UNIVERSITATEA LUCIAN BLAGA DIN SIBIU

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DOCTORAL THESIS

Research on the analysis and authentication of some products from food production

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CONTENTS

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INTRODUCTION

At the international level, even if concerns about the most complete physico-chemical characterization of a product are at an advanced stage, the use of this information for the certification of the quality and naturalness of food products, and in particular of products derived from fruits, is still an area of frontier. The use and combination of several analytical variables – information related to biologically active compounds (phenolic compounds, organic acids, amino acids), sugars, micro and macronutrients, isotopic fingerprint for the characterization of natural products in order to ensure their traceability and authenticity, as the present project proposes , represents a problem of real interest.

The plants are very important because they contain chemicals used in the pharmaceutical, cosmetic, chemical, but also food and agriculture industries. Many plants represent special reserves for obtaining volatile oils, essences, flavors, perfumes, resins, dyes, pesticides, rubber, medicines and other special products.

The studies show that more than 1500 new substances are discovered every year in plants and many of the substances used in the preparation of medicines have plant compounds in their composition. But many plants are disappearing and thus the genetic base of the plant kingdom on the globe is shrinking, which has determined the valorization of the genetic base but also the identification of new sources for obtaining the metabolites of interest.

Primary compounds (proteins, carbohydrates and fats) play a decisive role in the evolution of plants, but in addition to these, there are also secondary products such as: terpenes, steroids,

anthocyanins, anthraquinones, phenols and polyphenols. Secondary products are found in some plants, and can be located in certain organs or tissues, at a certain time of the plant's development or at a certain time of heat stress when the plant is in danger due to the presence of a pest. Obtaining these compounds may not be of vital importance to the cells that synthesize them, but may play a primary role in the growth and viability of the plant as a whole.

The plants remain the main sources of extraction of biologically active principles, because many secondary substances are important from a therapeutic point of view and cannot be obtained by chemical synthesis, because they have special structures containing chiral atoms of primary importance in the development of the plant.

Today's consumer is offered a wide range of both nutritious and non-nutritive food products that have the potential to improve the health status of the population, as well as to prevent or reduce the risk of the occurrence or development of certain conditions. In this context, the diversified diet can be of major importance, for the development of the functions of the human body within the normal parameters, a special role belonging to food of plant nature, such as fruits. Fruits are an important component in the human diet. Both fruits and vegetables are used in a balanced diet, and their chemical composition in terms of bioactive substances is very similar. The importance of fruits and vegetables has been recognized since the beginning by the first people who engaged in agriculture.

Recently, a lot of attention has been paid to functional foods from traditional raw materials, and at the same time, the identification of relevant markers that attest to the traceability and authenticity of these products is emphasized, their identification being possible through UHPLC-MS/MS methods (Geană et al. 2020).

In order to strengthen the knowledge about the chemical and nutritional composition of some foods (honey, wine and chestnuts) and to find distinctive characteristics useful for their authentication offering an important economic advantage, the biologically active compounds (total polyphenols, total flavonoids and antioxidant activity) were followed) examined by UV-Vis spectrophotometric methods, the profile of phenolic compounds UHPLC-MS/MS (phenolic acids, flavonoids), together with a non-targeted UHPLC-MS/MS, the screening profile, the carbohydrate profile (sucrose, fructose, glucose, maltose) by HPLC-ELSD and composition in mineral elements (Ca, Mg, Na, K, Fe, Pb, Ni, Cu, Cr) by F-AAS in the studied chestnut varieties (Ciucure et al. 2022).

PURPOSE AND SCIENTIFIC OBJECTIVES OF THE DOCTORAL THESIS

The doctoral thesis "Research on the analysis and authentication of some products from food production" aims to identify and establish markers of origin through the development and implementation of innovative fingerprinting methodologies with direct application in the investigation of traditional Romanian foods. The main object of study is natural products, with a particular emphasis on honey, wine and chestnuts, with the aim of obtaining reference data regarding their compositional, isotopic and protein profile, which in combination with multivariate statistical analysis techniques can provide valuable information with regarding the quality and authenticity of the products.

As main objectives, the thesis pursues:

♦ Establishing the most used analytical methods for authenticating the botanical and geographical origin of honey and identifying adulteration in order to identify the most effective method for distinguishing each possible fraud, especially advanced instrumental techniques, including spectrometric, spectroscopic and chromatographic methods coupled with chemometric interpretation of the data.

♦ Determination of the composition of individual phenolic compounds in bee honey from the Romanian flora and the use of analytical data and multivariate statistical analysis for the differentiation of bee honey according to botanical origin.

♦ Complete determination of general physico-chemical properties (water content, °Brix, electrical conductivity, free acidity, pH and 5-hydroxymethylfurfural (HMF) content) of different types of commercial honey or from local distributors.

♦ Characterization of bee honeys based on the major sugar composition (fructose, glucose, sucrose and maltose) and evaluation of the possibility of their differentiation.

♦ Verification of the authenticity of commercial bee honeys of different botanical origins (acacia, polyflora, honey, sunflower, rapeseed and linden), available on the market, based on the stable isotope method ($\delta^{13}C$) and evaluating the possibility of detecting the addition of sugar syrups from C4 plants (eg cane and maize) and confirmation of botanical origin.

♦ Investigation of the bioactive properties (total phenolic content, total flavonoids and antioxidant activity) of different Romanian red and white wine varieties with different aging periods, using UV-Vis quantitative spectrophotometric methods.

♦ Investigation of the bioactive characteristics (total polyphenols, total flavonoids and antioxidant activity) of six varieties of sweet chestnut.

♦ Investigating the individual polyphenolic profile (phenolic acids, flavonoids) along with the non-targeted UHPLC-MS/MS screening profile of the analyzed chestnut cultivars to find distinctive markers useful for authenticating a particular chestnut cultivar.

♦ Investigation of the sugar profile (sucrose, fructose, glucose, maltose) by HPLC-ELSD and the elemental composition (Ca, Mg, Na, K, Fe, Pb, Ni, Cu, Cr) by F-AAS were determined in the studied chestnut varieties to complete the information on the nutritional and bioactive composition of the six varieties.

♦ Study of the influence of the harvest year on the bioactive characteristics and the content of specific bioactive compounds in different chestnut varieties.

♦ Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used to distinguish between the different sweet cultivars grown in Romania.

♦ The use of stable isotopes as markers of origin in the study of the quality and authenticity of bee honey and chestnut fruits and the multivariate statistical analysis applied to the resulting parameters.

♦ The establishment of new and precise techniques for the compositional characterization of essential bee honey due to their nutritional and therapeutic qualities, through the development of an innovative analytical method that will allow the objective verification of the quality, authenticity and traceability of food products.

The novel elements of the doctoral thesis entitled "Research on the analysis and authentication of some products from food production" consist in the approach through advanced scientific methods of the physico-chemical composition of food products (honey, wine and chestnuts), methods that lead to establishing their authenticity and the possibility of identifying falsifications in the field. Also, the novelty of the thesis consists in the development of a method for the identification and quantification of phenolic compounds (UHPLC-MS/MS), establishing appropriate parameters for the method (recovery, precision, linearity and validation).

1. Current methodologies of authentication and control of food products

In the current global production and marketing, authentication of food is an important issue to ensure the quality of food (Aung and Chang 2014). Prevention of fraud in food sector and

promotion of authentic product is an essential element to ensure the commercial success of high-value agri-food products on the domestic and international markets. Fraudulent practices such as replacing the original products constituents with cheaper substituents will have a negative impact on consumer confidence and the competitiveness and profitability of honest producers. With the authenticity of food more and more worrying, the members of the European Parliament introduced honey in the list of products who are most exposed to the risk of food fraud, in most cases by the addition of sugars, as well as the false declaration of botanical or geographical origin. Therefore, perfect traceability is crucial for honey to ensure a fair and sustainable apiculture sector. European Commission encourages the use of analytical methods to determine the authenticity and quality of honey, both researchers and Regulatory Authorities are looking for newer, simpler, more sensitive and more economical procedures.

In this respect, European Commission with the scientific support of the JRC-Institute for Reference Materials and Measurements (JRC-IRMM), has recently organized a fraud detection control plan on EU markets (European Commission 2015), revealing that 19% of the tested honey (from almost 2200 analyzed) did not meet standard criteria for honey. The main identified nonconformities were: incompatible processing methods or inadequate storage conditions, identified based on physicochemical investigations (2%); false declaration of botanical (7%) and geographical (2%) origins, identified on the basis of pollen analysis; sugar adulteration based on exogenous sugar addition (6%); other labeling aspects (2%). 40% of the investigated honey samples, honeys that proved to be in compliance with the preliminary tests performed, were subjected to JRC-IRMM for EA/LC-IRMS analysis to detect the possible addition of certain sugar syrups to honey. The results of the coordinated control plan presented in the JRC's final report indicate that 14% of the checked honey samples do not meet the purity criteria, indicating that exogenous sugars may have been added (European Commission 2016).

According to the European Union Council Directive 2001/110/EC (Council Directive 2001/110/EC 2001) and FAO/WHO Codex Alimentarius (Codex Stan 12-1981 1981), honey is defined as a natural sweet product of *Apis mellifera* bees resulted from the nectar of plants or plant excretions, which the bees collect, combines them with their own specific substances and deposit in the honeycomb for maturation. By origin of provenance, honey is classified as: unifloral (rape, acacia, linden, sunflower, etc.) or polyfloral (coming from the nectar of several types of flowers)

honeys and forest honey or honeydew honey (which mainly comes from the secretion of other parts of plants, in combination with forest flora (Soares et al. 2017).

From chemical point of view, honey is a concentrated solution of carbohydrates (about 75% monosaccharides (fructose and glucose), 10-15% disaccharides (sucrose and maltose) and other oligo- and polysaccharides) which contains many bioactive compounds such as phenolic components (phenolic acids and flavonoids), organic acids (gluconic, oxalic, malic, lactic, ascorbic, maleic, citric, succinic, propionic, formic, fumaric, etc.), volatile compounds (monoterpenes, sesquiterpenes, benzene derivatives, superior alcohols, esters, aldehydes, ketones, fatty acids, etc.), vitamins (thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid, (B5), pyridoxine (B6), biotin (B7) , folic acid (B9), cyanocobalamin (B12), vitamin C), proteins (between 0.1% and 0.5% in honey) and amino acids (1% of honey constituents – proline, glutamine, histidine, glycine, threonine, alanine, arginine, tyrosine, valine, methionine, cysteine, isoleucine, tryptophan, ornithine, lysine, serine and glutamic, aspartic, amino butyric acids, etc.), minerals (K, Mg, Ca, Fe, P, Na, Mn, Li, Co, Ni, Cu, Cr, Ba, Se, etc.), pigments, waxes, pollen grains, enzymes (invertase, phosphatase, glucose oxidase) and other phytochemicals. Honey composition is affected by various factors including bee species, the plant from which the nectar was collected, specific for each season, geographical area, harvesting process and storage conditions (Machado De-Melo et al. 2018; da Silva et al. 2013).

Besides flavor and nutritional values, one of the most valuable honey quality is the therapeutic potential, honey being used from the oldest times for treating various human diseases and also for the promotion of a healthy life style through honey consumption (Bogdanov et al. 2008). Thus, honey used for this purpose should contain different bioactive compounds (phenolic compounds, organic acids, volatile compounds, vitamins, amino acids, etc.) with antioxidant potential and health-promoting capacities like heart protection, reducing cancer and immune system decrease, and control of various inflammatory processes (Khan et al. 2018). Unfortunately, similar to other high quality nutritional and medical food products, with a quite high price, honey is often subjected to direct or indirect adulteration with inexpensive sugar syrups leading to deterioration of bioactive constituent fingerprints (Soares et al. 2017).

For the detection of direct incorporation of foreign substances (sugar syrups) to honey or indirect adulteration of honey (bee-feeding with industrial sugars), different targeted or untargeted methods have been proposed (Ulberth, 2016). These approaches include the determination of

specific characteristics that are important in assessing the quality of honey and, implicitly, its authenticity (pollen analysis, organoleptic analysis, moisture, electrical conductivity, free acidity, diastase and invertase activities, proline content, etc.) (GULER et al. 2007), carbohydrate profiles (Cordella et al. 2005; Morales, Corzo, and Sanz 2008; Wang et al. 2015), NMR fingerprint (Bertelli et al. 2010; Spiteri et al. 2015), stable isotope ratios (Simsek, Bilsel, and Goren 2012; Tosun 2013). Some researchers emphasized that honey minority constituents responsible for its therapeutic potential, have been used mainly to characterize the honey floral source, allowing a clear discrimination (Dong, Zheng, and Xu 2011; Stanimirova et al. 2010). In another way, identification of marker compound for each monofloral honey represent an important issue for authentication of some valuable honeys, which are subject to adulteration by mixing with honey from other botanical sources, or false declaration of geographical provenance (Pita-Calvo and Vázquez 2018; Zhou et al. 2014)

Multivariate statistical evaluation of the analytical data is absolutely necessary in order to develop reliable methodologies that will be used for honey authenticity control. Statistical instruments such, analysis of variance (ANOVA), principal component analysis (PCA), cluster analysis (CA), principal component regression (PCR), stepwise discriminant analysis (SDA), partial least squares-linear discriminant analysis (PLS-LDA), partial least squares regression (PLSR), multiple linear regression (MLR), adaptive neuro-fuzzy inference system (ANFIS), least significant difference test (LSD) and artificial neural networks (ANN), were used for honey authenticity assessments (Amiry, Esmaiili, and Alizadeh 2017; Li et al. 2017; Oroian, Ropciuc, and Paduret 2018).

The aim of this work was to present a review of the analytical methods concerning the authentication of honey botanical and geographical origins and identification of adulteration in order to identify the most efficient method for each possible fraud. Characterization and classification studies of various botanical Romanian honeys were highlighted in the honey authenticity context and further investigations that would identify the best anti-fraud method for detection and elimination of prohibited practices in honey production process were suggested.

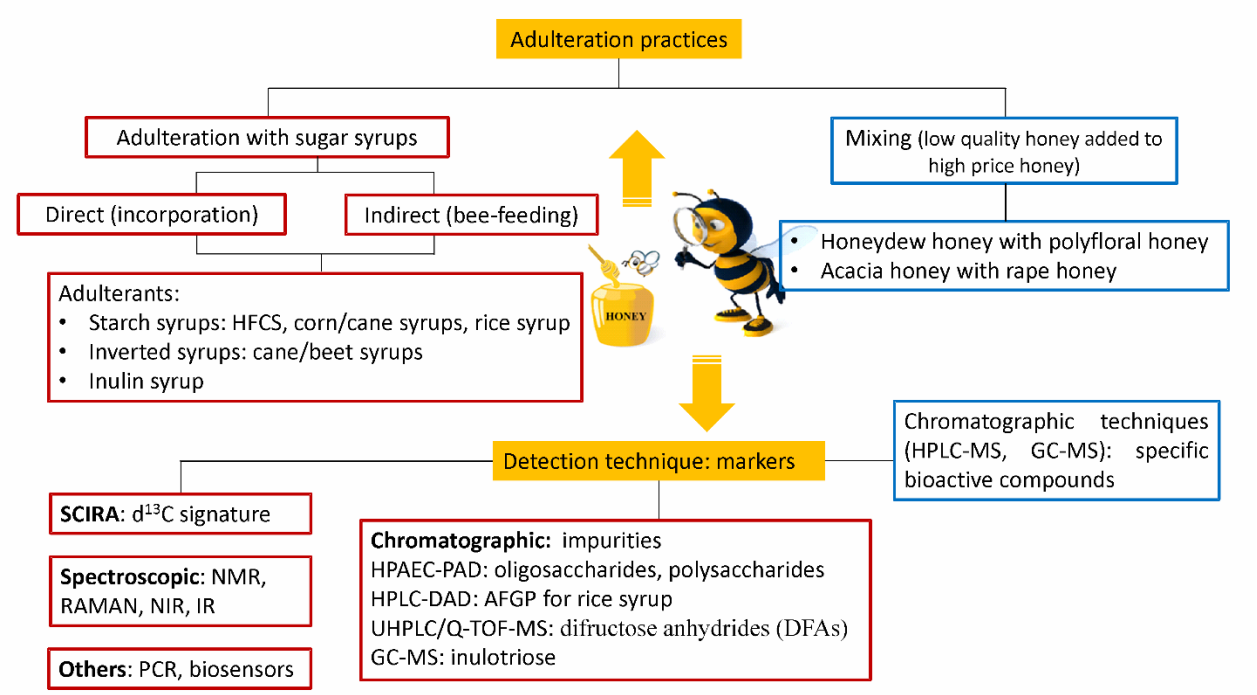

Figure 1. Types of honey adulteration, typical adulterants found in honey, detection techniques and specific markers for each type of adulteration.

2. Phenolic compounds profile and biochemical properties of honeys in relationship to the honey floral sources

Honey is produced and processed by honey bees (Apis mellifera) from the nectar and honeydew of plants. Thus, honey can be considered a natural product which contains predominantly a complex mixture of carbohydrates and small amounts of other constituents, including minerals, proteins, vitamins, organic acids, phenolic compounds, enzymes and other phytochemicals (Bertoncelj et al. 2011; Machado De-Melo et al. 2018). Phenolic compounds, mainly phenolic acids and flavonoids have been recognized as the major constituents responsible for health-promoting properties of honey, including antimicrobial, anti-inflammatory, antimutagenic, antitumor, antiviral, antioxidative activity, and many other effects on human health (Khan et al. 2018) The therapeutic potential of honey is associated with antioxidant capacity against free oxygen radicals, so honey is well known as a natural dietary antioxidant (Meo et al. 2017).

Identification and quantification of phenolic compounds in honey are of great interest as they make a significant contribution to the honey total bioactivity, their concentration reflecting the

quality of honey and being responsible for its colour, sensory features and antioxidant activity (Ciulu et al. 2016). There is an abundant literature regarding to the evaluation of antioxidant capabilities of unifloral honeys worldwide (Moniruzzaman et al. 2012; Petretto, Cossu, and Alamanni 2015). These contributions also describe the correlation with some spectrophotometric parameters like the total polyphenolic and total flavonoid, the colour and chromatographic phenolic profile (Can et al. 2015; Mărghitaş et al. 2009)

In the recent years, numerous studies have investigated the phenolic acid and flavonoid fingerprints of different types of honey to identify specific compounds that can be used as floral markers to discriminate the floral origin of honey (Consonni and Cagliani 2015; Gašić et al. 2015; Kaškonienė and Venskutonis 2010). Identification of these phenolic compounds appears to be one of the most promising techniques for determination of honey floral origin because these phytochemicals are dependent mainly, on the floral origin of melliferous plants (Bertoncelj et al. 2011), besides the origin from the propolis (Kečkeš et al. 2013; da Silva et al. 2013). Association of honey phenolic profile with pollen analysis and other physico-chemical analysis is practiced (Can et al. 2015; da Silva et al. 2013). Ellagic acid and pinocembrin were identified as floral markers for polyfloral Belgian honeys (Jasicka-Misiak et al. 2012), while kaempferol, morin and ferulic acid were used as floral markers to distinguish Chinese rape honey (Zhou et al. 2014). Bertoncelj el al. (Bertoncelj et al. 2011) and Oroian et al. (Oroian and Ropciuc 2017) did not show any specific compounds to be used as markers for determination of the floral origins of different types of Slovenian and Romanian honeys.

The determination of a phenolic profile of honey is a complex approach, so it is essential to develop separation and detection techniques which would enable an unambiguous determination of numerous compounds. The analytical procedures used to determine polyphenols in honey involve their extraction from honey matrix, and their chromatographic separation followed by quantification (Pascual-Maté et al. 2018). Numerous studies of honey phenolic acids and flavonoids have been focused on the extraction of the phenolic compounds from honey using the Amberlite XAD-2 resin (Mattonai et al. 2016; Zhao et al. 2016), solid-phase extraction (SPE) procedures with commercial cartridges (Bond Elut octadecyl C18, Sep-Pak RP C18, Oasis HLB and Strata-X) (Moniruzzaman et al. 2012; Sergiel, Pohl, and Biesaga 2014; Zhou et al. 2014) or liquid–liquid extraction methods (Karabagias et al. 2014; Kıvrak and Kıvrak 2017) prior to their identification and quantification. Liquid chromatography (LC) is considered to be the most useful

separation technique for the analysis of polyphenols in honey, including HPLC-DAD (Campone et al. 2014; Jasicka-Misiak et al. 2012; Oroian and Ropciuc 2017) and HPLC-MS for quantitative measurements (Gašić et al. 2014; Kečkeš et al. 2013; Zhou et al. 2014).

For the statistical modelling of the analytical data, chemometric techniques have been widely accepted as the most powerful tools to characterize and classify honey according to floral origins, of which principal component analysis (PCA) (Gašić et al. 2015; Kečkeš et al. 2013), partial least squares-discrimination analysis (PLSDA) (Gašić et al. 2014), linear discriminant analysis (LDA) (Bertoncelj et al. 2011) and hierarchical clustering analysis (HCA) (Shen et al. 2018) are commonly employed.

The European Commission has recommended setting up databases with reliable data of the characteristics for different types of honey (European Commission 2015). Accordingly, the purpose of this research was to study the phenolic compounds profile (phenolic acids and flavonoids) and bioactive properties (total phenolic content (TP), total flavonoids content (TF) and the DPPH radical-scavenging activity) of pure unifloral (acacia and rape), polyfloral, honeydew and mixed Romanian honeys in relation with the floral plants visited by the bees, so that it can contribute to honeys authentication. The possibility of verifying the floral origin of mixture honeys based on specific phenolic compounds, using multivariate statistical methods, was examined. The variables discriminating different pure honey samples were identified and successful models for further prediction were developed.

In this study, the quantification of phenolic acids and flavonoids in honey was performed by the UHPLC-DAD-ESI/MS technique after a preliminary step of isolating the compounds of interest from the honey matrix. A total of 32 compounds resulting from pollen, propolis and flower nectar were identified in honey samples and 24 of them were quantified by comparing retention times and MS spectra with available standards.

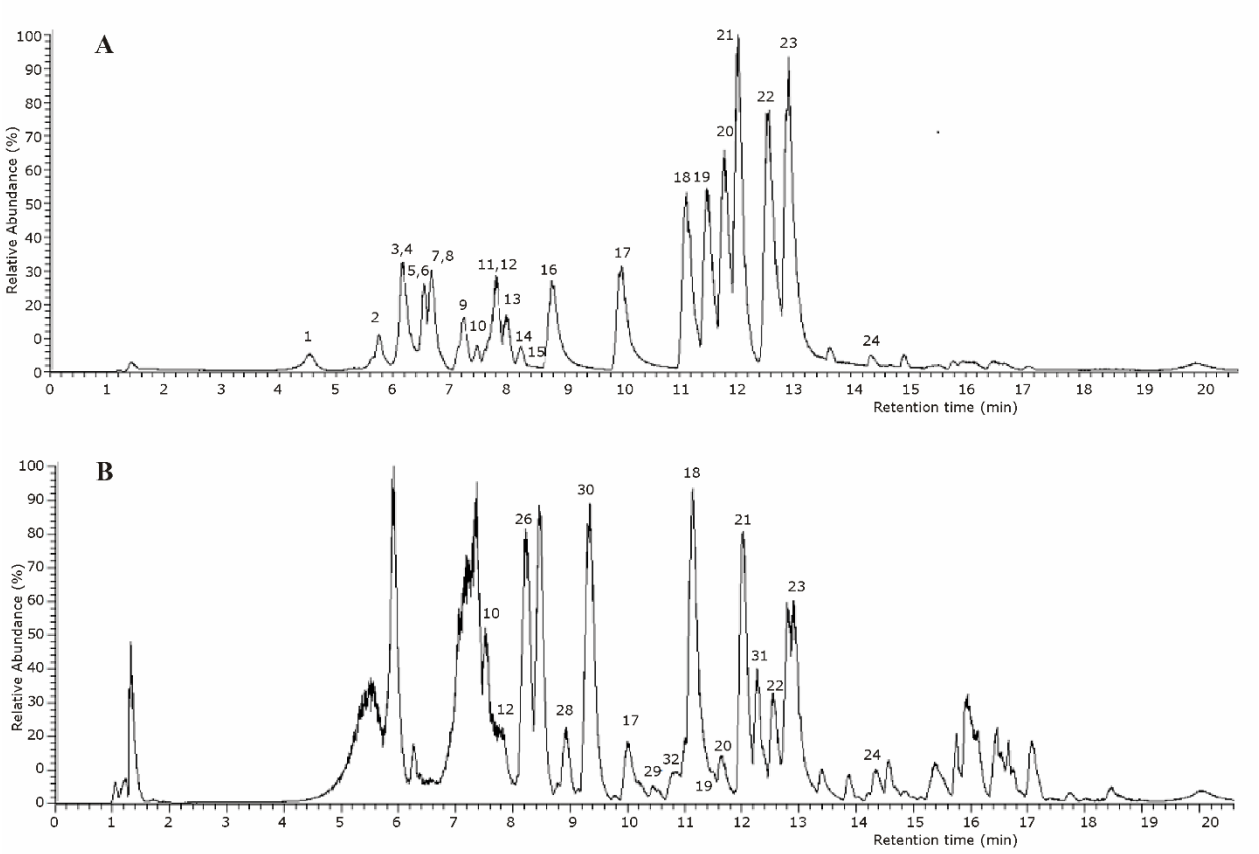

Figure 2: Base peak chromatogram of phenolic compounds standards solution (A) and rape honey aqueous extract (B): 1, gallic acid; 2, 3,4-dihydroxybenzoic acid; 3, catechin; 4, 4-hydroxybenzoic acid; 5, chlorogenic acid; 6, epicatechin; 7, caffeic acid; 8, syringic acid; 9, p-coumaric acid; 10, ferulic acid; 11, naringin; 12, rutin; 13, hesperitin; 14, trans-resveratrol; 15, trans-cinnamic acid; 16, myricetin; 17, quercetin; 18, kaempferol; 19, isorhamnetin; 20, apigenin; 21, pinocembrin; 22, galangin; 23, chrysin; 24, pinostrobin; 25, rhamnetin; 26, abscisic acid; 27, eriodictyol; 28, sakuranetin; 29, alpinetin; 30, pinobanksin; 31, pinobanskin-3-O-acetate, 32-luteolin

Of the 24 target compounds, only 17 were identified and quantified in all studied honey samples, 8 phenolic acids (3,4-dihydroxybenzoic, *p*-hydroxybenzoic, chlorogenic, caffeic, syringic, p-coumaric, ferulic and *t*-cinnamic acids) and 9 flavonoids (rutin, quercetin, kaempferol, isorhamnetin, apigenin, pinocembrin, galangin, chrysin, pinostrobin), while, catechin and epicatechin were found in negligible amount in the honeydew, polyfloral and rape honeys and gallic acid was found in negligible amount in sunflower and honeydew honeys. Naringin, hesperitin, myricetin and *t*-resveratrol have not been identified.

In the absence of standards, identification of another compounds in the honey extract was based on the search for the deprotonated molecule, [M–H] and the specific literature (Biesaga and

Pyrzynska 2009; Gašić et al. 2015). The exact mass search using ChemSpider reference library enabled us to identify rhamnetin, abscisic acid, luteolin, pinobanskin and pinobanskin-3-O-acetate in all studied honeys. Eriodictyol was identified only in rape honeys, while sakuranetin and alpinetin were identified in acacia and rape honeys.

Among numerous antioxidant constituents, phenolic compounds could be identified as components that account for the total reducing antioxidant capacity of investigated honey samples. Honeydew honeys showed higher total phenolic content, total flavonoid content and DPPH reducing antioxidant capacity mean values when compared to the other floral honeys.

The average TP and TF values were slightly higher than those reported for Romanian acacia, rape and honeydew honeys (Bobis et al. 2008; Dobre et al. 2014; Mărghitaş et al. 2009), Serbian polyfloral honeys (Gašić et al. 2014), Malaysian acacia honeys (Chua et al. 2013) and polyfloral, honeydew and acacia honeys from Burkina Faso (Meda et al. 2005). Even if the investigated honey samples are old samples, the bioactive properties are within the natural range of variation, storage condition affecting only to a minor degree the honey therapeutic potential (Chua et al. 2013).

Tabelul 1. Correlation matrix between honey phenolic compound profile and bioactive properties

	∑ Phenolic acids	∑ Flavonoids	TP	TF	DPPH
∑ Phenolic acids	**1.0000**				
∑ Flavonoids	-0.3081	**1.0000**			
TP	0.3082	-0.2845	**1.0000**		
TF	0.5616	0.1430	0.7512	**1.0000**	
DPPH	0.4943	-0.1691	0.9093	0.9150	**1.0000**

PCA analysis allows the reduction of the data dimension, showing the clustering into two main groups, coloured honeys (honeydew and polyfloral) and less coloured or uncoloured honeys (acacia and rape) (Fig. 3A). The clusters of acacia and rape honey samples partly overlap, but a certain separation can be observed here as well. Mixture honeys with nectar and/or pollen from other floral sources can be distinguished from pure acacia or rape honeys, less for AR, AT and RFT honeys, in which, probably, the percent of impurities in rape honey is very low. A clearly differentiation of RS and RA honeys from rape honeys was achieved, indicating the presence of

additional phenolic compounds amounts into the contaminated rape honeys compared with pure rape honeys.

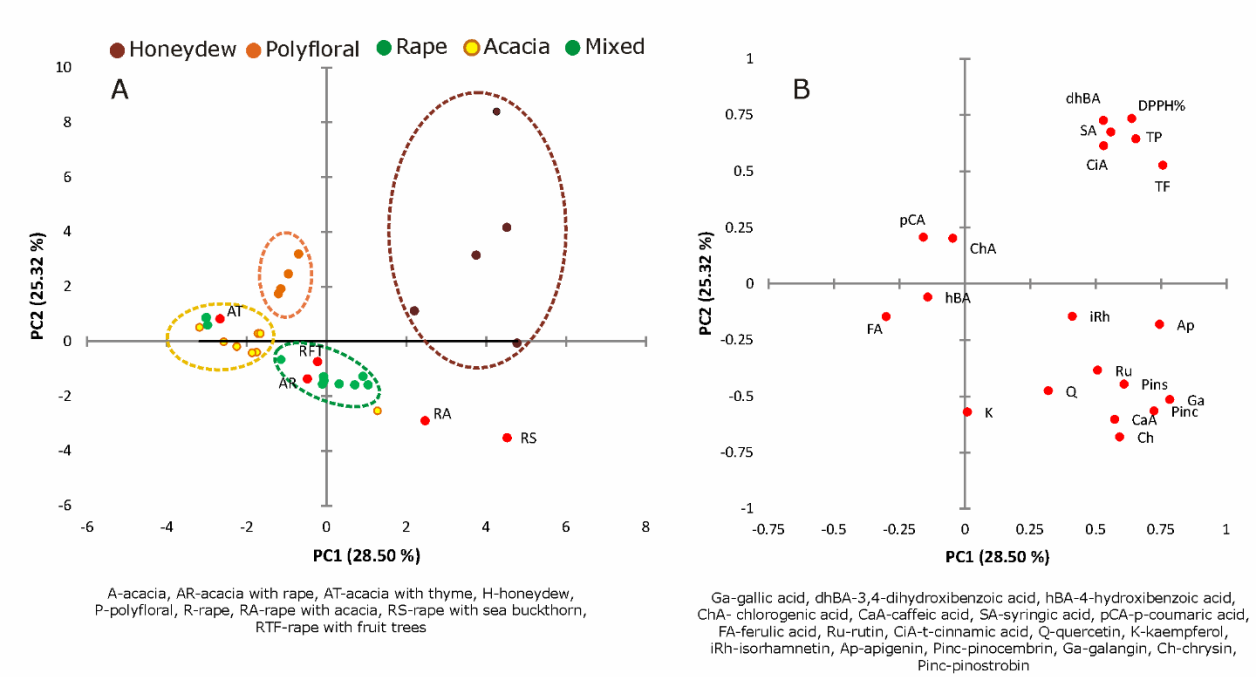

Figure 3: (A) Principal component analysis (PCA) scores plot showing separation between honey types; (B) distribution of variables generated from a correlation-matrix PCA

Our results showed that kaempferol, quercetin, isorhamnetin, rutin and apigenin could be suggested as floral markers for rape honey, while ferulic and p-hydroxybenzoic acids represent floral markers of acacia honey, p-coumaric and chlorogenic acids characterise polyfloral honey, while 3,4-dihydroxybenzoic, syringic and trans-cinnamic acids together with TP, TF and DPPH% bioactive properties are representative for honeydew honey (Figure 3B).

Particularly, cafeic acid and flavonoids like chrysin, galangin, pinocembrin and pinostrobin are considered as significant components that can be used to discriminate rape honey contaminated with nectar and pollen from other floral sources. Relevant results for honey floral origin discrimination were achieved for rape honeys contaminated with nectar and pollen coming from other floral sources like sea buckthorns.

3. Evaluation of honey in terms of quality and authenticity based on the general physicochemical pattern, major sugar composition and δ13C signature

Honey is defined as the natural sweet substance produced by honey bees (*Apis mellifera*), from the nectars and exudation of plants (Codex Stan 12-1981 1981) which possesses multiple therapeutic properties, such as antibacterial, prebiotic, antioxidant and antimutagenic (Meo et al. 2017). This natural product is an aqueous supersaturated sugar solution, mainly composed of fructose and glucose and other minor constituents, such as organic acids, amino acids, proteins, minerals, vitamins, lipids, aroma compounds, flavonoids, vitamins, pigments, waxes, pollen grains, several enzymes and other phytochemicals (Amiry, Esmaiili, & Alizadeh, 2017; de Almeida-Muradian et al., 2013; URAN, AKSU, & DÜLGER ALTINER, 2017). Properties and compositions of honey depend on the type of flowers from which bees collect the nectar, geographical origin and climatic conditions as well as the beekeeping practices, honey maturity, processing and storage conditions (de Almeida et al. 2016a; Kukurova et al. 2008; El Sohaimy, Masry, and Shehata 2015).

In addition to the natural valuable properties, honey is used as sweetener in a large number of processed food products, but its market value is significantly higher than other commonly utilized sweeteners, such as refined sugar syrups from corn, sugar cane, sugar beet and syrups of natural origin (rice, fruits, grapes) (Amiry et al. 2017). Therefore, for economic gains, there is the temptation to adulterate honey by dilution with cheap industrial sugar syrups (which simulate the honey carbohydrate profile), thus negatively affecting not only the consumers' nutrition and health, but also the honest beekeepers due to the impact of this fraud on the economy and market (M. F. Cengiz, Durak, & Ozturk, 2014). Thus, honey adulteration is an economically motivated adulteration for financial gain. In a top ten food products that are most at risk of fraud, honey reach the sixth position, highlighting that honey is highly vulnerable to food fraud. Beside the adulteration of honey with sugar syrups, tracing the botanical and geographical origins of honey represent important authenticity issues (Jandrić et al. 2017; Karabagias et al. 2018). Today, guaranteeing the authenticity and quality of honey has become a very imperative issue for the international honey market (processors, retailer and beekeepers), regulatory authorities and consumers and therefore establishing a comprehensive analytical procedure for detecting honey adulteration is a necessity (Çinar, Ekşi, and Coşkun 2014; Sobrino-Gregorio et al. 2017).

The EU regulation provides general quality criteria that the honey must meet, such as organoleptic characteristics (aspect, color, taste, consistency, flavour and aroma) and

physicochemical compositional parameters (sugars content, moisture content, mineral substances, acidity, vitamins, anorganic acids, proteins, proline, enzyme activity, electrical conductivity, hydroxymethylfurfural (HMF) content) (Council Directive 2001/110/EC 2001).

In recent years, several efforts have been made to address authenticity, traceability and intrinsic quality of honeys with the application of multivariate statistical techniques for processing different type of analytical data. Summarizing the researches in this direction, detecting honey adulteration in terms of sugar syrups addition is not easy to achieve. In this regard, different analytical techniques have been exploited including chromatography (TLC, thin-layer chromatography; HPLC, high- performance liquid chromatography; GC, gas chromatography; HPAEC, high-performance anion exchange chromatography) (Cordella et al. 2005; Puscas, Hosu, and Cimpoiu 2013; Ruiz-Matute et al. 2010), nuclear magnetic resonance (NMR) (Bertelli et al. 2010), isotopic ratios mass spectrometry (IRMS) (Simsek et al. 2012; Tosun 2013; Vetrova et al. 2017).

SCIRA (Stable Carbon Isotope Ratios Analysis) represent the standardised method capable to detect the adulteration of honey with cane or corn sugar syrups and is based on the fact that plants have distinct carbon isotope ratios that are produced by different photosynthesis cycles. There is a general agreement that plants with the Calvine Benson photosynthetic cycle (C3 plants: beet, wheat, grape, fruits, melliferous plants) have $\delta^{13}C$ values from -22‰ to -32‰, and plants with the Hatch-Slack photosynthetic cycle (C4 plants: corn, cane) have values from -9‰ to -18‰ (Vetrova et al. 2017). These differences in the isotope ratios are used to detect honey adulterated with sugar syrups that originate from C4 plants. As bees mainly produce honey from C3 plants, honey samples having $\delta^{13}C$ values smaller than -23.5‰ could be suspicious (Simsek et al. 2012). In order to establish the degree of adulteration with C4 sugar syrups it is necessary to determine the $\delta^{13}C$ value of proteins extracted from honey. Even though $\delta^{13}C$ value of honey fraction changes with the addition of sugar, the $\delta^{13}C$ value of protein fraction will not be affected and the difference between these two fractions will increase. The minimum difference in $\delta^{13}C$ between honey and its associated protein extract is expected to be -1.0‰, which correspond to 7% sugar added (Cengiz et al. 2014; Çinar et al. 2014). Instead, $\delta^{13}C$ as single parameter is not suitable to detect sugar beet addition to honey, since beet and melliferous plants belong to the same plants family (C3 plants), and therefore, the maximum $\delta^{13}C$ value in honey is anticipated to be -23.5‰ (Çinar et al. 2014).

These sophisticated analytical tools are time-consuming and expensive, being used to detect honey adulteration only in renowned laboratories. Several accessible methods are available, although these methods require systematization and validation as an important parts of authenticity assessments (de Almeida et al. 2016b). In this regard, the honey chemical characteristics, in conjunction with multivariate statistical analysis, have been found to be able to classify honeys according to geographical and botanical origins and possible adulteration. Physicochemical parameters such as moisture, ash, pH, total acidity, electrical conductivity, diastase number, proline content, sum of fructose and glucose, fructose/glucose ratio, HMF content are taken into account to determine the authenticity and quality of honey (Amiry et al., 2017; GULER, BAKAN, NISBET, & YAVUZ, 2007; KIVRAK et al., 2016; Popek, Halagarda, & Kursa, 2017; URAN et al., 2017). The new tendency in honey authentication is focused on the development of alternative analytical methods such as spectroscopic methods (Near-infrared spectroscopy (NIR), Mid-infrared Spectroscopy (MIR) and RAMAN) (Li et al. 2017; Oroian et al. 2018; Rios-Corripio, Rojas-López*, and Delgado-Macuil 2012), that allow a rapid screening, reducing the time and financial restrictions. Moreover, in order to get conclusive results for one sample it would be necessary to use the results obtained by applying the combination of several of these techniques.

The European Commission (European Commission 2016) recommended the development or construction of databases with information regarding certain physicochemical parameters for pure honeys, as well as for the common sugar syrup and bee feeding products used to stimulate the bee families in the accepted periods. Accordingly, the purpose of this study was to study the physicochemical characteristics, major sugar content and $\delta^{13}C$ isotopic signature of honeys from beekeepers and commercial honeys of different botanical origins (acacia, polyfloral, honeydew, sunflower and linden) and different types of industrial sugar syrups, so that it can contribute to the enhancement of the honey authenticity process. The capabilities of general physicochemical parameters (acidity, pH, electrical conductivity, refraction index, Brix, moisture content, HMF content), major sugar composition and $\delta^{13}C$ isotopic signature to distinguish between pure and adulterated honeys were compared, highlighting the advantages and limitation of each one. The analytical results were processed by multivariate statistical analysis in order to distinguish the adulteration of some honey samples in relation to sugar syrups addition or non-compliance with quality standards.

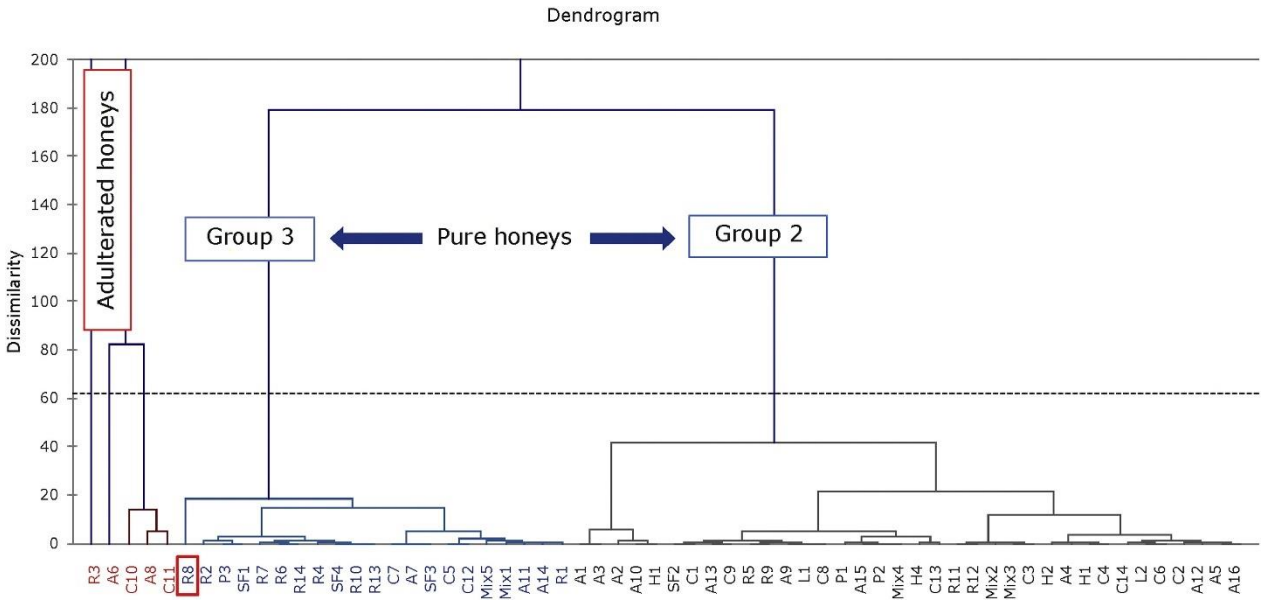

Figure 4: Dendrogram of the 62 honey samples (from beekeepers and commercial) represented by isotopic variables, obtained using Ward's hierarchical clustering method (HCA).

The method of stable carbon isotope ratio analysis (SCIRA) is a powerful technique for the detection of honey adulterated with C4 sugars (corn or cane), but it is not capable to detect honeys that do not complies with quality standards. Also, SCIRA method is not capable to detect the adulteration of honey with sugar syrups from C3 sources (beet, wheat, rice, etc.), this type of honey adulteration being identified by analysis of specific compounds presents as impurities resulted from sugar syrups (Du et al., 2015; Xue et al., 2013) or by $\Delta\delta^{13}C$ values analysis between fructose and glucose in honey and $\delta^{2}H$ values analysis of honey (Luo et al., 2016).

In order to differentiate between honeys with different botanical origins and to identify specific markers, multivariate statistical methods were applied to the obtained analytical data. The honeys that were identified as adulterated with C4 sugars were excluded from this differentiation. Principal components analysis (PCA) was carried out as an exploratory data analysis and it has resulted in a two principal components explaining 46.01 % of the total data variance. The first principal component (PC1) accounted 27.23 % of the total data variability, while the second one accounted 18.78 % (Fig. 5).

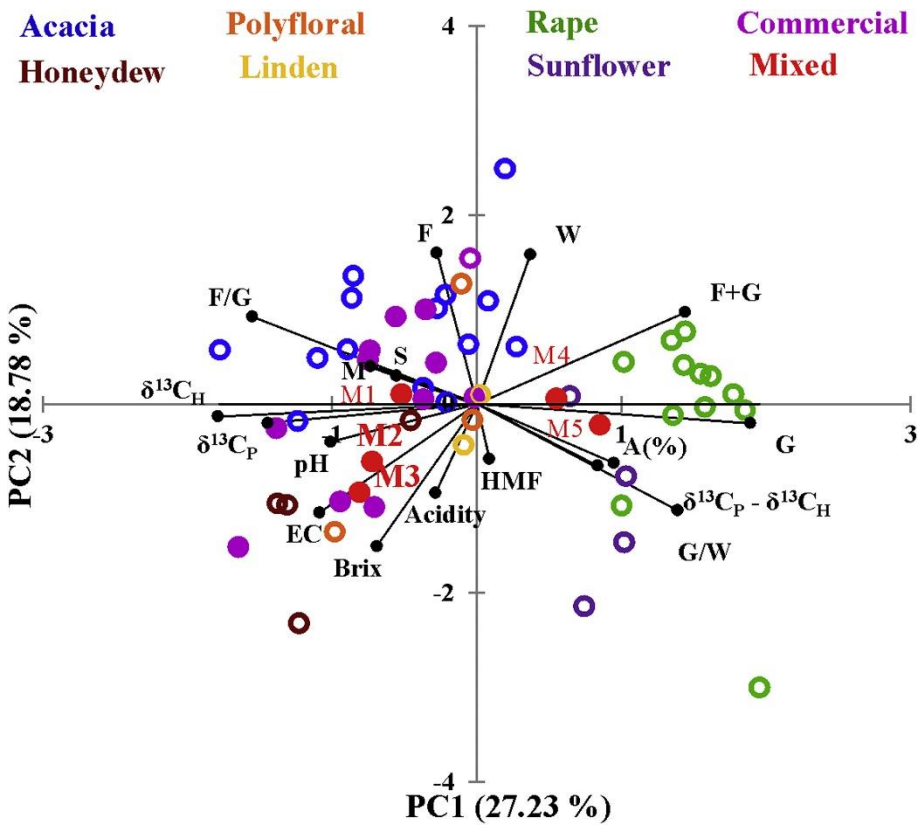

Figure 5: Principal component analysis (PCA) scores plot showing separation between honey types.

The acacia, rape and honeydew honey samples were grouped separately, exhibiting small internal variability, while rape with sunflower and linden with polyfloral honeys were overlapped indicating similar composition. The commercial honeys analyzed in this study seems to be of acacia, polyfloral or honeydew botanical origin, due to their overlapping with the corresponding regions in the PC1-PC2 score space. The mixed honeys were plotted as follows: M1 (declared by the beekeepers as acacia with some other floral impurities) was grouped in the acacia region, M2 (declared as polyfloral honey with small quantity of honeydew) and M3 (declared acacia with floral sources) were grouped in polyfloral/honeydew region, while M4 (declared as rape with linden floral sources) and M5 (declared as rape with polyfloral floral sources) were situated between rape and linden/polyfloral region. Among the investigated parameters, $\delta^{13}C$ isotopic signature, fructose (F) and water (W) contents and F/G ratio are specific markers for acacia honeys; glucose content (G), F+G content, G/W ratio, can be considered as markers for rape and sunflower honeys, while electrical conductivity (EC), pH, ºBrix and acidity characterized honeydew honeys.

Thus, general physicochemical parameters, major sugar composition and $\delta^{13}C$ isotopic fingerprint coupled with multivariate statistical analysis might be promising tools for honey botanical traceability, as other studies have shown (Dinca et al. 2014; Oroian, Ropciuc, Paduret, et al. 2017; Oroian, Ropciuc, and Buculei 2017). Exploring a larger set of samples with different floral origins in future studies will allow the model validation and prediction.

4. Characterization and classification of wines based on spectrophotometric determination of wine bioactive properties

Modern society encourages consumption of foods that can treat and prevent different disease and increase longevity, like foods and beverages rich in antioxidant compounds (Tarapatskyy et al. 2019; Wurz 2019). Wine is one of the oldest beverages and has been used as a medicine from ancient times in numerous countries. In France, it was conclusioned that moderate wine consumption leads to a low mortality rate from ischemic heart disease and the prevalence of other risk factors, such as smoking (French Paradox) (Renaud and de Lorgeril 1992).

The quality of wines is dictated by its color, smell and taste, rather than on its content of bioactive compounds (Stratil, Kubáň, and Fojtová 2008). From chemical point of view, wine is a hydro-alcoholic solution (~78% water)) with a great chemical complexity, including numerous minority bioactive phytochemical constituents and their metabolites which act synergistically on human health (Banc et al. 2014).

A wide variety of compounds contributes to the health benefits of wine, among them: phenolic compounds, soluble proteins, sugars, vitamins, volatiles, ketones, lipids and organic acids, the most representative being phenolic compounds. Polyphenolic compounds are commonly known as plant secondary metabolites and are directly associated with health-promoting properties of wines (Fernandes et al. 2017; Sartor et al. 2017). Among them, phenolic acids, stilbenes (e.g., resveratrol), flavonols (e.g., quercetin and myricetin), flavan-3-ols (e.g., catechin and epicatechin), procyanidins and anthocyanins represent the most valuable phenolic phytochemicals (Banc et al. 2014; Snopek et al. 2018).

In these latter days, phenolic compounds are the subject of increasing scientific interest due to their beneficial effects on human health (Tarapatskyy et al. 2019), among: cardio-protective, anti-cancer, anti-diabetic, anti-aging and neuro-protective effects (Fernandes et al. 2017) as results to their antioxidant character, associated with the presence of numerous antioxidant wines.

Antioxidants may be defined as inhibitors for the initiation and propagation of oxidative chain reaction, inhibiting the oxidation process of different molecules and protecting cells from oxidative stress. Also, they can also protect the human through the fight against free radicals in the body that cause disease and ageing.

The phenolic composition of wines is dependent on several factors; such as the grape variety, cultivation practices, winemaking techniques, ageing process and environmental factors (Fotakis et al. 2012; Sartor et al. 2017). Also, phenolic compounds play a major role in wine quality, contributing to the organoleptic properties such as color, flavor, astringency and to the oxidative stability (Fotakis et al. 2012).

Polyphenols are extracted during crushing and fermentation when the juice is in contact with the grape skins and seeds. Thus, the amount of phenolic compounds in red wine is higher compared with white wine because red juice has longer contact time with the grape skins and seeds (Yoo, Saliba, and Prenzler 2010).The phenolic content varies significantly in different types of wine depending on the presence of different classes of phenolic compounds (Snopek et al. 2018), leading to difference in the measured bioactive properties, including total phenolic content, total flavonoids content and antioxidant capacity (Fotakis et al. 2012), thus allowing the classification of wines according to the geographical and varietal origins and vintage year

The concentration of phenolic compounds in wines could be determined with low cost spectrophotometric methods, the most used being for the total phenolic content (Folin-Ciocalteu method), total flavonoid content assay, for the total anthocyanins quantification assay and for the antioxidant capacity estimation (Cassino et al. 2016; Pandeya et al. 2018).

The evaluation of the antioxidant capacity of wine is an indirect index of phenolic compounds present in wine. Several well established analytical methods for the evaluation of the antioxidant capacity were proposed: spectroscopic (colorimetric: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), FRAP (ferric reducing antioxidant power), CUPRAC (cupric reducing antioxidant power); fluorescence - ORAC (oxygen radical absorption capacity) and chemiluminescence), electrochemical (cyclic voltammetry, amperometry), chromatographic – HPLC (determination of target antioxidants) (Pisoschi and Negulescu 2012). Several studies on antioxidant capacity of wines have been published and among various analytical methods to evaluate total antioxidant capacity of wines, DPPH, ABTS and FRAP methods were commonly preferred (Sartor et al. 2017; Stratil et al. 2008).

Like any other food/feed matrix, wine requires authentication strategies based on suitable qualitative and quantitative analytical investigations of wine natural constituents which represents the specific fingerprint of each wine (Palade and Popa 2018).Various analytical approaches (chromatographic, spectroscopic, spectrometric, electrochemical) were applied in order to assess the profiles of wine bioactive constituents, including phenolic and volatile compounds, amino acids, thus, in combination with appropriate chemometric approaches contributing to the development of different methodologies for the assessment of wine authenticity (Rocchetti et al. 2018; Villano et al. 2017).

The present research aimed to evaluate the wine biochemical properties (total polyphenolic content, total flavonoids content and DPPH antioxidant capacity) of different red, rose and white wine varieties with different ageing times, produced at SCDVV Murfatlar, Romania during 9 years of production. All the data collected were analyzed by the multivariate statistical method of the Principal Component Analysis (PCA) in order to find the possible correlation between the total antioxidant activity measured and the concentration of each class of antioxidants analysed and for the classification of different red and white wine varieties and vintage years has been investigated.

Our results are in agreement with the available literature for Romanian wines (with 2455.9 mg/L GAE for red wines and 255.6 mg/L GAE for white wines) (Hosu et al., 2014, 2016), Spanish wines (1613.2 mg/L GAE for red wines and 240.8 mg/L GAE for white wines) (Gómez-Plaza et al., 2000) and wines from Czech Republic (1544.8 mg/L GAE

The most phenolic compounds from wines come from the grape skin and, therefore, higher concentrations of phenolics can be expected in red wines (Pandeya et al., 2018). According to Table 2, bioactive properties of red wines were higher compared with rose and white wines which is consistent to previous work reported (Pandeya et al., 2018; Stratil et al., 2008). The great differences in the contents of phenolic compounds in white and red wines indicate that anthocyanins (which are absent in white wines) represents the most important fraction of the phenolic compounds in red wines (Stratil et al., 2008).

The TP is an important parameter widely used for evaluation of wines and other foods. Wines with higher TPC are considered to be better quality, in our case, Pinot Noire and Feteasca Neagra for red wines and Muscat Ottonel and Riesling Italian for white wines. The wines with the higher TP tends to provide the higher antioxidant capacity indicating that the TP is responsible for the antioxidant capacity of the wine.

The antioxidant activity is a very relevant parameter to evaluate wine quality and its bioactive properties. For the analyzed wines, antioxidant capacity of red wines (expressed as μmoli/L Trolox) decrease in the order: Feteasca Neagra > Cabernet Sauvignon > Pinot Noire > Merlot, while for the white wines decrease in the order: Muscat Ottonel > Riesling Italian > Feteasca Regala > Chardonnay > Pinot Gris > Columna > Sauvignon Blank.

In the case of red wines, concentrations of total phenolics and antioxidant capacity were higher in wines from the 2010 and 2011 vintage years, with the exception of Pinot Noire wine from 2015. Young wines from 2017 presented the lowest values of antioxidant capacity (Fig. 6).

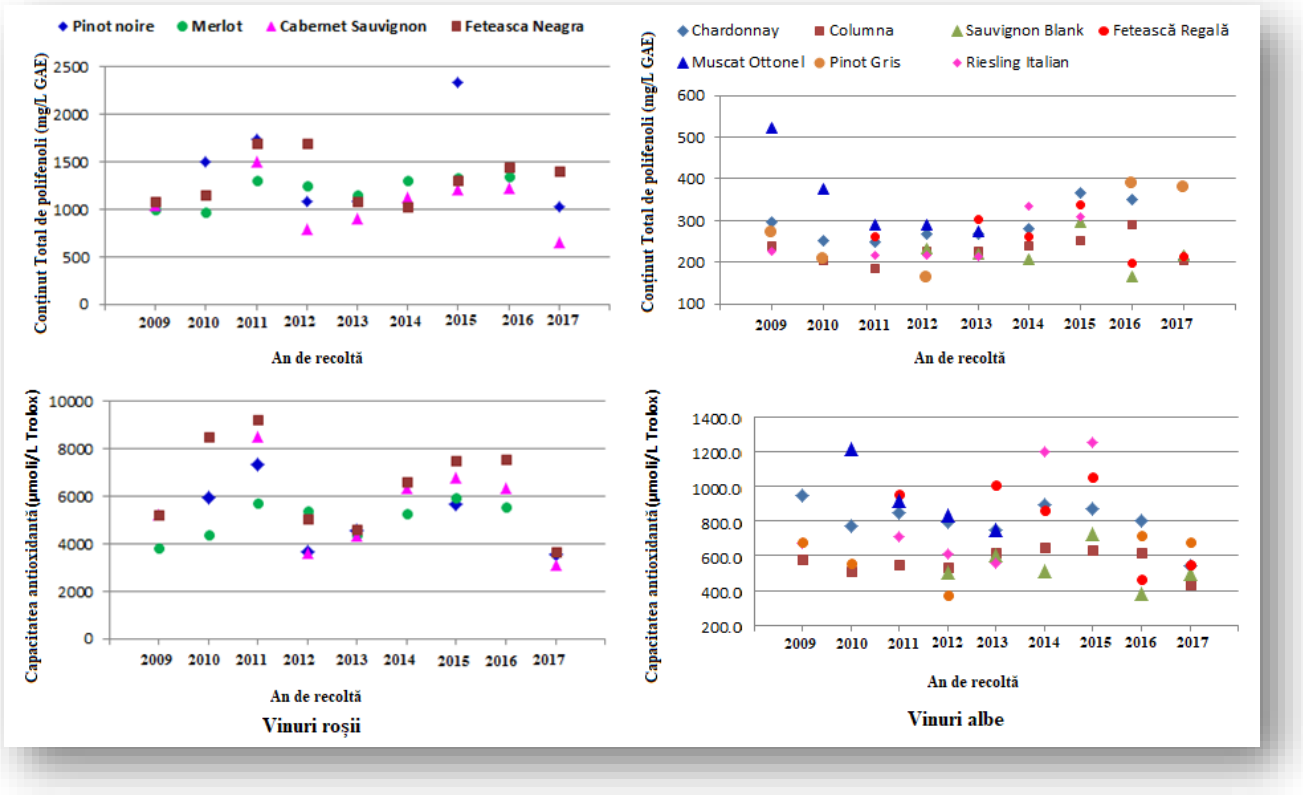

Figure 6. Conținutul total de polifenolici (TP) și capacitatea antioxidantă (AC) ale vinurilor roșii și albe, în funcție de anul de recoltă

5. Compoziția profilului fitochimic și nutrițional în fructele diferitelor soiuri de castan dulce (Castanea Sativa MILL.)

The sweet chestnut is the fruit of the Castanea sativa Mill., which belongs to the genus Castanea, of the Fagaceae family, and is cultivated especially in Mediterranean Europe (Míguez-Soto et al., 2019). Sweet chestnut is an important resource in Europe due to its economic value associated with fruit, wood and tannin production and indirectly with honey production, but also due to its cultural value (Beccaro et al., 2020a). Castanea sativa Mill. is also commonly known as European chestnut and has a large distribution in Spain, France, Greece, Italy, Portugal, and Turkey (Choupina, 2019). Nutritionally, chestnuts have interesting characteristics, containing significant amounts of carbohydrate dietary fiber, but small amounts of crude protein (2–4%) and low levels of crude fat (predominated by unsaturated fatty acids) (2–5%) compared to typical walnuts (walnuts, almonds, hazelnuts) (Akbulut et al., 2017; Otles & Selek, 2012a), thus being a good source of energy with multiple health-beneficial effects. Chestnuts are also low in fat, thus helping to decrease cholesterol levels and they contain a high amount of vitamin C, macro- (K, P, Mg, Ca, Na) and micro-nutrients (Mn, Fe, Zn and Cu) (Poljak et al., 2021). The fruits also have a significant antioxidant activity associated with polyphenolic contents (gallic acid and ellagic acid being predominant) and organic acid contents (ex. oxalic, cis-aconic, citric, ascorbic, malic, quinic and fumaric acids) (B. Gonçalves et al., 2010). Thus, chestnut fruits have become very important in the human diet due to their nutritional composition and health benefits, for example, their use in gluten-free diets in celiac disease (El Khoury et al., 2018), reducing abdominal adiposity (Rodrigues et al., 2020) and reducing coronary heart disease and cancer rates (Choupina, 2019). The growing demand for traditional foods has converted chestnuts into a value-added resource with considerable potential as functional foods or food ingredients. The nuts are consumed in roasted or boiled form or for the development of different added value products in the cake and candy industry (Mert & Ertürk, 2017a). Considering the fact that cooked chestnuts are a good source of phenolics (gallic and ellagic acids) and organic acids (citric acid) and have low fat contents (B. Gonçalves et al., 2010), properties that are associated with positive health benefits, the development of new products based on chestnuts should be encouraged (da & Silva, 2016). Therefore, over the last few decades, the chestnut industry has significantly grown in Europe, especially in the production of marron glacé, purées and chestnut flour, which find increasing application as an ingredient in gluten-free diets (Vella et al., 2017), such as the production of pasta

by incorporating chestnut flour and bee pollen (Brochard et al., 2021). In addition, chestnut extracts can be used in the food industry as functional ingredients and natural preservatives aiming to replace the synthetic ones capable of improving the shelf-life and nutritional value of products (Pinto et al., 2017; V. Silva et al., 2020). Furthermore, chestnut shells as the main byproduct generated from chestnut processing are currently used as fuels (You et al., 2014), for the production of lignin biopolymer and bioethanol following a biorefinery approach (A. Morales et al., 2018), but also can be a source of hydrolyzable tannins as natural pigments for food and pharmaceutical industries (Pinto et al., 2021), as a bioactive ingredient for nutraceutical and cosmetic industries (Pinto et al., 2017).

Therefore, due to the increased economic interest in the use of sweet chestnuts in the food industry, there was a need to develop selected cultivars with enormous potential on human health associated with the consumption of chestnuts and processed products based on chestnuts (Corona et al., 2021). Additionally, in order to increase chestnut production and resistance to chestnut-specific diseases, some hybrids have appeared over time (da & Silva, 2016).

The nutritional composition and bioactivity of fresh sweet chestnuts show differences between cultivars (Barreira, Casal, et al., 2009; Beccaro et al., 2020a), producing regions, harvesting year (Barreira et al., 2012), soil and climatic conditions (temperature, sun exposure and precipitation) (Peña-Méndez et al., 2008), but also cultivation techniques, for example, nutrients, minerals, irrigation and diseases and pests (M. C. B. M. de Vasconcelos et al., 2010; Mota et al., 2018).

The increased market demand and consumer awareness imposes the development of reliable methods able to distinguish between different cultivars, highlighting high quality products in terms of sensorial and qualitative properties and high bioactive composition. In the last few decades, different methodologies have been used to characterize and distinguish between different sweet chestnut cultivars, including morphological characteristics (Furones-Pérez & Fernández-López, 2009) and chemical composition addressing the proximate analysis including dry mass, ash quantity, total fat, total protein (M. D. C. B. M. De Vasconcelos et al., 2007; B. Gonçalves et al., 2010), total carbohydrates, total sugar, invert sugar, starch, sucrose (Choupina, 2019), but also mineral contents (Ca, Mg, Fe, Mn, Cu, Zn, P, Na and K) (Akbulut et al., 2017; Choupina, 2019; Ertürk et al., 2006), total polyphenols (mg GAE/g) and antioxidant activity (μmol Trolox equivalent/g) dry weight basis (Akbulut et al., 2017; Otles & Selek, 2012a) and organic acids

(oxalic, cis-aconitic, citric, ascorbic, malic, quinic and fumaric acids) (Delgado et al., 2018; B. Gonçalves et al., 2010), free amino acids (M. D. C. B. M. De Vasconcelos et al., 2007), sugars profile (Barreira, Pereira, et al., 2009; Hernández Suárez et al., 2012a).

For decades, conventional extraction methods including maceration or Soxhlet extraction using different polar solvents (methanol, ethanol, chloroform and petroleum ether) were the most used to extract bioactive compounds from a natural matrix, and which, due to environmental, economic and safety concerns, presents a huge limitation for an industrial application. Given these disadvantages, more sustainable extraction methods, including ultrasonic extraction, microwave extraction, supercritical fluid extraction and enzymatic extraction were promoted for the extraction of polar bioactive compounds from natural sources (Leichtweis et al., 2021; Pinto et al., 2021).

Chestnut growing areas in Romania cover a total area of 3160 ha distributed on a discontinuous area, consisting of long bands situated on the foothills of the Carpathians, mostly in the west part of Romania, where the moderate-continental climate has a slight Mediterranean influence. Chestnut natural distribution cover two principal centers, namely Maramureș (the hilly foothills of Baia Mare) and Oltenia (subcarpathian hills of Oltenia on the territory of Gorj, Mehedinţi and Vâlcea counties) and other several small areas on Southeast of the Oriental Carpathians and Northwest and Southwest of Transylvanian plateau (Chira et al., 2013). The semi-spontaneous flora of Northern Oltenia contains many biotypes of sweet chestnut (Castanea sativa Mill). Since 1998, several French cultivars, which are hybrids between Japanese chestnut (Castanea crenata Siebold & Zucc.) and European chestnut (Castanea sativa Mill.) were introduced at the Fruit Growing Research—Extension Station (SCDP) Vâlcea for testing and they have proved to yield well in the given conditions. Physical characteristics (diameters, height, shape index and size index, mass, volume and specific weight), nutritional composition (water (%), titrable acidity (g malic acid/100 g), lipids (%), proteins (%) and bioactive characteristics (total polyphenols, total flavonoids, antioxidant activity and some individual polyphenolic compounds) of these cultivars were previously addressed (Cosmulescu et al., 2020a) and supplementary characteristics are required in order to make a detailed characterization and comparison of these six chestnut cultivars, in order to provide valuable information for selection of the chestnut cultivar with high quality bioactive characteristics that can be cultivated for the development of different value-added food products with multiple benefits on human health.

Therefore, this work aims to strengthen our knowledge about the chemical and nutritional composition of the six sweet chestnut cultivars of French origin, namely ‘Marsol’, ‘Maraval’, ‘Bournette’, ‘Précoce Migoule’ and ‘Marissard’ in order to find distinctive features useful for authenticating a certain chestnut cultivar, providing an important economic advantage. For that, the bioactive characteristics (total polyphenols, total flavonoids and antioxidant activity) examined by UV-Vis spectrophotometric methods, UHPLC-MS/MS phenolic compound profile (phenolic acids, flavonoids), together with a non-target UHPLC-MS/MS screening profile, the sugar profile (sucrose, fructose, glucose, maltose) by HPLC-ELSD and elemental composition (Ca, Mg, Na, K, Fe, Pb, Ni, Cu, Cr) by F-AAS were determined in the studied chestnut cultivars in order to complete the information about the nutritional and bioactive composition of all these cultivars. In addition, we studied the influence of the harvest year on the bioactive characteristics and the content of the specific bioactive compounds. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used in order to discriminate between the different sweet cultivars grown in Romania.

The bioactive properties of plant materials are closely related to the different classes of biologically active chemical compounds found in their natural composition. These are expressed by the total content of polyphenols expressed in gallic acid equivalents, the total content of flavonoids expressed in units of rutin or quercetin and the antioxidant activity, expressed mostly in Trolox equivalents.

Mean TP, TF and AA values for the studied chestnut cultivars during two consecutive years, 2016 and 2017, investigated in this study and for 2015 reported in a previous study (Cosmulescu et al. 2020) are presented in Figure 6.

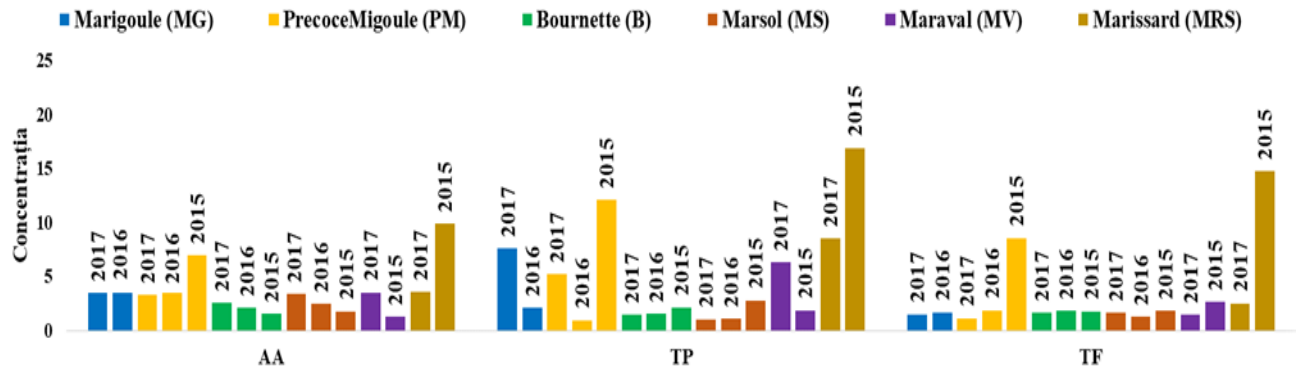

Figure 6: Antioxidant activity (AA) expressed as μmoli Trolox equivalents/g, Total polyphenols (TP) expressed as mg GAE equivalents/g and Total Flavonoids (TF) expressed as mg rutin equivalents/g of the six chestnut cultivars for three consecutive years.

High contents of TP correspond to 'Marissard', with average values of 12.69 mg GAE equivalent/g DW, followed by and 'Précoce Migoule', 'Marigoule' and 'Maraval' cultivars with average values of 6.11, 4.84 and 4.10 11 mg GAE equivalent/g DW, respectively. The obtained values were in agreement with data from other studies (Otles and Selek 2012a). Average values of TF ranged between 1.57 mg rutin equivalent/g DW in 'Marsol' and 8.64 mg rutin equivalent/g DW in 'Marissard'.

To evaluate the contribution of each class to the total polyphenolic composition, the phenolic bioactive compounds were grouped in the following classes: benzoic acids (gallic, protocatechuic, p-hydroxybenzoic and syringic acids), cinnamic acids (caffeic, p-coumaric, ferulic, cinnamic and chlorogenic acids), catechins ((+)-catechin and (−)-epicatechin) and flavanols (naringin, rutin, myricetin, quercetin, isorhamnetin, apigenin, pinocembrin) and t-resveratrol) (Figure 7). Apigenin, hesperidin and kaempferol were not identified in chestnut extracts, while chrysin and galangin were quantified in very low amounts only in 'Marigoule' or quantified below LOQs.

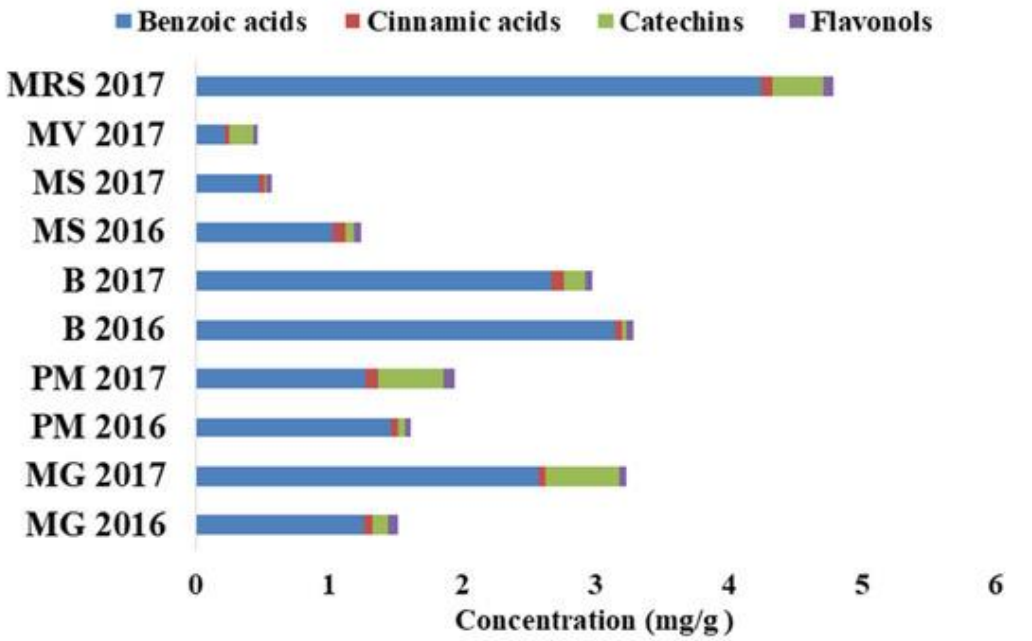

Figure 7: Polyphenolic profile of the analyzed chestnut cultivars harvested in 2016 and 2017.

Within the polyphenolic groups, differences were observed among chestnut cultivar and harvest year, higher amounts of polyphenolic compounds corresponding to ‘Marissard’, ‘Bournette’ and ‘Précoce Migoule‘.

Benzoic acids and catechins, recognized for their antioxidant, anticancer, anti-inflammatory and antimicrobial potential (Dinis et al. 2011; Zavalloni, Andresen, and Flore 2006), represented the main component of the polyphenolic chestnut extract and among them, gallic and ellagic acids were quantified between 0.162–2.077 mg/g DW and 0.046–2.928 mg/g DW, while (+)-catechin was quantified between n.d. and 0.547 mg/g DW. p-Coumaric, ferulic and cinnamic acids were the representative for the cinnamic acids class, with values between 0.008–0.045 mg/g DW for p-coumaric acid, 0.008–0.036 mg/g DW for ferulic acid and 0.002–0.028 mg/g DW for cinnamic acid. Among flavanols, myricetin, naringin, rutin and quercetin were quantified in higher amounts, with values ranging between 0.036–0.051 mg/g DW for myricetin, n.d.—0.018 mg/g DW for naringin, n.d.—0.011 mg/g DW for rutin and n.d.—0.009 mg/g DW for quercetin. t-Resveratrol was quantified with values between 0.001 and 0.014 mg/g DW, with higher amounts in “Marigoule” and “Marissard” in the 2017 harvest year. The results of the quantitative analysis are similar to those obtained in other studies (Beccaro et al., 2020a; da & Silva, 2016; Otles & Selek, 2012a).

Identification and quantification of polyphenols in fruits are of major importance because of their beneficial impact on human health due to their additive and synergistic effects on radical scavenging (Hohrenk et al. 2020). Thus, an analytical approach based on a non-target UHPLC-Q-Orbitrap HRMS method was carried out aiming to identify other bioactive compounds and specialized metabolites that occur in methanolic extracts of chestnut fruits, which are responsible for their antioxidant and anti-inflammatory activities, and to highlight the differences between extracts of different chestnut cultivars. Data processing analysis used Compound Discoverer software using an untargeted metabolomics working template, which comprises an untargeted workflow which includes options for peak picking performs RT alignment, prediction of the molecular formula, evaluation of adducts, the assignment and comparison of fragmentation pattern (including dealkylation and dearylation products and bio-transformation products), background annotation, an automated library and database search for identification purposes, including mzCloud (ddMS2), Chemspider, MzVault and Mass List Matches (Hohrenk et al. 2020). An overview of the Compound Discoverer workflow and parameters can be found in Figure 8. The

output of this is a feature list, which includes accurate mass, retention time, m/z adducts and their areas and intensities. Only features that were five times the intensity of the blank were considered.

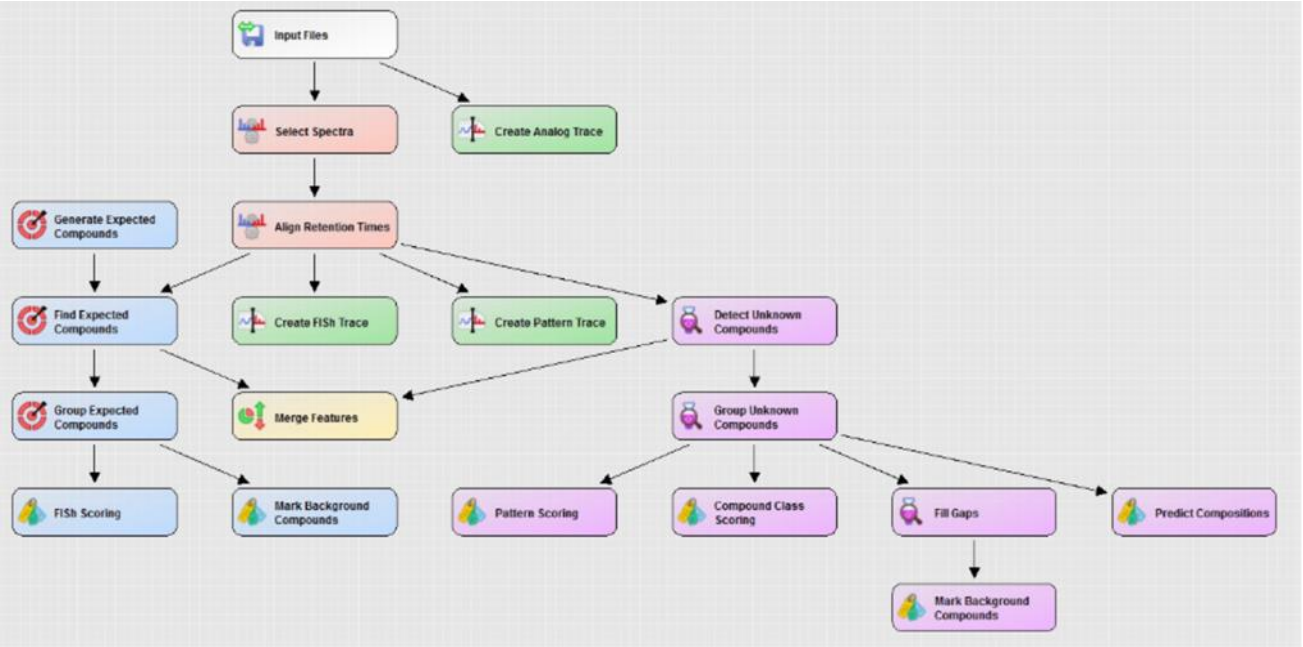

Figura 8: Untargeted Metabolomics Workflow: Finding and Identifying Differences Between Samples

Based on Compound Discoverer processing results, the identity of most of the peaks was attributed. In particular, five main classes of compounds, i.e., phytochemical compounds (flavanols, flavanols, isoflavones, calchones, anthocyanidin derivatives, terpenoids and sesquiterpenoids, vitamins, gibberellin plant hormones, metabolites), fatty acids (saturated and unsaturated fatty acids and derivatives), amino acids, organic acids, but also various sugars and sugar derivatives could be identified in chestnut hydro-methanolic extracts.

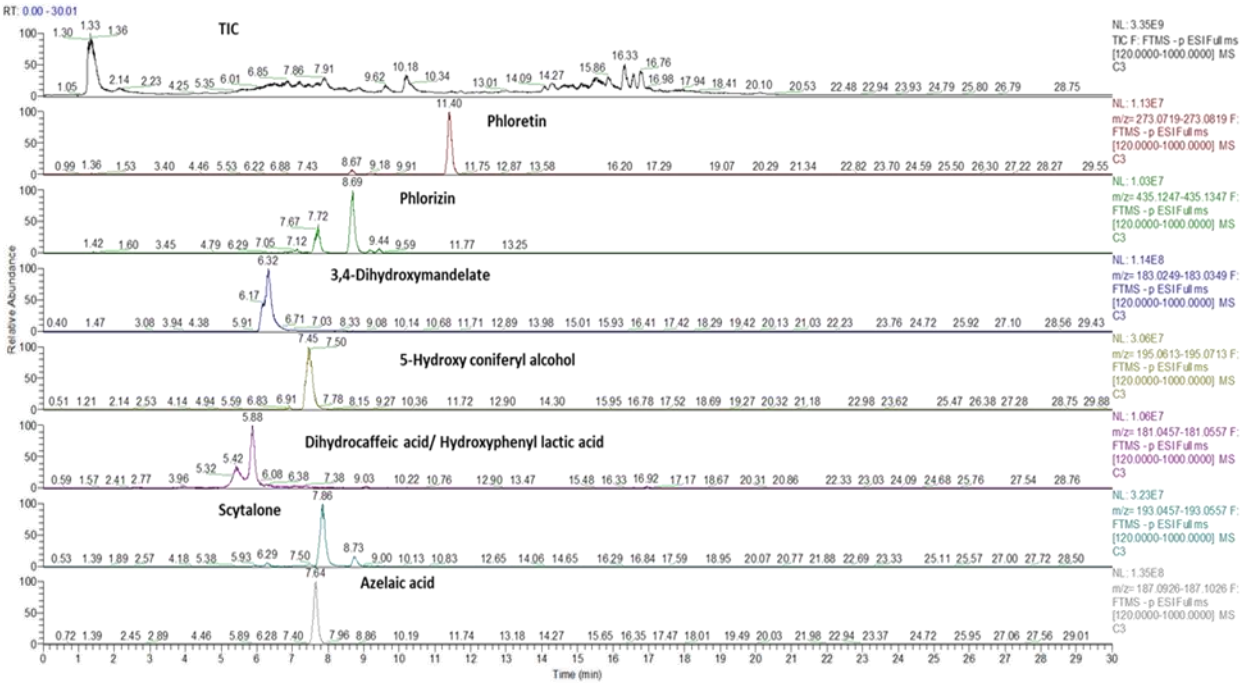

Figura 9. Identification of fatty acids and some derivatives in chestnut extract by UHPLC-Q-Exactive high-accuracy analysis of deprotonated precursors and fragment ions of specific components combined with data processing using Compound Discoverer software.

Carbohydrates are relevant components in chestnuts, with sucrose being an important parameter in assessing the quality of the fruit. Together with sucrose, glucose and fructose are present in significant amounts in chestnuts, the profile of free sugars contributing to the identification of chestnut cultivars. Sugar profile can be influenced by several conditions, such as cultivars, genotypes, environmental factors (climatic conditions, soil characteristics) technical and cultural practices, and harvest time (Mert & Ertürk, 2017).

The highest quantity of sugars was observed for “Bournette” in 2017, followed by “Marigoule” and “Précoce Migoule” in the 2016 harvest year (Figure 10a). Sucrose was the most abundant sugar in the analyzed chestnuts with values ranging from 20.34–154.94 g/kg DW, the lower value corresponding to ‘Maraval’, while the highest value corresponds to “Bournette” in 2017. The sucrose level of chestnut fruits cultivated in Romania is similar to those grown in Turkey (68.20–174.00 g/kg DW) (Mert & Ertürk, 2017), but lower compared with chestnut fruits cultivated in Italy (2.98 –245.09 g/kg DW) (Beccaro et al., 2020) and Portugal (40.30–233.00 g/kg DW) (Beccaro et al., 2020) and higher compared with chestnut fruits cultivated in Tenerife (Spain) (31.10–99.40 g/kg DW) (Hernández Suárez et al., 2012).

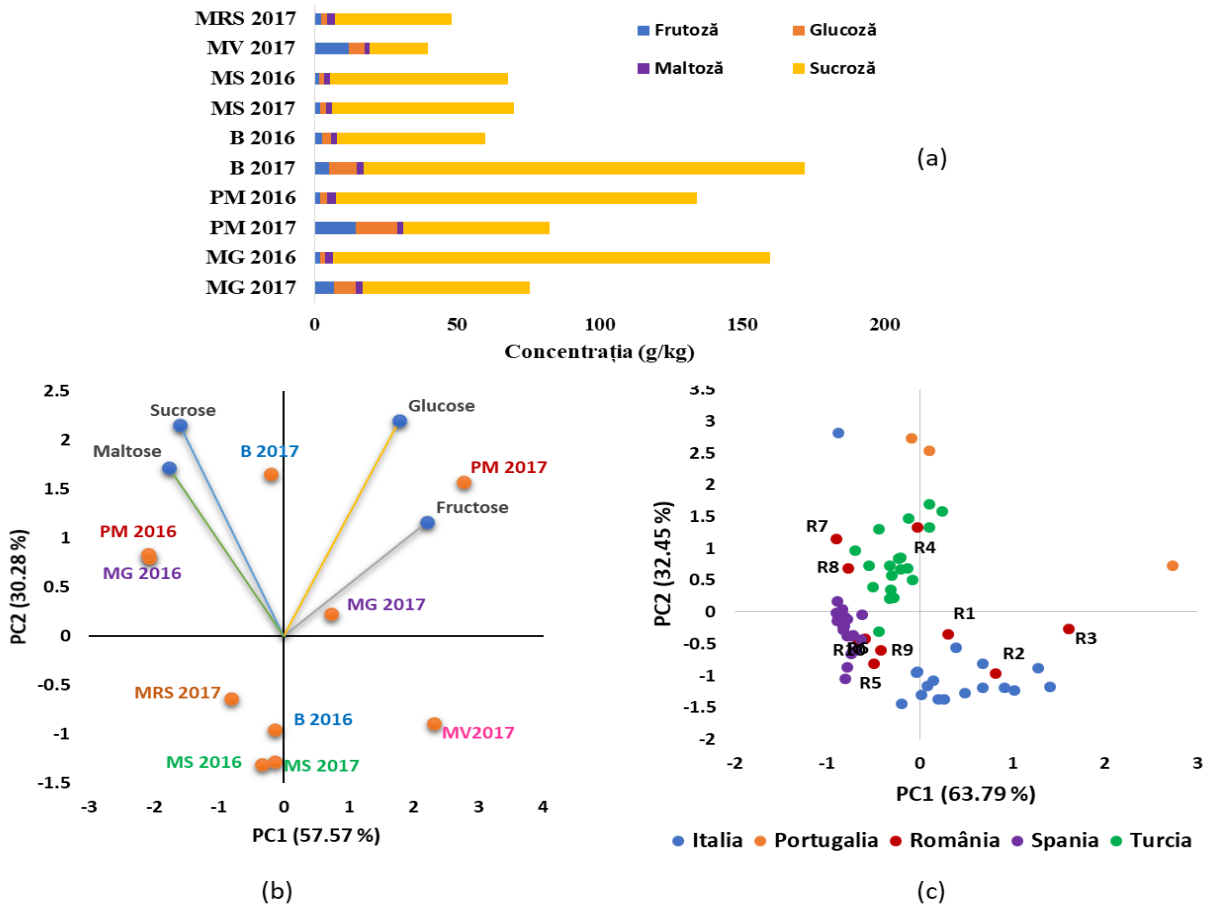

Figura 10. (a) Sugar profile of the analyzed chestnut varieties harvested in 2016 and 2017; (b) Principal component analysis (PCA) of the fruits of different chestnut cultivars based on individual phenolic compounds ("Précoce Migoule"—PM, "Bournette"—B, "Marsol"—MS, "Marissard"—MRS, "Marigoule" —MG and "Maraval"—MV); (c) PCA analysis based on sugar composition of chestnut fruits harvested in some European countries.

In general, fructose and glucose were quantified in equal amounts in the studied chestnut cultivars, with fructose ranging between 1.55–14.35 g/kg DW and glucose ranging between 1.56–14.46 g/kg DW, the highest contents corresponding to "Précoce Migoule" harvested in 2017 (Figure 10a). Our data were higher than those reported by other authors which found glucose and fructose concentrations between not detected and 3.1 g/kg DW for both monosaccharides (De La Montaña Míguelez, Míguez Bernárdez, and García Queijeiro 2004) or between 0.56–2.40 g/kg DW for fructose and 0.49–1.90 g/kg DW for glucose (Hernández Suárez et al., 2012), but in the same range as Mert et al. who report fructose between 1.5–8.0 g/kg DW and glucose between 4.0–

11.3 g/kg DW (Mert & Ertürk, 2017). Maltose was quantified in low amounts, with values between 1.77 and 3.16 g/kg DW. It can be concluded that the 2017 harvest year, in which there were higher temperatures and more abundant rainfall, was more favorable to accumulate sugars in "Bournette", while the 2016 harvest year was more favorable for "Marigoule" and "Précoce Migoule" cultivars. PCA analysis based on sugar profile (sucrose, fructose and glucose) of chestnut fruits grown in the main European chestnut producing countries (Italy, Portugal, Turkey, Spain) and the chestnut cultivated in Romania (Figure 10c), indicate a good correlation between the sugar content of chestnuts and climatic conditions, temperate continental corresponding in Romania and Turkey (around the Black Sea) characterized by an average temperature of 11.94°C and 566.07 mm precipitation in Turkey and 10.5°C and 669.15 mm precipitation in Romania, compared with the temperate oceanic climate in Portugal (average temperature of 16.11°C and 677.93 mm precipitation), the Mediterranean climate in Italy (average temperature of 13.35°C and 791.32 mm precipitation) and the subtropical climate with some influences from Sahara in Tenerife (Spain) (average temperature of 14.47°C and 518.24 mm precipitation). The chestnuts from Portugal Italy, Spain and Turkey were grouped in distinct groups, while the chestnuts cultivated in Romania were overlapped on the others.

FINAL CONCLUSIONS

All honey samples presented substantial values of bioactive compounds, the values of polyphenols and flavonoids being in an optimal ratio, as demonstrated by the results regarding their antioxidant activity. It was noted that there is a correlation between the three types of analysis, and the fact that in all the analyzed samples they have promising results, practically the honey from our country is of optimal quality. The resulting values are consistent with the variety of honey, harvest year and area, the most significant results being noted in the samples from the north and west of the country. The obtained results showed that the profile of phenolic compounds in Romanian honey seems to be useful for determining their floral origin, because these compounds demonstrate a significant variation in their quantitative composition in honey with different floral origin. It is difficult to differentiate honey contaminated with phenolic compounds originating from other floral sources due to the similarity of the phenolic compound fingerprints.

Authentication of bee honey and identification of adulterations through physicochemical determinations demonstrated that the fructose/glucose ratio was in the range of 0.32-1.97, with a value below 1 for rapeseed honey indicating its rapid crystallization.

The authentication of bee honey and the identification of falsifications through isotopic determinations established that 3 samples (R3, C10 and C11), representing 4.8% of the analyzed honey, had a sugar content of C4 > 7%, which were immediately classified as adulterated honey , the remaining honey samples being considered pure.

The multivariate statistical analysis applied to the isotopic parameters proved to be adequate to differentiate the adulterated honey, but did not allow the identification of honey not conforming to the quality standards. The $\delta^{13}C$ values of the protein extracted from honey ($\delta^{13}CP$), the general physico-chemical parameters and the major sugar composition depend on the botanical origin of the honey.

Based on the polyphenol profile and the different ratios between polyphenols and total flavonoids, it was also possible to differentiate some red wine varieties, separating Feteasca Neagră and Pinot Noire wines from Cabernet Sauvignon and Merlot wines, being wines in which significant amounts of total phenolic compounds are found.

It can be seen that older wines, mainly the red wine varieties Fetească Neagră and Pinot Noire and the white wine varieties Muscat Ottonel and Riesling Italian show a higher content of polyphenols and antioxidant capacity and are considered to be of better quality.

This study highlights, for the first time, a comprehensive bioactive characterization of the sweet chestnut varieties „Marsol”, „Maraval”, „Bournette”, „Précoce Migoule” and „Marissard” grown at the Vâlcea Pomiculture Research Station (SCDP), in Oltenia de Nord, Romania to provide valuable information for the selection of chestnut variety with high quality bioactive characteristics that can be cultivated for the development of various food products with added value and multiple benefits on human health.

Based on total polyphenol content, total flavonoids, antioxidant activity and individual phenolic compounds profile, no significant differences were observed between the varieties studied, a higher bioactive potential corresponding to „Marissard”, followed by „Précoce Migoule”, "Marigoule" and "Bournette". Based on gallic and cinnamic acids and rutin, some differences can be observed between the variables of the varieties studied, „Bournette” showing a higher content of gallic acid and a lower cinnamic acid, while „Marissard” showed the highest

amounts of cinnamic acid and rutin. Discriminant analysis shows a clear discrimination between chestnut fruits harvested in different years, indicating that climatic conditions have a significant contribution to the synthesis of chestnut bioactive compounds.

HRMS screening coupled with statistical data processing using the Compound Discoverer software based on an untargeted metabolomic working template allows the identification of other bioactive compounds from chestnut hydro-methanolic extracts, such as phytochemical compounds (flavanols, isoflavones, chalchones, anthocyanidin derivs. , terpenoids and sesquiterpenoids), vitamins, plant hormones gibberellin, metabolites, fatty acids (saturated and unsaturated fatty acids and derivatives), amino acids and organic acids that are probably responsible for their antioxidant and anti-inflammatory activities.

Statistical analysis based on qualitative data on phytochemical and saturated and unsaturated fatty acid fingerprints indicates a clear discrimination of chestnut fruits harvested in different years, indicating that chestnut phytochemical and fatty acid fingerprints depend primarily on climatic conditions and subsequently of genotype. Based on HRMS phytochemical fingerprints, „Marigoule" and „Marissard" were grouped separately from the other cultivars, while based on fatty acid and derivative fingerprints, „Précoce Migoule" and „Bournette" form a distinct group from the other cultivars . Quantitative data would be very useful for identifying chestnut varieties with high phytochemical composition and high fatty acids.

Large and significant differences ($p < 0.0001$) in sugar and mineral composition were detected among different cultivars and harvest years. The variety and climatic conditions influence the sugar composition, with the highest amount of sugars being observed in the variety „Bournette" in 2017, which was warmer and rainier than 2016, while the 2016 harvest year was more favorable for the accumulation of sugars in the Varieties „Marigoule" and „Précoce Migoule". The variety and climatic conditions influence the elemental composition of chestnut fruits, the mineral composition of the soil being the same for all studied varieties. The variety "Précoce Migoule" has a higher content of macro and micronutrients, with K being the most abundant nutrient. Higher amounts of Na were identified in „Bournette" and „Marissard".

PCA analysis based on the sugar profile (sucrose, fructose and glucose) and mineral composition of chestnut fruits grown in Romania and those grown in the main European chestnut producing countries (Italy, Portugal, Turkey, Spain) indicates a good correlation between sugar and minerals. the content of chestnuts and the climatic conditions corresponding to different

geographical areas. It can thus be concluded that the climatic and pedoclimatic characteristics corresponding to the North of Oltenia, Romania favor the accumulation of sugars and minerals, and thus, crops with nutritional characteristics similar to those obtained in the specific crop area can be obtained.

Analytical investigations have revealed that the sweet chestnut varieties grown in Romania present a bioactive, phytochemical and nutritional composition similar to the varieties grown in the major European chestnut-producing countries, indicating the great adaptation potential of the chestnut in the continental temperate zone with small characteristic Mediterranean influences, from the southwest area of Romania.

The results presented in the PhD thesis constitute the starting point for the construction of databases for chestnut fruits in Romania, based on different classes of chemical compounds found in the natural chemical composition of chestnuts (individual phenolic compounds, sugars, minerals, polyphenols total, total flavonoids).

The classification methodologies of bee honey, wines and chestnuts proposed in this doctoral thesis can also be applied to products from other varieties (varieties, floral origin) from Romania, thus providing a relevant footprint of Romanian products, being useful for the prevention fraudulent practices in the food industry.

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