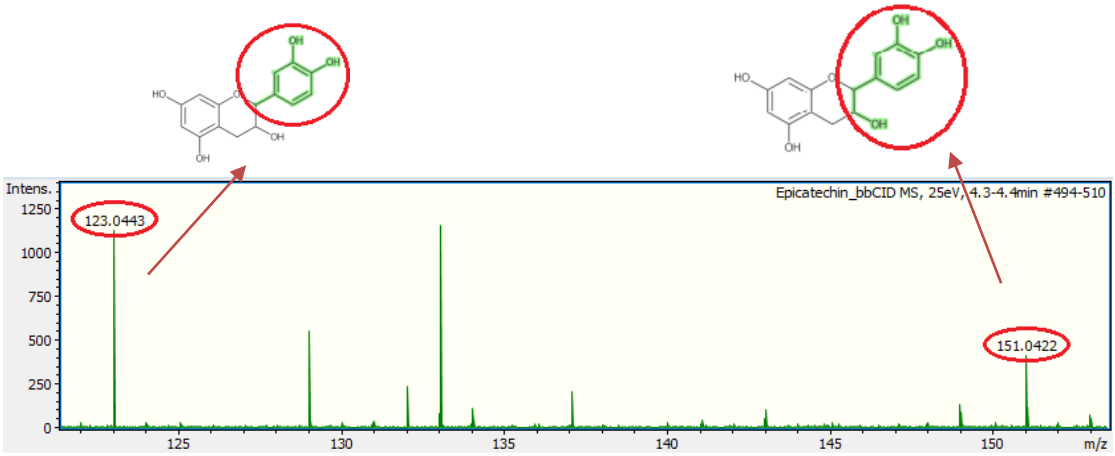


Figure 22: MS/MS spectra of Epicatechin

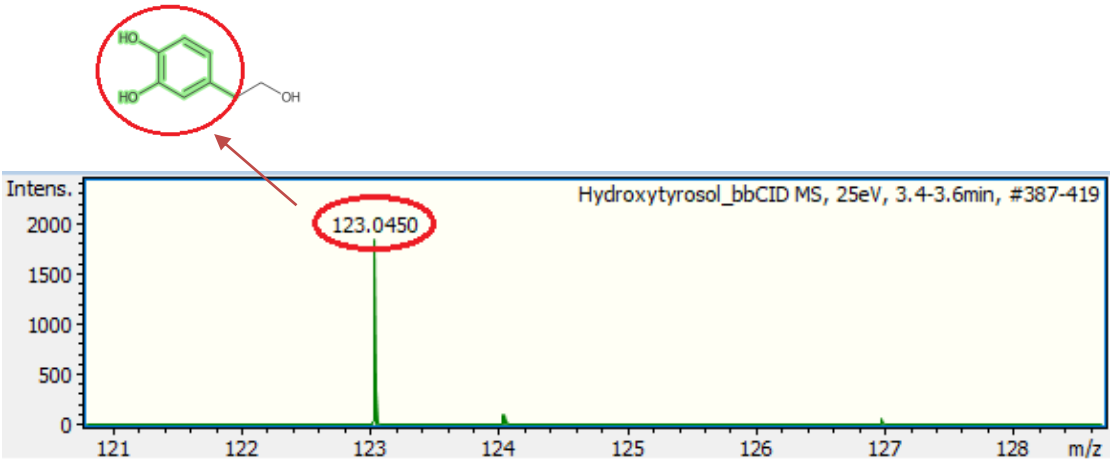


Figure 23: MS/MS spectra of Hydroxytyrosol

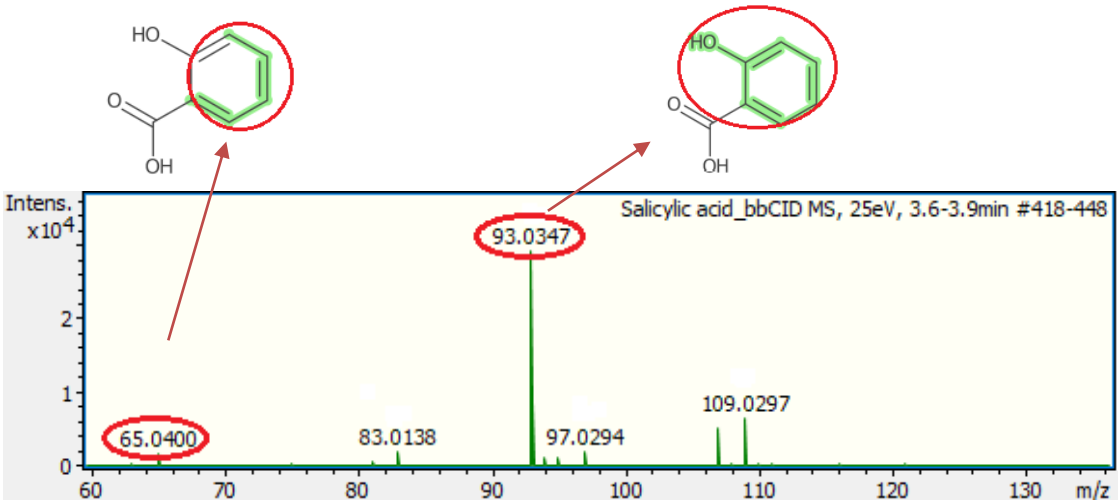


Figure 24: MS/MS spectra of Salicylic acid

5.4 Data exported from PCA

5.4.1 PCA in pure fruit juices

In order to correlate and in sometimes confirm the results from the experiments of adulteration, PCA was performed in the ‘Statistica’ program.

Firstly, all the codes of pure fruit juices available: pomegranate (L1, L2), apple (N1, N4), white grape (I1, I2, I4, I5) and red grape (TH1, TH2, TH3, TH4, TH5) were inserted in the program. The scree plot taken (**Figure 25**) shows the contribution of each component, and more specifically in this case the first two components (PC1, PC2) seem to explain the majority of the results (87%).

In the loading scatterplot (**Figure 26**) we can see the differentiation among the matrixes. We also observe that all the codes of each matrix are found in the same coordinates in the plot, which is expected.

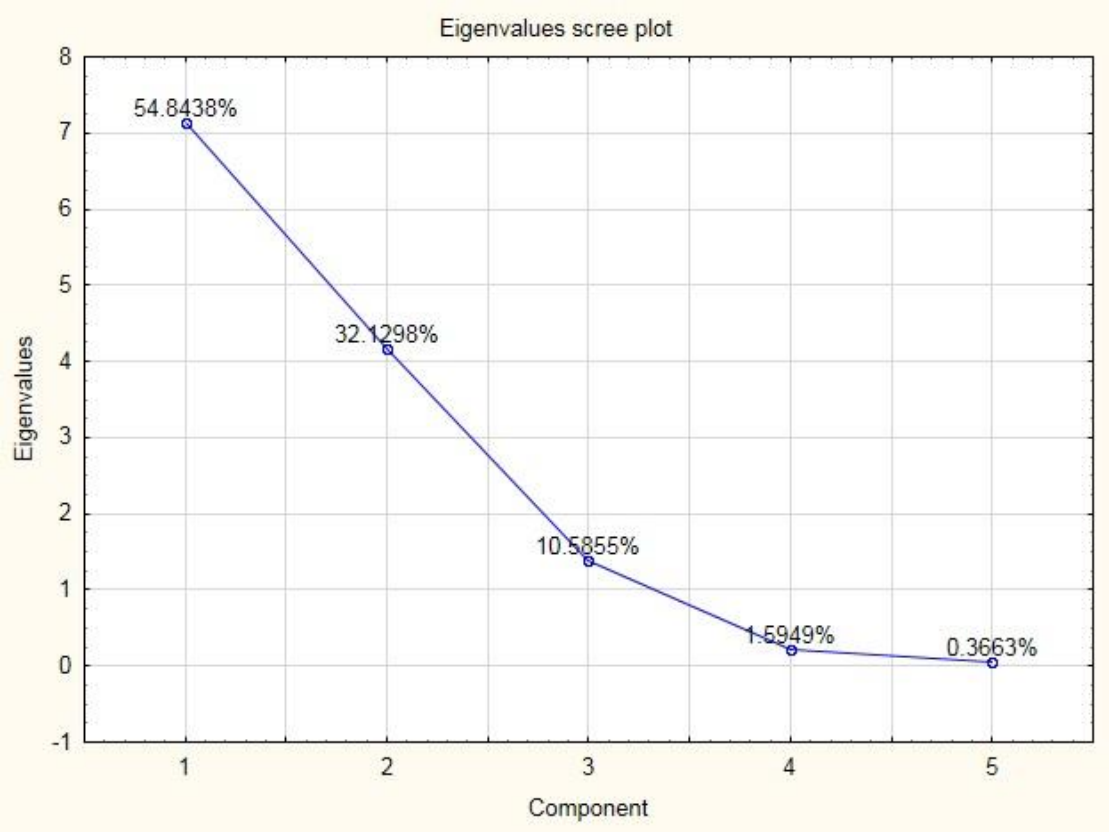


Figure 25: Scree plot of pure fruit juices

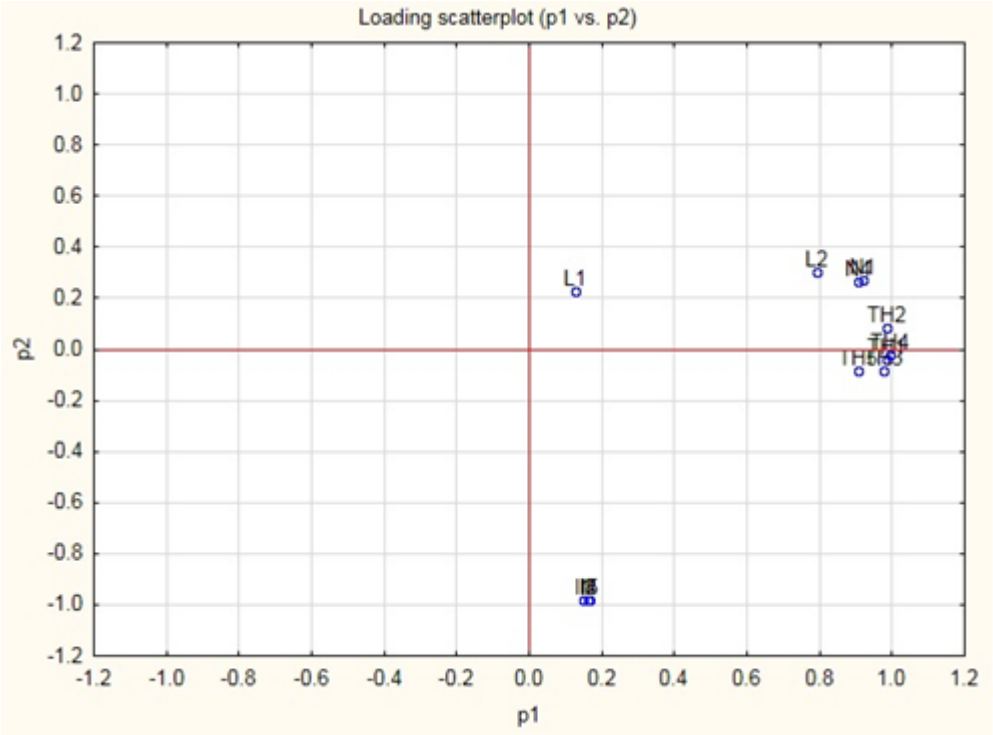


Figure 26: Loadings scatterplot of pure fruit juices

5.4.2 PCA in grape and pomegranate juices

The grape juice codes both red and white that used in adulteration experiments (codes TH2 and I1 respectively), as well as the two codes of pomegranate juice (codes L1 and L2) were included in the analysis. Aim of the analysis is the detection of possible differentiation between red and white grape juices and pomegranate juices as well. In **Figure 27** the scree plot is presented, from which is shown that the three first components (PC1, PC2, PC3) explain the results credibly (96%).

In the loading scatterplots different combinations of the three components are presented, combinations which are necessary in order to ameliorate the presentation of the results. In **Figure 28**, the axes are the PC1 and PC3, while in **Figure 29** the PC2 and PC3.

In green circle are presented the data from the white grape adulteration in L1 and L2 pomegranate matrix, in blue circle the data from the red grape juice adulteration in L1 pomegranate matrix and in res circle the data from the red grape juice adulteration in L2 pomegranate matrix.

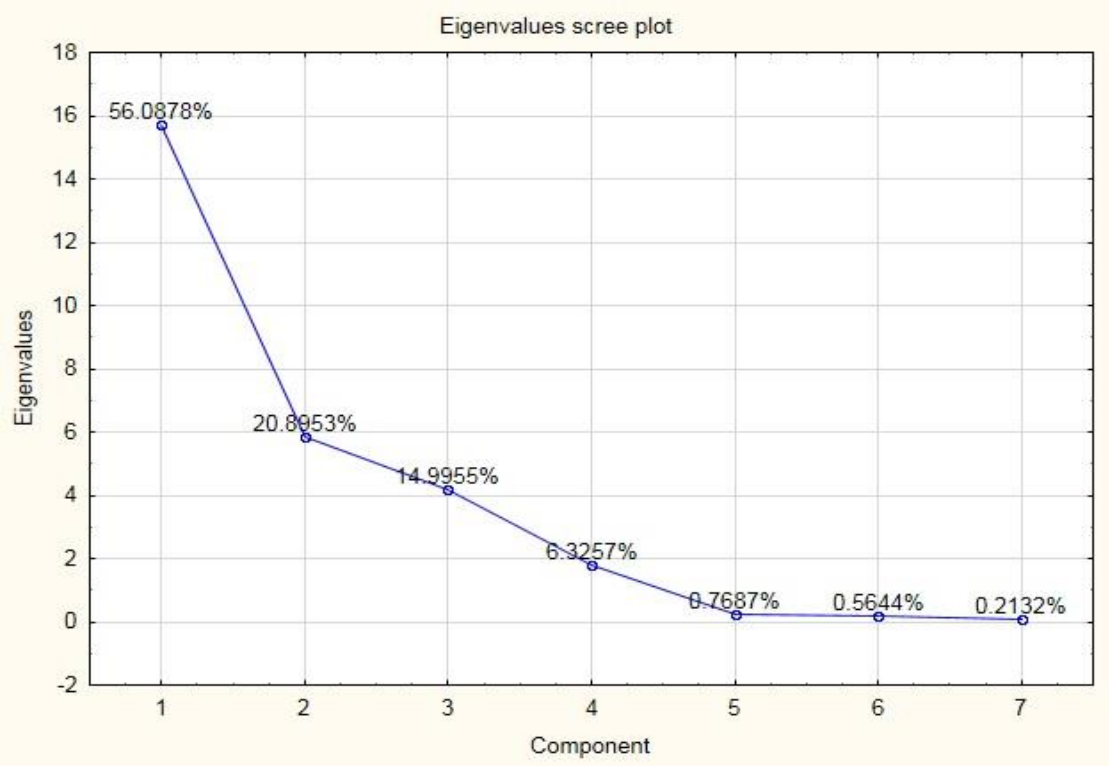


Figure 27: Scree plot of grape and pomegranate juices

From the results taken follow, three different groups of data is observed, which are circled. Thus, we conclude that we can separate the white grape adulteration from the red grape one. Moreover, we can separate the different matrices of pomegranate juice and their ratios of adulteration, but only in the case of red grape adulteration. Finally, the pure matrices are clearly differentiated in grapes, but not in pomegranates, as the codes of the two pomegranates' juices have the similar coordinates with the results of the red grape adulteration (Table 24), not being able to differentiate from them.

In **Table 24** are presented the analytical loadings from the grape analysis.

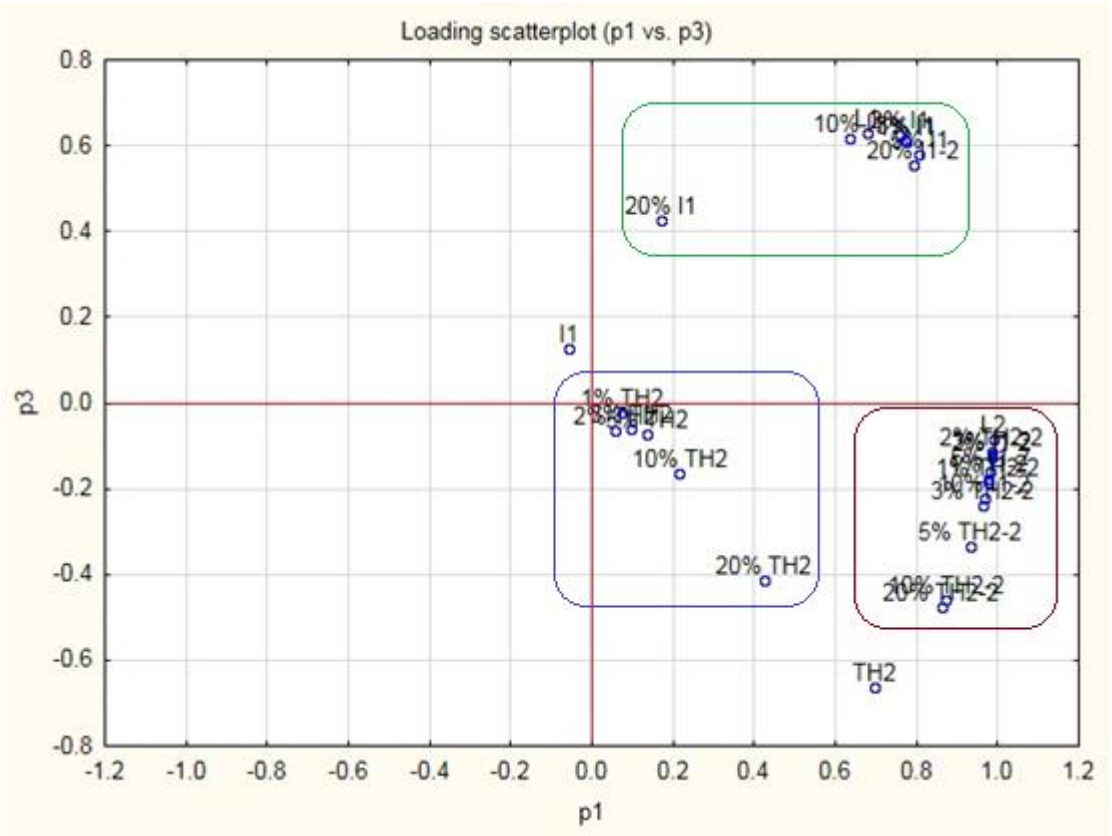


Figure 28: Loadings scatterplot PC1 and PC3 of grape and pomegranate juices

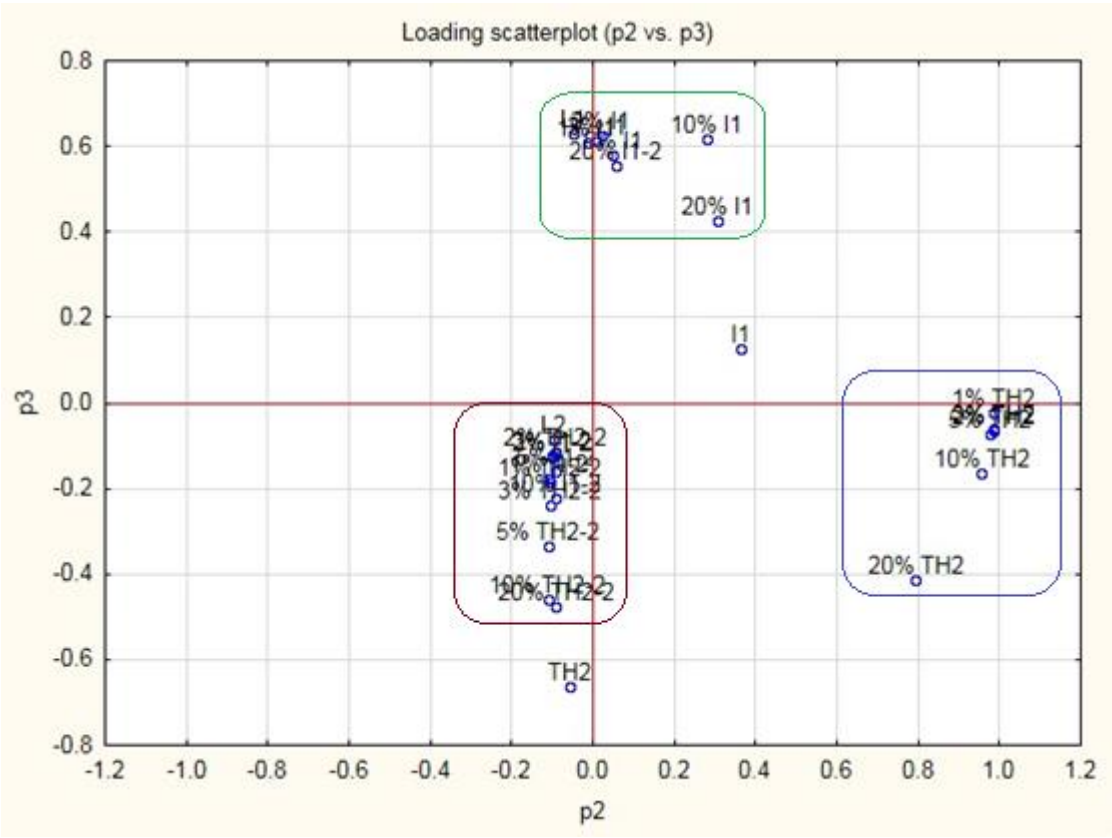


Figure 29: Loadings scatterplot PC2 and PC3 of grape and pomegranate juices

Table 24: Loading spreadsheet of red grape and pomegranate juices analysis

	Variable no	Component 1	Component 2	Component 3
L1	1	0.676981	-0.047583	0.629215
TH2	2	0.696069	-0.055150	-0.661653
I1	3	-0.057899	0.365567	0.124833
20% TH2	4	0.424708	0.794798	-0.415085
10% TH2	5	0.215862	0.955350	-0.163401
5% TH2	6	0.138062	0.975444	-0.073648
3% TH2	7	0.097169	0.983603	-0.058408
2% TH2	8	0.058919	0.986616	-0.062329
1% TH2	9	0.073689	0.987300	-0.023161
20% I1	10	0.170493	0.306818	0.425837
10% I1	11	0.635912	0.281664	0.615598
5% I1	12	0.807799	0.050475	0.579595
3% I1	13	0.773033	0.009783	0.613064
2% I1	14	0.759351	0.020635	0.624808
1% I1	15	0.775881	-0.014688	0.608282
L2	16	0.988297	-0.097795	-0.084477
20% TH2-2	17	0.861581	-0.089964	-0.475993
10% TH2-2	18	0.873209	-0.111287	-0.458830
5% TH2-2	19	0.934084	-0.107959	-0.334090
3% TH2-2	20	0.964655	-0.103820	-0.238708
2% TH2-2	21	0.986173	-0.090299	-0.115470
1% TH2-2	22	0.975444	-0.106389	-0.185919
20% I1-2	23	0.793549	0.059011	0.555146
10% I1-2	24	0.969013	-0.090640	-0.220756
5% I1-2	25	0.981783	-0.091197	-0.159215
3% I1-2	26	0.984204	-0.099343	-0.122228
2% I1-2	27	0.983716	-0.100201	-0.127437
1% I1-2	28	0.976855	-0.107630	-0.174864

5.4.3 PCA in apple and pomegranate juices

The apple juice that used in the adulteration experiments (N1 code) as well as the two codes of pomegranate juice (codes L1 and L2) were included in the analysis. Aim of the analysis is the detection of possible differentiation between the two codes of pomegranate juices and their adulterations. In **Figure 30** the scree plot is presented, from which is shown that the two first components (PC1, PC2) provides a credible analysis (94%) of the data.

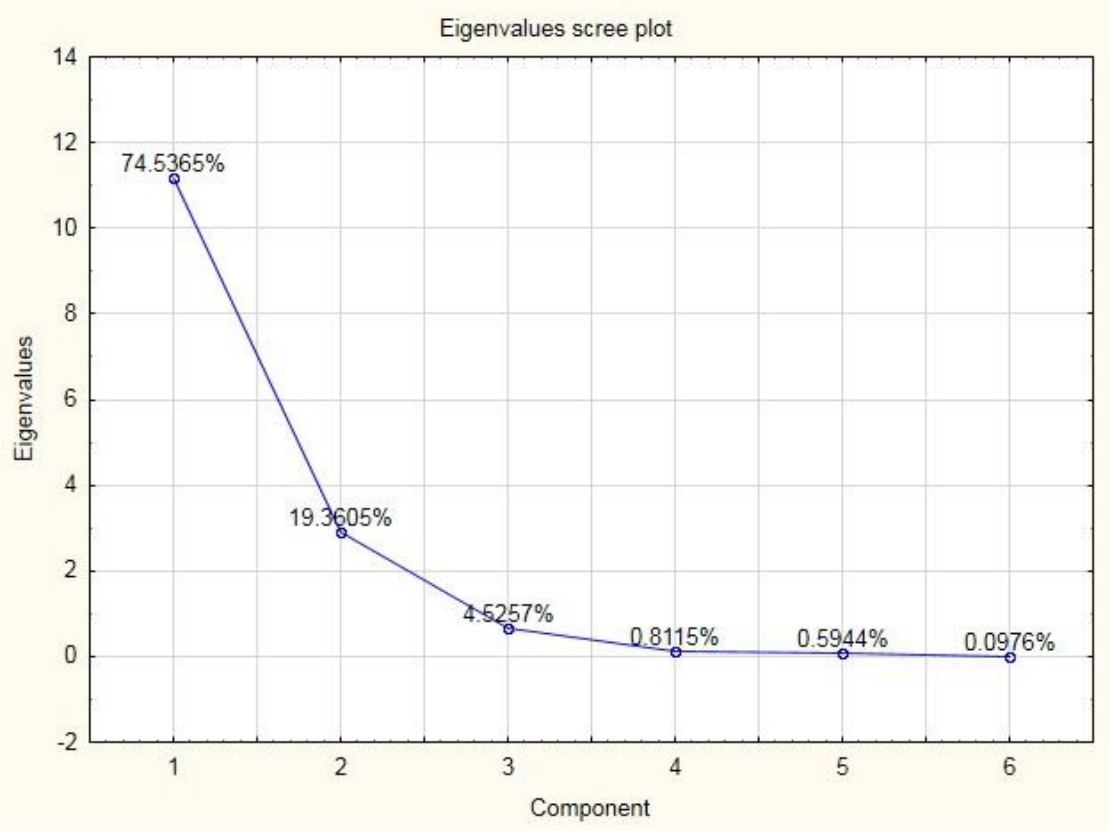


Figure 30: Scree plot of apple and pomegranate juices

From the loadings scatterplot of apple juices (**Figure 31**) we observe a differentiation between the two different pomegranates matrices and their ratios of adulteration. In green circle the apple adulterations in L1 matrix are presented, while in blue circle those in L2 matrix. Finally the pure apple is clearly differentiated, but the pomegranates coincide with the results of the apple adulteration.

In **Table 25** are presented the analytical loadings from the apple analysis.

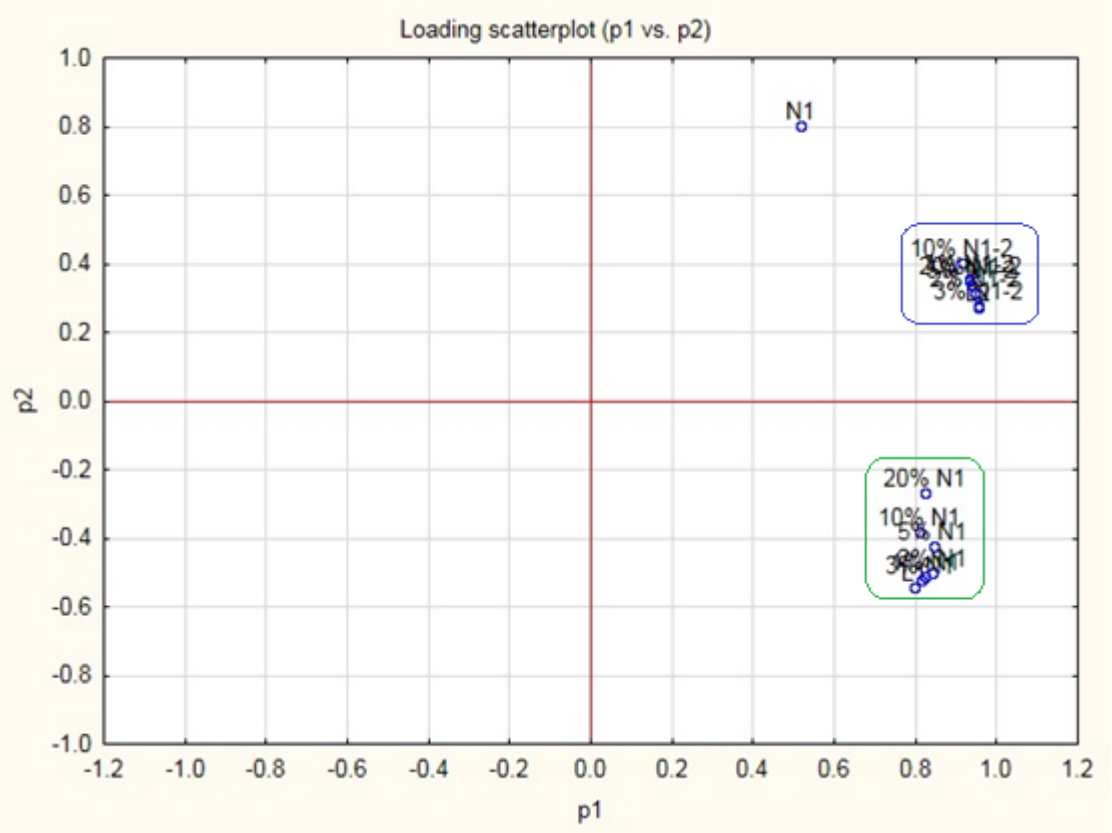


Figure 31: Loadings scatterplot of apple and pomegranate juices

Table 25: Loading spreadsheet of apple and pomegranate juices analysis

	Variable no	Component 1	Component 2
L1	1	0.796796	-0.542871
N1	2	0.519075	0.804037
20% N1	3	0.824023	-0.266641
10% N1	4	0.808881	-0.382648
5% N1	5	0.843704	-0.420995
3% N1	6	0.814483	-0.523188
2% N1	7	0.842649	-0.502061
1% N1	8	0.823367	-0.513510
L2	9	0.956498	0.272427
20% N1-2	10	0.934733	0.349704
10% N1-2	11	0.913955	0.402002
5% N1-2	12	0.938097	0.340164

3% N1-2	13	0.955460	0.278493
2% N1-2	14	0.945444	0.316535
1% N1-2	15	0.932157	0.356484

CHAPTER 6

Conclusions

Fruit juices are often subjected to economically motivated adulteration. One of the most frequent profit-driven fraudulent procedures is the extension of authentic pomegranate juice with cheaper alternatives (typically juices obtained from apples, grapes and others). Aim of this particular thesis was the detection of this kind of fraud and the differentiation of the juices that were added as adulterants based on the antioxidants' pattern of each juice.

The analysis of all pure fruit juices available was the first step of the analysis. In cases of more than one code in each juice (i.e. apple, red and white grape), a variation of the antioxidants was found. Target screening was applied based on some performance criteria, such as mass accuracy, retention time, isotopic pattern and MS/MS information in order to facilitate confidence. Secondarily, adulteration experiments were performed for the identification of the proposed-from-observation markers in percentages of 20, 10, 5, 3, 2 and 1%. Finally, the results received were processed in a PCA model in order to export possible correlations between the samples.

The results taken from the target screening analysis gave some possible markers that can be identified in most cases until 1% percentage of adulteration, results that were verified using the PCA model.

Thus, it came clear that in all cases we can differentiate the pure fruit juices from the adulterated ones.However, the exact percentages of adulteration was not possible to detected, except in somes cases those of 20 and 10%.

There is also a differentiation between the two varieties of pomegranate juices. In other words, we can presume the variety of each pomegranate juice from the results. We also observe, in the loadings scatterplots of grapes, that the freshly-squeezed pomegranate juice has similar coordinates with those of red grape juice and its relative percentages of adulteration, thus, it can be categorised in the same group of results. This obvious differentiation between the two different

codes of pomegranate juice may be due to the different variety of pomegranate at first sight. Another possible explanation may be that the freshly-squeezed pomegranate has a higher amount of Epicatechin, antioxidant- marker which is found in red grape juice that may come from the sqeezing of the pomegranate, as some parts of the internal cortex ended up in the juice. Epicatechin is an antioxidant found mainly in the cortex of fruit, that is the reason why in red grape juice is found in larger amount than in white grape one.

To concude, the results of this study can be used to indicate the presence of adulterants in pomegranate juice. In some cases, also varieties can be discriminated. However, the identification of the exact amount of adulterant added cannot yet be achieved in the majority of the samples. The results are mainly qualitative and give information about the presence or not of adulterant juices in different matrices of pomegranate juice.

In order to quantify the exact percentages of adulteration suspect screening and non-target screening experiments and research have to be conducted.

ABBREVIATIONS – ACRONYMS

AAC	Administrative Assistance and Cooperation System
APCI	Atmospheric pressure chemical ionisation
bbCID	broad-band Collision Induced Dissociation
DAD	Diode Array Detector
DDA	Data Depended Acquisition
DIA	Data Independent Acquisition
EC	European Council
EIC	Extracted Ion Chromatogram
EU	European Union
EMA	Economically Motivated Adulteration
ESI	Electrospray Ionisation
EU	European Union
FDA	Food and Drugs Administration
FFN	Food Fraud Network
FLD	Fluorescence Detector
HACC	Hazard Analysis and Critical Control Points
HE	High Energy
HRMS	High-Resolution Mass Spectrometry
IS	Internal Standard
LC-HRMS	Liquid Chromatography – High-Resolution Mass Spectrometry
LC-MS	Liquid Chromatography – Mass Spectrometry
LE	Low Energy
LOD	Limit of Detection
ME	Matrix Effect
META-PHOR	Metabolomics for Plant, Health and OutReach
MLOD	Method limit of Detection
MLOQ	Method limit of Quantification
MS/MS	Tandem mass spectrometry
MVA	Multi-Variate Analysis
OCR	Official Controls Regulation
OLAF	European Anti-Fraud Office

PC	Principal Component
PCA	Principal Component Analysis
QC	Quality Chart
QqQ	Triple quadrupole
QTOF	Quadrupole-Time-of-flight
RC	Regenerated Cellulose
RP	Reversed-Phase
RT	Retention time
SD	Standard Deviation
TOF	Time-of-flight
UHPLC	Ultra High Performance Liquid Chromatography
US	United States
USC	United States Code

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