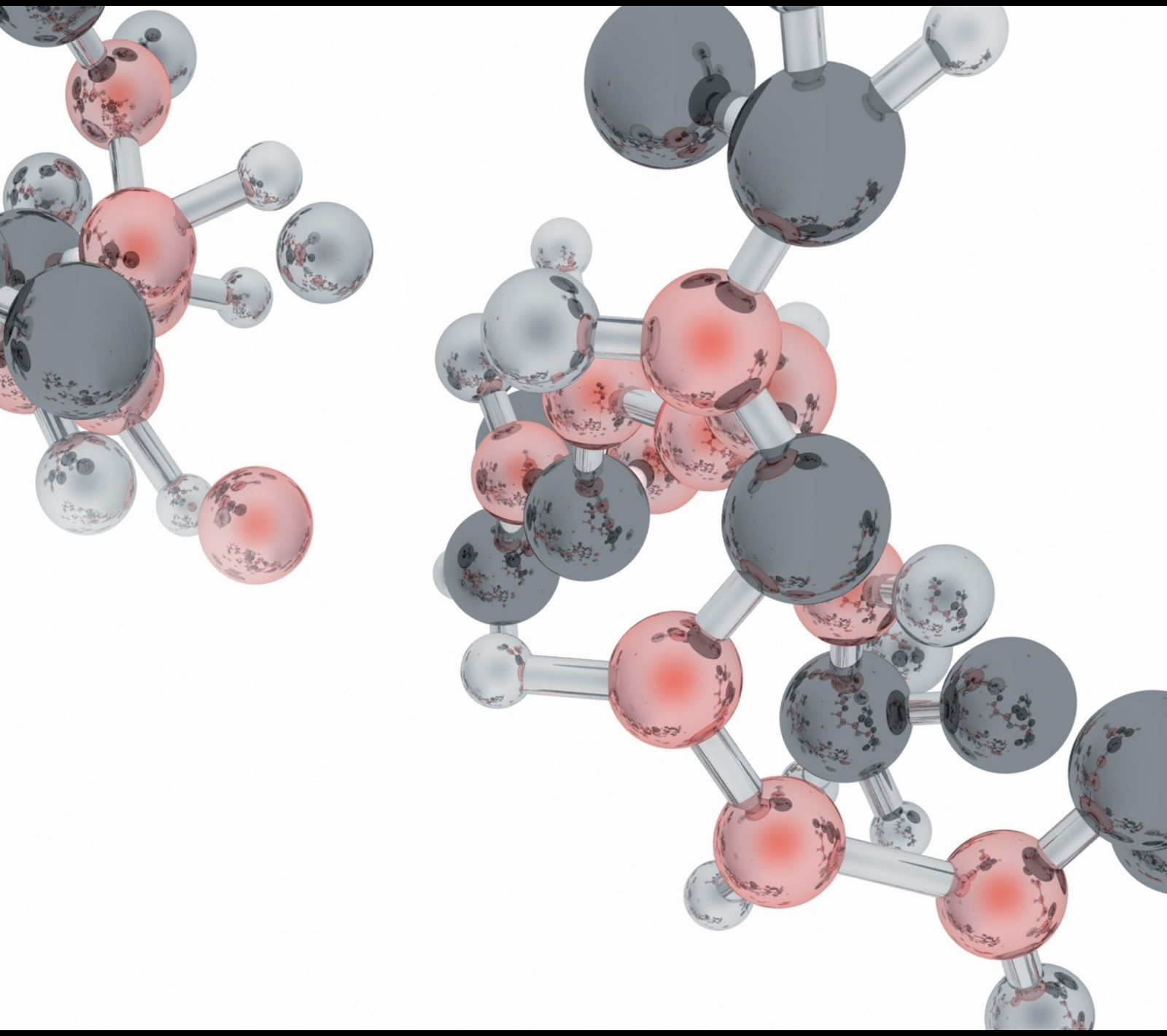
Journal of Analytical Methods in Chemistry

Chemistry of Medicinal Plants, Foods, and Natural Products 2015

Guest Editors: Shixin Deng, Shao-Nong Chen, and Jian Yang



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Editorial Board

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 121849, 2 pages
http://dx.doi.org/10.1155/2015/121849

Editorial

Chemistry of Medicinal Plants, Foods, and Natural Products 2015

Shixin Deng,[1] Shao-Nong Chen,[2] and Jian Yang[3]

[1]*Research and Development Department, Morinda Inc., American Fork, UT 84003, USA*
[2]*UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois, Chicago, IL 60612, USA*
[3]*Western Pacific Tropical Research Center, College of Natural and Applied Sciences, University of Guam, Mangilao, GU 96923, USA*

Correspondence should be addressed to Shixin Deng; shixin_deng@morinda.com

Received 20 September 2015; Accepted 21 September 2015

Medicinal plants and natural products have been demonstrated to possess diversified health benefits for thousands of years by traditional uses and modern scientific research. An increased global demand has been observed over years. In 2014, we published the first special issue of Chemistry of Medicinal Plants, Foods, and Natural Products, aiming to address safety, identity, and efficacy of the natural entities. As a continuing program, we edited a special issue for 2015. The challenge for raw materials and finished products of botanicals attracts public and scientific communities. The safety and efficacy of these medicinal natural products are closely associated with their identity, authenticity, and quality, which in turn relate to many factors, such as geographical conditions (soil, sunlight, precipitation, and air) and postgrowth factors (harvesting, storage, transportation, manufacturing processes, etc.). In this special issue, we have invited original research articles addressing the novel analytical method development and validation, methodology and instrumentation improvement, chemical characterization, and biological activities of plant materials, extracts, and pure phytochemicals.

Sesquiterpene lactones represent a large group of natural compounds with diversified biological activities. B. Ivanescu et al. presented an overview of methodology on chemical extraction, identification, and quantification, as well as a literature review of biological activities of sesquiterpene lactones found in *Artemisia* genus. Z. Nagy et al. investigated variation of polyphenols, capsaicinoids, and vitamin C in six hybrids of chili peppers with HPLC during ripening period. By means of different detectors, 7 major capsaicinoids, many polyphenols, and vitamin C were identified and quantified. Based on these results, authors observed that the amounts of vitamin C increased and most of polyphenols kept the same level in all hybrids, while major capsaicinoids variation depended on different hybrid peppers during ripening. J. K. Kim et al. addressed the comparison of the nutritional and chemical properties and sensory attributes of *Seomae* mugwort and the commonly consumed species *Artemisia princeps* Pamp. and concluded that *Seomae* mugwort had higher contents of polyunsaturated fatty acids, total phenolic compounds, vitamin C, and essential amino acids and a better radical scavenging activity and more diverse volatile compounds than *A. princeps*.

Q. Wu and X. Gong used LabVIEW, a G language-based virtual instrument software, to establish a system for determination of sugar contents in honey. They stated that the new system may improve the accuracy of the measurement results by avoiding the artificial operation, cumbersome data processing, and the artificial error in optical activity measurement, and thus, it may apply to the analysis of the batch inspection on the sugar degree of honey. M. Dyduch-Siemińska et al. presented their research on the chemical composition of three wild strawberry cultivars fruits. They observed significant differences among these cultivars in terms of physicochemical property, flavonoids contents, phenolic acids, and total tannins and anthocyanins, as well as their antioxidant activity by means of the DPPH method. C. Corsaro et al. presented a method of using ^{1}H HR-MAS NMR technique to investigate metabolites of Mediterranean diet. Authors processed and analyzed the HR-MAS solid-state

NMR data with different software for PCA and quantitative analysis. By combination with qNMR techniques and PCA analysis, authors could distinguish the characterization of Protected Geographical Indication (PGI), Protected Designation of Origin (PDO), and Traditional Italian Food Products (PAT) vegetables from other non-PGI, non-PDO, and non-PAT. The advantage of HR-MAS NMR methodology is the rapidity and simultaneity of the qualitative and quantitative analyses without additional sample treatment. Although its sensitivity is not very high, the advantage of this methodology will make it very attractive for food industry.

G. Negri et al. discussed safety issue of homemade alcoholic beverages (unrecorded) in Brazil. By means of different measurements, including GC-FID/MS, FT-IR, and ICP-ASE, authors investigated a total of 152 samples collected from two cities of Sao Paulo State and seven cities of Minas Gerais State, Brazil. The results revealed that most of these unrecorded alcohol beverages have exceeded the regulatory limitation, including methanol and cyanates. Authors suggested more severe Quality Control (QC) and regulations on these unrecorded beverages are needed. S. Bado et al. investigated physical and chemical variability of tigernuts (*Cyperus esculentus*) cultivated in Burkina Faso. They reported that three *Cyperus esculentus* morphotypes studied are important source of macronutrients (starch, fat, and sucrose) and minerals (potassium, phosphorus, silicon, chlorine, sulfur, and magnesium). Genetic variability exists among cultivated tigernuts from Burkina Faso and from others grown worldwide. M. Godlewska et al. developed a precolumn derivatization method to determine the quantity of α-lipoic acid (LA), an organosulfur compound. In this method the LA degraded product, DHLA, was converted to 2-S-pyridinium derivative with 2-chloro-1-methylquinolinium tetrafluoroborate. It not only increases sensitivity with spectrophotometric assay, but also stabilizes the reaction product to increase reliable results for the determination of LA with HPLC method.

The paper authored by J.-H. Kim et al. developed and validated a quantitative analytical method by using high-performance liquid chromatography equipped with a photodiode array detector for determination of 19 marker compounds in herbal preparations. The method also was combined with a chemometric analysis involving principal component analysis and hierarchical clustering analysis. J. Chen et al. described that a Rapid-Resolution Liquid Chromatography- (RRLC-) Triple Quadrupole Mass Spectrometry with MRM has been developed for characterization of Deer-Horn Glue. It could be used for detection of gelatin adulteration quickly as QC of Deer-Horn Glue, a traditional Chinese medicine. M. M. Celik et al. addressed the protective effects of caffeic acid phenethyl ester (CAPE) and intralipid (IL) on nephrotoxicity caused by acute dichlorvos toxicity on rats. They concluded that both CAPE and IL may prevent the renal injuries with their antioxidant activities.

Shixin Deng
Shao-Nong Chen
Jian Yang

Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 916346, 9 pages
http://dx.doi.org/10.1155/2015/916346

Research Article

Characterization of Nutritional Composition, Antioxidative Capacity, and Sensory Attributes of *Seomae* Mugwort, a Native Korean Variety of *Artemisia argyi* H. Lév. & Vaniot

Jae Kyeom Kim,[1] Eui-Cheol Shin,[2] Ho-Jeong Lim,[2] Soo Jung Choi,[3] Cho Rong Kim,[4] Soo Hwan Suh,[5] Chang-Ju Kim,[6] Gwi Gun Park,[7] Cheung-Seog Park,[8] Hye Kyung Kim,[9] Jong Hun Choi,[10] Sang-Wook Song,[11] and Dong-Hoon Shin[4]

[1] *Department of Food Science and Nutrition, University of Minnesota, Saint Paul, MN 55108, USA*
[2] *Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 660-758, Republic of Korea*
[3] *Functional Food Research Center, Korea University, Seoul 136-701, Republic of Korea*
[4] *Department of Food and Biotechnology, Korea University, Seoul 136-701, Republic of Korea*
[5] *National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong 363-700, Republic of Korea*
[6] *Department of Physiology, Kyung Hee University, Seoul 130-701, Republic of Korea*
[7] *Department of Food Science and Biotechnology, Gachon University, Seongnam 461-701, Republic of Korea*
[8] *Department of Microbiology, Kyung Hee University, Seoul 130-701, Republic of Korea*
[9] *Department of Food Biotechnology, Hanseo University, Seosan 356-706, Republic of Korea*
[10] *Natural Way Company, Pocheon 83-135, Republic of Korea*
[11] *Department of Family Medicine, The Catholic University of Korea, Suwon 442-723, Republic of Korea*

Correspondence should be addressed to Dong-Hoon Shin; dhshin@korea.ac.kr

Received 16 March 2015; Revised 21 May 2015; Accepted 25 May 2015

Academic Editor: Mengxia Xie

Few studies have investigated *Seomae* mugwort (a Korean native mugwort variety of *Artemisia argyi* H. Lév. & Vaniot), exclusively cultivated in the southern Korean peninsula, and the possibility of its use as a food resource. In the present study, we compared the nutritional and chemical properties as well as sensory attributes of *Seomae* mugwort and the commonly consumed species *Artemisia princeps* Pamp. In comparison with *A. princeps, Seomae* mugwort had higher contents of polyunsaturated fatty acids, total phenolic compounds, vitamin C, and essential amino acids. In addition, *Seomae* mugwort had better radical scavenging activity and more diverse volatile compounds than *A. princeps* as well as favorable sensory attributes when consumed as tea. Given that scant information is available regarding the *Seomae* mugwort and its biological, chemical, and sensory characteristics, the results herein may provide important characterization data for further industrial and research applications of this mugwort variety.

1. Introduction

Mugworts (the genus Artemisia) have been widely used as tea, spices, and food ingredients in East Asia. Much attention has been recently paid to their multiple health benefits including anti-tumor-promoting effects [1], induction of apoptosis in various types of cancer cells [2, 3], antidiabetic effects [4], anti-inflammatory effects [5], and anticoagulant/antiplatelet activities [6]. Amongst a plethora of *Artemisia* species, *Artemisia princeps* Pamp., which is widely consumed in Korea, and its bioactive compounds (e.g., eupatilin and jaceosidin) have been most extensively studied in various experimental models [5, 6], yet little information is available regarding the Korean native mugwort variety (also known as *Seomae* mugwort) of *Artemisia argyi* H. Lév. & Vaniot, cultivated in the southern Korean peninsula.

Considering that (1) environmental factors play a significant role in growth as well as the content of active

compounds of *Artemisia* species [7], (2) diverse *Artemisia* species have been demonstrated to have varying biological effects [4], and (3) scant information is available regarding the native variety of *A. argyi* (exclusively cultivated in Namhae County, Republic of Korea) and its biological, chemical, and sensory characteristics, it would be important and timely to report the chemical composition and functionality of this variety and the possibility of its use as a food ingredient. Specifically, in the present study both fatty acids and amino acids profiles were analyzed in order to compare contents of essential fatty acids (e.g., linoleic acid) and essential amino acids. Further, antioxidative capacity, vitamin C contents (i.e., a major vitamin of mugworts), and total phenolic compounds were assessed to address potential health promoting effects thereof. In addition, mugwort teas were prepared using *Seomae* mugwort and *A. princeps* and their sensory attributes were analyzed to test potential for the practical use of *mugwort tea*. All parameters of *Seomae* mugwort analyzed in the study were compared with those of *A. princeps*.

2. Materials and Methods

2.1. Materials. The *Seomae* mugwort (a Korean native variety of *A. argyi*) was kindly provided by the Namhae Agricultural Association Corporation (Namhae, Republic of Korea) where all *Seomae* mugworts harvested in the entire Namhae County are collected. This variety was specifically cultivated in Namhae County, Republic of Korea. *A. princeps* was purchased from a local store (Jinju, Republic of Korea). After being obtained, both mugworts were identified and specimen vouchers were issued by the Department of Agriculture and Herbal Resources of the Gyeongnam National University of Science and Technology (GFA-006 and GFA-007 for *A. princeps* and *Seomae* mugwort, resp.). Leaf samples were completely dried at room temperature, ground, and then stored at −80°C until being analyzed. Heptadecanoic acid (98% purity) and a lipid standard mixture (37 fatty acid methyl esters (FAME)) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals were of analytical grade.

2.2. Analysis of Free Amino Acids. Briefly, 1 g of each sample was added to 20 mL of pure ethanol and agitated for 10 min. After agitation, samples were centrifuged at 3000 ×g for 20 min and the supernatants were evaporated using a rotary evaporator (R-III; BÜCHI, Postfach, Switzerland). The residues were dissolved in 25 mL of Lithium Loading Buffer (Biochrom Ltd., Cambridge, UK) and incubated for 1 h at 4°C after addition of 20 mg of sulfosalicylic acid. Subsequently, samples were centrifuged at 3000 ×g for 20 min and filtered through a 0.2 μm membrane filter. Samples were analyzed using an amino acid analyzer (L-8900; Hitachi High Tech, Tokyo, Japan) equipped with an ion exchange column (2622PF, 4.6 mm × 60 mm; Hitachi High Tech). The column temperature ranged between 30°C and 70°C and the detection wavelengths were 570 nm and 440 nm.

2.3. Fatty Acid Composition

2.3.1. Lipid Extraction. Total lipids were extracted as reported elsewhere with slight modifications [8]. Ten grams of ground samples was suspended in 20 mL of deionized water, 50 mL of methanol, and 25 mL of chloroform, and ~10 mg of hydroquinone was subsequently added. The contents were agitated on an orbital shaker for 2 min at 3000 ×g and the resulting slurry was filtered using a filter paper (Whatman No. 1; GE Healthcare, Little Chalfont, UK). One gram of sodium chloride was added to the filtrate to facilitate phase separation and the filtrate was placed at room temperature overnight. The resulting chloroform phase was then evaporated and samples were stored under a nitrogen headspace at −80°C until being further analyzed.

2.3.2. Fatty Acid Methylation. To analyze fatty acid profiles, FAME were prepared as described previously [9]. In brief, extracted lipids (25 mg) were transferred into a Reacti-Vial (Thermo Fisher Scientific, Rockford, IL, USA) and their mass was accurately measured. The internal standard (heptadecanoic acid in hexane, 1 mg/mL) was added and samples were mixed with 0.5 N sodium hydroxide in methanol followed by flushing with nitrogen gas. The mixtures were then placed in a heating block set at 100°C for 5 min. After cooling, 2 mL of 14% boron trifluoride solution (in methanol) was added to each vial equipped with a Reacti-Vial magnetic stirrer. The vials were vortexed and placed in the Reacti-Block B-1 aluminum block within a Reacti-Therm III Heating/Stirring Module (Thermo Fisher Scientific) at 100°C for 30 min. After derivatization, each sample was extracted with 1.5 mL of hexane.

2.3.3. GC Analysis. An Agilent Technologies (Santa Clara, CA, USA) 7890A Network GC system equipped with a flame ionization detector (FID) was used to quantify fatty acids. Chromatography was performed on an SP-2560 capillary column (100 m × 0.25 mm *i.d.*, 0.25 μm film thickness; Sigma-Aldrich Co.). The analyses were performed in the constant flow mode. A split liner with glass wool was installed in the injector and the injector temperature was set at 220°C for injection. The FID temperature was set at 240°C, and ultra-high purity hydrogen (flow rate: 40 mL/min) and scientific-grade air (flow rate: 450 mL/min) were used as the FID fuel gases. The temperature of the oven was initially held at 140°C for 5 min and then was ramped up at 4°C/min to 230°C and maintained at 230°C for an additional 35 min. Triplicate readings were taken.

2.3.4. Fatty Acid Identification. Using the internal standard (heptadecanoic acid), the relative response factor for each FAME was calculated by using the following equation:

$$R_i = \frac{(Ps_i \times Ws_{C17:0})}{(Ps_{C17:0} \times Ws_{is})}, \quad (1)$$

where R_i is the relative response factor for fatty acid i, Ps_i is the peak area of individual FAME i in the FAME standard solution, $Ws_{C17:0}$ is the mass (mg) of heptadecanoic acid

FAME in the injected FAME standard solution, $Ps_{C17:0}$ is the peak area of heptadecanoic acid FAME in the FAME standard solution, and Ws_{is} is the mass (mg) of individual FAME i in the injected FAME standard solution.

2.3.5. Method Validation for Fatty Acid Analysis. The relative repeatability standard deviation and % relative standard deviation were determined to validate the method for fatty acid analysis in lipid extracts of *Seomae* mugwort and *A. princeps* by assaying Standard Reference Material (SRM) 1849a (Infant/Adult Nutritional Formula) purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.4. Determination of Total Phenolic Compounds. The total phenolic contents of *Seomae* mugwort and *A. princeps* were compared using a previously described spectrophotometric method with slight modifications [10]. The dried samples were prepared at a concentration of 1 mg/mL of water and then a 40 μL aliquot of each sample was diluted with 200 μL of distilled water. Folin-Ciocalteu's reagent (200 μL; Sigma-Aldrich Co.) was added to the mixture, followed by the addition of 600 μL of sodium carbonate solution (30%, w/v) and 160 μL of distilled water. The mixture was thoroughly mixed and kept in the dark for 2 h at 25°C, after which the absorbance was read at 750 nm. The total phenolic compounds in each sample were determined from interpolation of the calibration curve constructed by using gallic acid solution (0–500 μg/mL).

2.5. Analysis of Vitamin C Contents. One gram of ground mugwort sample was added to 1 mL of 10% formic acid and then diluted with 19 mL of 5% formic acid. Samples were thoroughly vortexed and placed at room temperature for 20 min followed by centrifugation (1,000 ×g; 10 min). Resulting supernatants were filtered through a HPLC membrane filter (Sigma-Aldrich Co., Nylon 66 Filter Membranes, 0.45 μm) and injected to a HPLC system (10 μL injection; Shimadzu, Kyoto, Japan). Isocratic method (0.05 M of phosphate buffer and acetonitrile, 60 : 40) was used for the separation of vitamin C using the Bondapak C18 column (Waters, Milford, MA, USA) which was utilized for separation and analytes were monitored at 245 nm wavelength. The standard curve was constructed using the authentic standard for quantification of vitamin C. The r-squared value of the standard curve was greater than 0.99.

2.6. Antioxidative Capacity Measurement. The antioxidant capacity of *Seomae* mugwort and *A. princeps* was compared using a typical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described elsewhere [11]. In brief, serial dilutions of samples were prepared (200, 400, 600, and 800 μg/mL) and then 80 μL of each sample was added to 320 μL of DPPH solution (0.2 mM, dissolved in pure ethanol). The reactions were performed in an incubator at 37°C and the absorbance was measured at 517 nm. The IC_{50} of each sample was calculated by using the following equation:

$$\text{Radical scavenging activity (\%)} = \left(1 - \frac{\text{the absorbance of the treated sample}}{\text{the absorbance of control sample}}\right) \times 100. \quad (2)$$

2.7. Analysis of Volatile Compound Composition. A Likens and Nickerson–type simultaneous steam distillation and extraction apparatus (SDE) was used for the extraction of volatile compounds according to the method reported elsewhere [12]. Ground samples (100 g) were mixed with distilled water (1 L) followed by the addition of internal standard (1 mL of n-pentadecane, 1 mg/mL, Sigma-Aldrich Co.). Atmospheric steam distillation was performed to collect sample volatiles in a 100 mL mixture of n-pentane and diethyl ether (1 : 1, v/v) over 3 h at 110°C. Anhydrous sodium sulfate (10 g) was added to the extracts, which were then placed at 4°C overnight. Samples were then filtered and reduced to a volume of 1 mL using a nitrogen evaporator. Concentrated samples were analyzed using a GC fitted with a mass spectrometer (Agilent 7890A and 5975C, resp.), which was operated in electron impact ionization mode (70 eV), scanning a mass range (m/z) from 30 to 550 amu. An HP-5MS column (30 m × 0.25 mm, *i.d.* × 0.25 μm film thickness, Agilent Technologies) was used for the analysis. The temperature of the column was maintained at 4°C for the first 5 min and then increased to 200°C at a rate of 5°C/min. The analysis was carried out in the splitless mode, using helium as the carrier gas (1 mL/min flow rate). The injector temperature was 220°C. Separated peaks in the total ionization chromatogram were identified using a database (The NIST 12 Mass Spectrum Library; Gaithersburg, MD, USA) and then confirmed by matching the retention indices (RI) with data from published literature. RI were calculated according to the following formula [13] and based on a series of n-alkanes (C8–C20):

$$\text{RI}_x = 100n + 100\left(\frac{t_{Rx} - t_{Rn}}{t_{Rn+1} - t_{Rn}}\right), \quad (3)$$

where RI_x is RI of the unknown compound, t_{Rx} is retention time of the unknown compound, t_{Rn} is retention time of the n-alkane, and t_{Rn+1} is retention time of the next n-alkane. Each t_{Rx} is between t_{Rn} and t_{Rn+1} (n = number of carbon atoms).

2.8. Olfactometry Analysis. Separated volatile compounds were further analyzed through an olfactory detection port with a heated mixing chamber (ODP 3; Gerstel, Linthicum, MD, USA). In advance of performing experiments, panels were trained with the instrument operation and data collection. Specifically, they were asked to respond to their perceived intensity of odor through the detection port using a signal generator. The intensity scales of the signal generator ranged from 0 (no perception) to 5 (the strongest perception).

Table 1: Demographic information of study participants and frequency of tea consumption[(a)].

	Percentage (n)
Gender	
Male	33 (5)
Female	67 (10)
Age	
19–29	87 (13)
30–40	13 (2)
≤40	0
Frequency of tea consumption (per month)	
Never	13 (2)
≥5 times	60 (9)
≥10 times	27 (4)
≥20 times	0 (0)
Daily	0 (0)

[(a)]A total of 15 participants were recruited from the Gyeongnam National University of Science and Technology through fliers. The protocol was approved by the University Institutional Review Board and written consent forms were obtained from the participants in advance ofcollecting data.

To take into account individual variations, 3 trained panels performed an identical experiment and recorded the intensity of each volatile compound isolated from the samples.

2.9. Evaluation of Sensory Attributes of Seomae Mugwort and A. princeps

2.9.1. Study Participants. A total of 15 participants evaluated the sensory attributes of the two *Artemisia* species. All subjects were recruited from the Gyeongnam National University of Science and Technology through fliers and received a gift card incentive for participation. People who discovered themselves having allergy to either *Seomae* mugwort or *A. princeps* were screened prior to the sensory evaluation. The study was approved by the University Institutional Review Board and consent forms were provided to participants. The *demographic* characteristics *are summarized in* Table 1.

2.9.2. Tea Preparation and Sensory Evaluation. To prepare mugwort tea, 5 g of a dried sample was added to 1 L of boiling water and brewed for 5 min. All preparation steps were performed by a professional cook and samples were prepared about 10 min before sensory evaluation. Teas prepared from both species (100 mL each) were provided to each subject. Participants evaluated the teas for perceived color acceptability, flavor acceptability, saltiness, bitterness, sourness, astringency, sweetness, and overall preference using labeled affective magnitude (LAM) scales; the scales were labeled with the phrases "greatest imaginable like," "like extremely," "like very much," "like moderately," "like," "neither like nor dislike," "dislike moderately," "dislike very much," "dislike extremely," and "greatest imaginable dislike." The scales ranged from 0 (greatest imaginable dislike) to 15 (greatest imaginable like) [14].

Table 2: Free amino acids profile of *Artemisia princeps* Pamp. and *Seomae* mugwort[(a)].

	A. princeps	*Seomae* mugwort
Essential amino acid (mg/100 g of dried material)		
Histidine	7.18 ± 0.16^{a}	2.54 ± 0.06^{b}
Phenylalanine	66.05 ± 0.26^{b}	93.78 ± 0.74^{a}
Valine	102.71 ± 1.97^{b}	167.07 ± 0.85^{a}
Leucine	59.26 ± 0.65^{a}	44.51 ± 0.60^{b}
Isoleucine	61.56 ± 0.89^{a}	47.62 ± 00.57^{b}
Threonine	22.20 ± 1.03^{a}	15.12 ± 0.29^{b}
Nonessential amino acid (mg/100 g of dried material)		
Arginine	29.47 ± 0.68^{a}	20.55 ± 0.32^{b}
γ-Aminobutyric acid	12.60 ± 0.18^{b}	48.52 ± 0.87^{a}
Alanine	86.90 ± 0.88^{a}	34.29 ± 0.60^{b}
Cysteine	4.42 ± 0.30	4.52 ± 0.25
Glutamic acid	23.59 ± 0.68^{b}	33.45 ± 0.36^{a}
Tyrosine	7.62 ± 0.13^{b}	10.77 ± 0.38^{a}
Glycine	4.57 ± 0.25^{b}	11.39 ± 0.35^{a}
β-Alanine	16.18 ± 0.78	15.96 ± 0.09
α-Aminobutyric acid	2.53 ± 0.44^{b}	6.54 ± 0.37^{a}
Aspartic acid	7.01 ± 0.51^{b}	8.68 ± 0.29^{a}
Serine	47.52 ± 0.61	47.48 ± 0.65
Total essential amino acid	318.93 ± 1.22^{b}	370.64 ± 0.27^{a}
Total nonessential amino acid	242.35 ± 2.24	242.15 ± 2.80
Total free amino acid	561.28 ± 3.30^{b}	612.79 ± 2.97^{a}

[(a)]Data represents the mean ± SD ($n = 3$). Different superscript letters indicate statistical significance of the differences between *Seomae* mugwort and *A. princeps* groups, tested by Student's t-test using the SAS. P values less than 0.05 were considered statistically significant.

2.10. Statistical Analysis. All results were expressed as the mean ± standard deviation (SD). The statistical significance between groups (i.e., *Seomae* mugwort versus *A. princeps*) was tested via Student's t-test, using the Statistical Analysis System (SAS; Cary, NC, USA). A P value less than 0.05 was considered to be statistically significant.

3. Results and Discussion

To compare general nutritional compositions of *Seomae* mugwort and *A. princeps*, we analyzed the content of free amino acids, fatty acids, vitamin C, and total phenolic compounds. First, we found that the content of free amino acids of *A. princeps* was significantly different from that of *Seomae* mugwort (Table 2). Specifically, the content of the essential amino acids valine and phenylalanine was significantly higher in *Seomae* mugwort (by approximately 63% and 41%, resp.) than in *A. princeps*. The content of total essential amino acids was approximately 57% in *A. princeps* and 61% in *Seomae* mugwort. Notably, it has been reported that γ-aminobutyric acid (GABA), a nonprotein amino acid, is beneficial for

Table 3: Method validation of fatty acids analysis: % accepted values and % relative standard deviations (RSD) determined using SRM 1849a.

Fatty acids	% weight			% of accepted value[d]	% RSD[e]
	Accepted value[a]	Analytical value[b]	Bias[c]		
C14:0	4.76 ± 0.14	4.79 ± 0.13	−0.03	100.63	2.71
C16:0	9.89 ± 1.10	9.81 ± 0.21	0.08	99.19	2.14
C16:1 ω-7	0.12 ± 0.01	0.13 ± 0.01	−0.01	108.33	7.69
C18:0	4.21 ± 0.10	4.25 ± 0.05	−0.04	100.95	1.18
C18:1 ω-9	50.37 ± 5.51	50.45 ± 2.72	−0.08	100.16	5.39
C18:1 ω-7	1.02 ± 0.03	1.03 ± 0.05	−0.01	100.98	4.85
C18:2 ω-6	25.95 ± 2.11	25.82 ± 1.10	0.13	99.50	4.26
C18:3 ω-3	0.42 ± 0.01	0.46 ± 0.02	−0.04	109.52	4.35
C20:0	0.24 ± 0.03	0.26 ± 0.01	−0.02	108.33	3.85
C20:1 ω-9	2.51 ± 0.26	2.52 ± 0.05	−0.01	100.40	1.98
C22:0	0.34 ± 0.01	0.32 ± 0.01	0.02	94.12	3.13
C24:0	0.17 ± 0.01	0.16 ± 0.01	0.01	94.12	6.25

[a]The accepted value was calculated using the certified fatty acids content of SRM 1849a based on % weight.
[b]Data represents the mean ± SD ($n = 3$). [c]Bias = accepted value − analytical value. [d]The ratio of the analytical value to accepted value expressed as a percentage. [e]RSD indicates interday relative standard deviation (SD × 100/mean) of analytical values.

treatment of general anxiety and anxiety disorders [15, 16]. We found that *Seomae* mugwort had approximately 3.8-fold higher content of GABA than *A. princeps*, indicating potential benefits of this variety in medicinal psychopharmacology, which warrants further investigations.

The fatty acid analysis method was validated before determination of the fatty acid composition of *Seomae* mugwort and *A. princeps* (Table 3). The accuracy of the method was calculated based on the percentage of the certified fatty acid content in SRM 1849a and expressed as the percentage of the accepted value. The accuracy ranged from 94.12 to 108.33%, while the reproducibility of the method, indicated by the relative standard deviation (RSD), was higher than 90% for all fatty acids. The complete fatty acid profiles of *Seomae* mugwort and *A. princeps* are shown in Table 4. In total, nine fatty acids, ranging from C16 to C24, were detected based on retention mapping with external standards. These fatty acids were quantified relative to the internal standard (heptadecanoic acid). In *A. princeps*, C18:1 and C18:2 were the most prevalent fatty acids (34.91% and 27.56%, resp.), followed by C18:3 ω-6 (9.83%), C16:0 (8.73%), and other fatty acids. Interestingly, the content of C18:3 ω-6 was much higher in *Seomae* mugwort (36.36%, Table 4). *Artemisia princeps* had a lower total content of saturated fatty acids than *Seomae* mugwort (27.47% versus 40.79%), while the content of polyunsaturated fatty acids was higher in *Seomae* mugwort, likely due to C18:3 ω-6 (Table 4).

The amount of phenolic compounds in *A. princeps* was 49.12 ± 1.23 mg per 100 g of dried material whilst it was much higher (by approximately 50%) in *Seomae* mugwort (74.53 ± 2.08 mg per 100 g, Table 5). Further, the vitamin C content of *Seomae* mugwort was 2-fold higher than that in *A. princeps*. Specifically, it was found that *Seomae* mugwort contains 209.1 ± 3.2 mg of vitamin C per 100 g of dried sample materials (Table 5). We compared the antioxidative capacities of

Table 4: Comparison of fatty acid profiles between *Artemisia princeps* Pamp. and *Seomae* mugwort[a].

Fatty acids	*A. princeps*	*Seomae* mugwort
C16:0	8.73 ± 0.06^{b}	18.82 ± 0.15^{a}
C16:1	0.23 ± 0.01^{b}	2.04 ± 0.05^{a}
C18:0	3.54 ± 0.04^{a}	1.66 ± 0.07^{b}
C18:1	34.91 ± 0.06^{a}	5.09 ± 0.09^{b}
C18:2	27.56 ± 0.07^{a}	15.73 ± 0.12^{b}
C20:0	2.53 ± 0.04^{b}	3.63 ± 0.13^{a}
C18:3 ω-6	9.83 ± 0.06^{b}	36.36 ± 0.20^{a}
C22:0	8.58 ± 0.14^{b}	10.91 ± 0.09^{a}
C24:0	4.08 ± 0.14^{b}	5.76 ± 0.07^{a}
SFA[b]	27.47 ± 0.08^{b}	40.79 ± 0.10^{a}
MUFA[c]	35.14 ± 0.03^{a}	7.12 ± 0.07^{b}
PUFA[d]	37.39 ± 0.06^{b}	52.09 ± 0.16^{a}

[a]Data represents the mean ± SD ($n = 3$). Different superscript letters indicate statistical significance of the differences between *Seomae* mugwort and *A. princeps* groups, tested by Student's t-test using the SAS. P values less than 0.05 were considered statistically significant. [b]SFA: saturated fatty acids. [c]MUFA: monounsaturated fatty acids. [d]PUFA: polyunsaturated fatty acids.

the two mugwort species using the DPPH radical scavenging assay and found that the IC_{50} value of *Seomae* mugwort was 0.55 ± 0.09 mg, whereas *A. princeps* extract required a higher concentration, 0.82 ± 0.12 mg, which is expected given the significantly higher amounts of vitamin C/total phenolic compounds in the *Seomae* mugwort. Generally, the antioxidant activity is closely correlated with the amount of phenolic compounds [17, 18]; this trend was also observed in the present study (Table 5). However, due to the inherent limitations of the method (e.g., nonspecific oxidation by

Table 5: Total phenolic contents, vitamin C contents, and antioxidative capacities of *Artemisia princeps* Pamp. and *Seomae* mugwort[(a)].

	A. princeps	*Seomae* mugwort
Total phenolic content (mg/100 g of dried sample)[(b)]	49.12 ± 1.23^{b}	74.53 ± 2.08^{a}
IC_{50} in DPPH radical scavenging (mg)[(c)]	0.82 ± 0.12^{a}	0.55 ± 0.09^{b}
Vitamin C content (mg/100 g of dried sample)[(d)]	100.6 ± 2.2^{b}	209.1 ± 3.2^{a}

[(a)]Data represents the mean ± SD ($n = 3$). Different superscript letters indicate statistical significance of the differences between *Seomae* mugwort and *A. princeps* groups, tested by Student's t-test using the SAS. P values less than 0.05 were considered statistically significant. [(b)]The total phenolic contents of samples were measured using Folin-Ciocalteu's reagent as described in the Materials and Methods. [(c)]The IC_{50} values of *A. princeps* and *Seomae* mugwort were calculated and compared using a typical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. [(d)]The vitamin C was analyzed using the HPLC as described in the Materials and Methods.

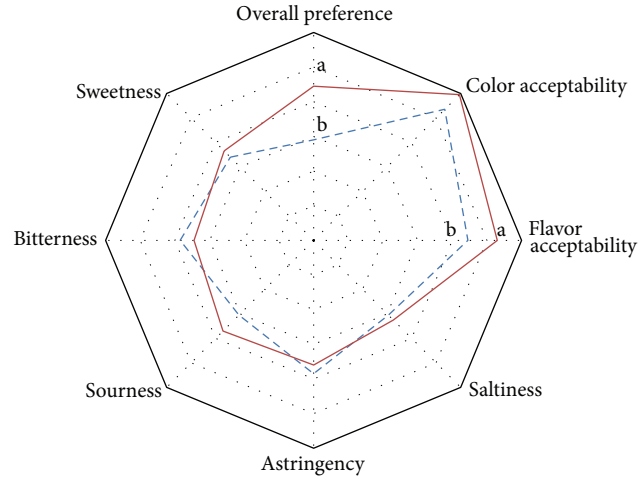

Figure 1: Comparison of the sensory profiles of mugwort tea prepared with either *Artemisia princeps* Pamp. or *Seomae* mugwort (a Korean native variety of *Artemisia argyi* H. Lév. & Vaniot). A total of 15 participants used LAM scales for perceived color acceptability, flavor acceptability, saltiness, bitterness, sourness, astringency, sweetness, and overall preference. Dashed line and solid line indicate *A. princeps* and *Seomae* mugwort, respectively. Preference scales ranged from 0 (greatest imaginable dislike) to 15 (greatest imaginable like). Different superscript letters indicate statistical significance of the differences between *Seomae* mugwort and *A. princeps* groups, tested by Student's t-test using the SAS. P values less than 0.05 were considered statistically significant.

Folin-Ciocalteu's reagent), identification of specific phenolic constituents was not possible in our experiments. Their identification in the future might further elucidate the health benefits of these mugwort species.

Using SDE, 43 volatile compounds were identified in *A. princeps* and 50 in *Seomae* mugwort (Table 6). Representative chromatograms of both mugwort species are shown in Supplemental Figure 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/916346. Intuitively, it is clear that *Seomae* mugwort should have more diverse profiles of volatiles given the numbers of compounds listed in the table and identified in chromatograms, as well as their peak areas. This was further supported by olfactometry analysis by three trained panels. Strong intensities of *Seomae* mugwort were recorded mostly between 12 min and 21 min of the aromagram (Supplemental Figure 1(C)). Notably, within this range of retention times, a few volatile chemicals present in *Seomae* mugwort had significantly higher peak areas. For instance, terpenic compounds (e.g., α-terpinolene and α-terpinene) were significantly more abundant in *Seomae* mugwort than in *A. princeps*; most of these compounds were not detected in *A. princeps* (e.g., α-terpinene, 1,8-cineole, camphor, and 4-terpineol; Table 6). It has been reported that these terpenic compounds possess characteristic woody, citrus, floral, and herbal flavors [19], which possibly confer more favorable sensory characteristics when consumed in the present study. Importantly, the sensory attributes of volatile compounds are difficult to predict due to potential associations between aromas of different compounds (e.g., synergistic or masking effects) [19]. In the olfactometry analysis, we only recorded the aroma intensities but were unable to assess their flavor descriptions and acceptability. Hence, comparative sensory evaluation of *Seomae* mugwort and *A. princeps* was performed.

To examine the potential for the practical use of *mugwort tea* as a *nutritious drink, we prepared tea samples from both mugwort species.* As mentioned above, the participants were asked to evaluate perceived preference for each mugwort tea. Subjects evaluated color acceptability prior to consuming the samples. Then, other perceived qualities (flavor acceptability, saltiness, bitterness, sourness, astringency, sweetness, and overall preference) were evaluated after sample consumption by using the LAM scales of 0–15 points. We did not find any differences in sweetness (6.8 ± 1.8 and 7.3 ± 1.2 for *A. princeps* and *Seomae* mugwort), bitterness (7.7 ± 1.5 and 6.9 ± 2.1 for *A. princeps* and *Seomae* mugwort), sourness (6.1 ± 2.2 and 7.4 ± 2.4 for *A. princeps* and *Seomae* mugwort), astringency (7.7 ± 2.1 and 7.2 ± 2.2 for *A. princeps* and *Seomae* mugwort), and saltiness (6.1 ± 2.4 and 6.5 ± 1.8 for *A. princeps* and *Seomae* mugwort) between samples. There were, however, significant differences in overall preference (5.8 ± 0.9 and 8.9 ± 1.1 for *A. princeps* and *Seomae* mugwort; $P < 0.05$) and flavor acceptability (8.9 ± 1.1 and 10.6 ± 1.0 for *A. princeps* and *Seomae* mugwort; $P < 0.05$, Figure 1), which may be due to the differences in the profiles of volatile compounds between *Seomae* mugwort and *A. princeps*, in particular the difference in terpenic compounds (Table 6). Of many properties, we were specifically interested in "bitter taste" and "astringency," which may impact consumers' preference and palatability. In the analysis of free amino acids, we found that the content of branched-chain amino acids was slightly higher in *Seomae* mugwort than in *A. princeps* (259.20 mg/100 g versus 223.53 mg/100 g; Table 2). Branched-chain amino acids (leucine, isoleucine, and valine) are known to confer bitter taste [20]. However, our results indicate that there was no

Table 6: Volatile compounds present in *Artemisia princeps* Pamp. and *Seomae* mugwort[(a)].

Peak number[(b)]	Compounds[(c)]	Retention time (min)	Peak area $\times 10^3$	
			A. princeps	*Seomae* mugwort
1	Propanoic acid methyl ester	3.32	4,655.5 ± 502.1[b]	43,759.7 ± 1,202.3[a]
2	Acetic acid ethyl ester	3.91	873.6 ± 90.2[b]	8,365.1 ± 902.1[a]
3	2,3-Dimethyl pentane	4.79	89.1 ± 84.3[b]	984.5 ± 42.5[a]
4	Butyl ethyl ether	5.39	478.3 ± 63.1[b]	4,321.9 ± 472.5[a]
5	Diethyl sulfide	5.51	61.5 ± 33.2[b]	1,687.2 ± 202.9[a]
6	Acetal	6.29	1,122.9 ± 172.6[b]	13,265.2 ± 1,502.5[a]
7	2-Methyl-2-hexanol	6.98	Not detected[b]	1,178.4 ± 227.3[a]
8	Valeric acid methylbutyl ester	7.07	1,646.2 ± 216.2[b]	19,573.6 ± 1,312.4[a]
9	Methylbenzene	7.32	372.7 ± 39.3[b]	3,455.6 ± 482.1[a]
10	2-Furancarboxaldehyde	9.45	53.3 ± 32.1[b]	953.3 ± 113.2[a]
11	Chlorobenzene	9.87	59.5 ± 29.6[b]	757.16 ± 221.5[a]
12	2-Hexenal	10.13	Not detected[b]	1,323.3 ± 160.5[a]
13	Ethyl benzene	10.40	1,253.9 ± 264.3[b]	15,135.1 ± 1,302.1[a]
14	*m*-Xylol	10.67	42.9 ± 40.7[b]	624.01 ± 129.4[a]
15	*o*-Xylol	11.47	89.3 ± 66.2[b]	1,628.5 ± 278.4[a]
16	α-Terpinolene	12.62	Not detected[b]	60,468.9 ± 2,532.8[a]
17	α-Pinene	12.85	252.9 ± 102.5[b]	5,512.4 ± 762.0[a]
18	Camphene	13.35	Not detected[b]	2,842.3 ± 388.7[a]
19	Sabinene	14.18	49.8 ± 36.1[b]	357.3 ± 94.4[a]
20	β-Pinene	14.29	220.3 ± 100.5[b]	1,473.5 ± 233.5[a]
21	1-Octen-3-ol	14.34	Not detected	3,433.3 ± 582.3[a]
22	β-Myrcene	14.72	119.4 ± 84.5[a]	Not detected[b]
23	Yomogi alcohol	15.08	Not detected[b]	288,651.3 ± 1,321.1[a]
24	α-Terpinene	15.57	Not detected[b]	1,863.8 ± 282.4[a]
25	*o*-Cymene	15.83	Not detected[b]	1,483.3 ± 248.5[a]
26	D-Limonene	15.96	75.9 ± 63.1[b]	750.3 ± 121.5[a]
27	1,8-Cineole	16.05	Not detected[b]	32,351.2 ± 1,321.8[a]
28	2,4-Hexadiene	16.17	Not detected[b]	5,933.3 ± 567.3[a]
29	Phenyloxirane	16.42	695.1 ± 111.8[a]	Not detected[b]
30	Benzeneacetaldehyde	16.43	Not detected[b]	5,493.2 ± 484.3[a]
31	γ-Terpinene	16.90	Not detected[b]	1,384.6 ± 233.2[a]
32	*cis*-β-Terpineol	17.21	Not detected[b]	617.9 ± 171.3[a]
33	Artemisia alcohol	17.78	Not detected[b]	533,734.3 ± 8,242.0[a]
34	β-Linalool	18.15	136.7 ± 70.3[b]	17,562.3 ± 1,382.4[a]
35	Nonanal	18.25	80.7 ± 29.5[a]	Not detected[b]
36	Camphor	19.57	Not detected[b]	4,463.87 ± 529.4[a]
37	4-Terpineol	20.49	Not detected[b]	4,215.52 ± 498.5[a]
38	β-Fenchyl alcohol	20.88	Not detected[b]	2,583.98 ± 200.4[a]
39	Indole	23.69	144.0 ± 101.1[b]	1,073.78 ± 218.3[a]
40	δ-Elemene	24.87	75.4 ± 43.1[a]	Not detected[b]
41	Eugenol	25.34	Not detected[b]	13,037.30 ± 1,009.3[a]
42	α-Copaene	25.90	325.0 ± 112.2[b]	2,643.10 ± 183.6[a]
43	β-Bourbone	26.16	65.25 ± 45.6[b]	2,933.01 ± 438.3[a]
44	β-Elemene	26.27	845.4 ± 205.1[a]	Not detected[b]
45	Caryophyllene	27.06	13,728.3 ± 1,225.3[b]	85,473.18 ± 5,384.5[a]
46	β-Copaene	27.26	Not detected[b]	1,417.52 ± 135.8[a]
47	α-Amorphene	27.65	60.5 ± 60.9[a]	Not detected[b]

Table 6: Continued.

Peak number[(b)]	Compounds[(c)]	Retention time (min)	Peak area $\times 10^3$	
			A. princeps	*Seomae* mugwort
48	*cis*-β-Farnesene	27.75	1,890.6 ± 210.9[b]	2,483.2 ± 499.3[a]
49	α-Humulene	27.88	3,921.7 ± 673.3[b]	9,065.3 ± 886.1[a]
50	γ-Muurolene	28.37	Not detected[b]	1,646.7 ± 245.3[a]
51	γ-Curcumene	28.38	998.1 ± 89.0[a]	Not detected[b]
52	β-Cubebene	28.54	16,826.3 ± 1,533.2[b]	29,434.57 ± 5,553.7[a]
53	β-Selinene	28.68	Not detected[b]	8,386.7 ± 1,334.3[a]
54	Zingiberene	28.75	6,225.4 ± 562.1[a]	Not detected[b]
55	Germacrene B	28.91	1,347.8 ± 113.2[a]	Not detected[b]
56	α-Farnesene	28.99	889.5 ± 82.0[a]	Not detected[b]
57	β-Bisabolene	29.07	200.2 ± 121.2[a]	Not detected[b]
58	γ-Cadinene	29.29	353.5 ± 178.1[b]	3,976.39 ± 529.9[a]
59	δ-Cadinene	29.47	1,345.4 ± 203.1[b]	4,073.7 ± 587.9[a]
60	α-Cadinene	29.82	211.4 ± 52.2[a]	Not detected[b]
61	*trans*-β-Farnesene	30.33	158.2 ± 78.0[a]	Not detected[b]
62	Nerolidol	30.34	Not detected[b]	3,122.5 ± 443.9[a]
63	Caryophyllene oxide	30.95	315.3 ± 192.1[a]	Not detected[b]
64	Diethyl phthalate	31.02	233.6 ± 54.2[a]	Not detected[b]
65	α-Guaiene	31.30	Not detected[b]	1,347.3 ± 309.4[a]
66	tau-Muurolol	32.20	349.1 ± 120.2[a]	Not detected[b]

[(a)] Data represents the mean ± SD ($n = 3$). Different superscript letters indicate statistical significance of the differences between *Seomae* mugwort and *A. princeps* groups, tested by Student's t-test using the SAS. P values less than 0.05 were considered statistically significant. [(b)] Peak numbering was determined by the order of elution. [(c)] The gas chromatographic retention data and mass spectral data were compared to those of authentic samples and library compounds, respectively.

difference in such unfavorable tastes between these mugwort species. No significant correlation was found between tested sensory attributes and the frequency of tea consumption as well as participants' sex (data not shown). Considering the small number of participants, further investigations may be warranted to clarify and confirm the observed trends. Furthermore, it would be interesting to include another type of tea (e.g., green tea) in sensory evaluation as a control for a direct comparison with its sensory attributes. Lastly, given the nature of sensory evaluation, it is also possible that perceived attributes relatively vary with individuals; thus, descriptive sensory evaluation with trained panelists might be warranted in the future.

4. Conclusions

In the present study, we compared the nutritional characteristics and sensory attributes of *Seomae* mugwort, a native mugwort variety of *A. argyi* cultivated in Namhae County in South Korea, and those of *A. princeps*. The native variety showed (1) higher contents of essential amino acids without compromising flavor, (2) higher amount of polyunsaturated fatty acids, likely due to an increased content of C18:3 ω-3, (3) better radical scavenging activity against DPPH and higher vitamin C/total phenolic compound contents, and (4) more diverse volatile compounds with more favorable sensory attributes when consumed as tea. Given that scant information is available regarding the *Seomae* mugwort and its biological, chemical, and sensory characteristics, the results of this study may provide important preliminary data for further industrial and research applications of this mugwort variety.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jae Kyeom Kim and Eui-Cheol Shin equally contributed to this work.

Acknowledgment

This research was supported by High Value-Added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 831238, 8 pages
http://dx.doi.org/10.1155/2015/831238

Research Article

The Content of Secondary Metabolites and Antioxidant Activity of Wild Strawberry Fruit (*Fragaria vesca* L.)

Magdalena Dyduch-Siemińska,[1] Agnieszka Najda,[2] Jan Dyduch,[2] Magdalena Gantner,[3] and Kamila Klimek[4]

[1]*Department of Genetics and Horticultural Plant Breeding, University of Life Sciences in Lublin, Akademicka Street 15, 20-068 Lublin, Poland*
[2]*Department of Vegetable and Medicinal Plants, University of Life Sciences in Lublin, K. Leszczyńskiego Street 58, 20-068 Lublin, Poland*
[3]*Division of Engineering in Nutrition, Warsaw University of Life Sciences, Nowoursynowska Street 159, 02-776 Warsaw, Poland*
[4]*Department of Applied Mathematics and Informatics, University of Life Sciences in Lublin, Głęboka Street 28, 20-612 Lublin, Poland*

Correspondence should be addressed to Agnieszka Najda; agnieszka.najda@up.lublin.pl

Received 1 March 2015; Accepted 20 May 2015

Academic Editor: Constantinos Georgiou

Chemical analyses carried out in 2011–2013 aimed at evaluating the contents of flavonoids, free phenolic acids, tannins, anthocyanins, and antioxidant activity (%) by means of DPPH radical neutralization ability in fresh and air-dried fruits of three wild strawberry cultivars. Examinations revealed differences in contents of biologically active substances determined in raw versus dried material depending on the cultivar. Mean concentrations of flavonoids and tannins were highest in raw fruits of "Baron von Solemacher" cv., which amounted to 1.244 mg·g^{-1} and 6.09%, respectively. Fresh fruits of "Regina" cv. were characterized by the highest average content of phenolic acids and anthocyanins: 4.987 mg·g^{-1} and 0.636 mg·100 g^{-1}. The pattern of mean contents of biologically active substances analyzed in air-dried fruits was similar. Significant differences in abilities to neutralize the DPPH radical to diphenylpicrylhydrazine by extracts made of examined wild strawberry fruits were also indicated.

1. Introduction

Fragaria vesca L. is a well-known and valuable plant species; however, its cultivation is still much poorly spread [1]. Currently grown cultivars of *Fragaria vesca* are derived from a wild species, which can be found in woods and grasslands in Europe, Western Asia, North America, and temperate areas in Chile [2]. *Fragaria vesca* ssp. *vesca* have been cultivated for centuries in European gardens. Their widespread temperate growing range, self-compatibility, and long history of cultivation, coupled with selection for favorable recessive traits such as day neutrality, nonrunnering, and yellow-fruited forms, offer extensive genotypic diversity [3]. Despite high price, wild strawberries fruits are a product that is highly appreciated by consumers [1]. Their natural aspect, color, nutritional values, and high natural antioxidant compounds content are their most attractive characteristics. A high percentage of these fruits are sold as a frozen product, which is used in the manufacturing of cakes, ice creams, or milk desserts [1, 4]. There are an increasing demand for fresh berries and, consequently, a need to increase their distribution ratio and shelf life [1]. *Fragaria* spp. is unusual in what is called the fruit actually originates from the expansion of the flower base (the receptacle) as a pseudocarp, with the real one seeded fruits (achenes) on the epidermal layer [5–7]. Wild strawberry fruits ripen during 3-4 weeks after flowers develop, although the period greatly depends on the weather conditions. Many epidemiological studies have shown that a higher consumption of fruit and vegetables is associated with the prevention of chronic diseases such as diabetes, heart

disease, and certain cancers. Apart from essential nutrients, fruit and vegetables also contain a variety of different phytochemicals that can act as antioxidants, prevent oxidation, and also exhibit other bioactive physiological properties. Different antioxidants, such as flavonoids, phenolic acids, carotenoids, and ascorbic acid, have been proposed to act anticarcinogenically [8–13]. Red, spherical, sweet taste fruits (*Fragariae fructus*) with unforgettable flavor are one of the raw materials achieved from wild strawberry [13]. According to much of the research done, wild strawberry fruits have high antioxidant activity, which has been linked to their content of phenolic compounds [4, 14].

Small fruit breeding programs are currently used to acquire new cultivars improved for specific agronomic (yield and size), qualitative (firmness, sugars content, and acidity), and sensorial (colour and aroma) characteristics, all combined to increased disease resistance and plant adaptability. Nowadays, besides all these parameters, it is necessary to look for the specific bioactive components well known for their effect on human health. This aspect is now highly requested by the consumer [15]. Flavonoids and phenolic acids are the most common phenolic compounds in small fruits with strong antioxidant capacity [13, 16–18]. Taking this into account, the following study was carried out to make chemical analyses aim at evaluating the contents of flavonoids, free phenolic acids, tannins, anthocyanins, and antioxidant activity (%) by means of DPPH radical neutralization ability in fresh and air-dried fruits of three wild strawberry cultivars. The aim of these studies was to identify cultivars which were characterized by the highest content of investigated biologically active substances and the highest ability to free radicals elimination. Fruits of these cultivars will be characterized by the highest prohealth properties. The results from the performed experiments will also have practical application during the breeding program preparation in order to obtain new cultivars of this species.

2. Experiment

The study material consisted of fruits collected from three cultivated wild strawberry cultivars: "Baron von Solemacher," "Yellow Wonder," and "Regina" originating from agrotechnical experiments carried out at Department of Vegetable and Medicinal Plants, University of Life Sciences, Lublin (Poland, 51°23′ N, 22°56′ E). Seeds of tested cultivar were sown manually on March 5th, 2010, into boxes filled with a substrate (peat substrate) and covered with thin sand layer. After emergence and forming 2-3 true leaves, the seedlings were transferred into boxes at 5 × 3.5 cm spacing. Plants were set into their permanent place on June 20th, 2010, in plots of 7.5 m^2 area (2.0 × 3.75 m) at 40 × 25 cm spacing (14 plants per row, i.e., 9.3 plants·m^{-2}) in three replicates. The agrotechnical experiment was carried out on dusty soil characterized by good abundance in nutrients and neutral reaction. Soil under wild strawberry cultivation was prepared according to commonly accepted recommendations applying manure (40 kg·ha) for the forecrop (onion). Phosphorus, potassium, and magnesium fertilizers were used before seedling planting at the following amounts: 80 kg·ha—P_2O_5; 100 kg·ha—K_2O. Starter rate (N—30 kg·ha) was applied, when the seedling was taken the roots. The wild strawberry plantation was regularly manually deweeded. First harvest was in second growing years. The fruit harvest was carried out at the full of fruiting stage in years 2011–2013. Fruits were harvested once in the early morning, in June every year.

Table 1: Mean monthly air temperatures, amount, and total hours of precipitation at ES Felin in the years 2011–2013.

Month	2011	2012	2013	Mean for 1951–2000
		Temperature °C		
IV	8.8	8.7	9.3	7.5
V	14.9	15.0	12.8	13.0
VI	18.1	18.1	17.7	16.5
VII	19.1	19.2	18.3	17.9
		Amount of precipitation mm		
IV	17.4	17.4	55.8	40.6
V	80.5	81.5	101.6	58.3
VI	87.8	87.8	25.9	65.8
VII	87.0	87.0	77.1	78.0
		Total hours in sunshine hrs.		
IV	238.8	139.6	291.5	156.6
V	268.6	183.0	274.6	280.9
VI	272.2	316.6	200.3	228.7
VII	236.7	242.3	279.6	158.0

Weather conditions during growth and studies upon *Fragaria vesca* are presented in Table 1.

Directly after the harvest, part of material was subject to laboratory analyses as raw, while another part was dried out. The drying process was performed in a drying facility at 40°C till the moment of a constant air-dried fruit weight achievement. Raw material was subject to determinations of dry matter (%) by means of drier method [19], weight loss after drying-moisture (%) [20] content flavonoids (mg·g^{-1}) [20], sum of phenolic acids (mg·g^{-1}) [21], tannins (%) [20], anthocyanins (mg·100 g^{-1}) [22], and antioxidant capacity (%) as an ability to neutralize the DPPH radicals [23]. Biochemical analysis was performed in Laboratory for Vegetable and Herbal Material Quality at the Department of Vegetable and Medicinal Plants, University of Life Sciences, Lublin.

2.1. Dry Matter. Dry matter determination was carried out by means of drier method according to Charłampowicz [19]. Aliquots of about 1 g (0.0001 g accuracy) of raw and ground fruits were weighed. Samples were placed in a drier and dried at 105°C for 6 hours. The drying process was repeated till the constant weight of samples (difference between two subsequent weighings should not be greater than 0.5 mg). The difference of weights before and after drying was the water loss, and then the result was recalculated onto the percentage of dry matter. Determinations were made in three replicates.

2.2. Weight Loss after Drying-Moisture Content. The loss after drying was determined by means of gravimetric method

according to Polish Pharmacopoeia VII [20]. Samples of 1 g of three cultivars of ground wild strawberry fruits were weighed in vessels. Samples were then placed in a drier at 105°C and dried for 2 hours; after that they were cooled to ambient temperature in desiccator over silica gel and weighed again. The drying was repeated until the constant weight (difference between two subsequent weighings should not be greater than 0.5 mg). The difference of weights before and after drying was the water loss (moisture content); all determinations were made in 3 replicates.

2.3. Total Flavonoids Estimation. Total flavonoids were estimated according to the spectrophotometric method of Christ and Müller [20] after their extraction, as recommended by the European Pharmacopoeia. For this purpose, 2.0 g of crushed fruit was added to a round-bottomed flask; 20 mL of acetone, 2 mL of HCl (281 g·L^{-1}), and 1 mL of methenamine (5 g·L^{-1}) were then added and the mixture was maintained for 30 min under reflux on a water bath. The hydrolysate was filtered through cotton wool into a volumetric flask of 100 mL, then placed in a flask together with the cotton pellet and 20 mL of acetone, and refluxed for 10 min. Next, 20 mL of solution was dispensed into a separatory funnel with 20 mL of water and extracted with ethyl acetate in 15 mL portions 3 times with 10 mL. The combined organic layers were washed twice with 40 mL of water, filtered into a volumetric flask of 50 mL, and supplemented with ethyl acetate. To determine flavonoid content, 2 samples were prepared: to 10 mL of the stock solution 2 mL of a solution of aluminum chloride (20 g·L^{-1}) was added, supplemented with a mixture (1 : 19) of acetic acid (1.02 kg·L^{-1}) and methanol (25 mL). To prepare the comparative solution, stock was supplemented with 10 mL of a mixture (1 : 19) of acetic acid (1.02 kg·L^{-1}) and methanol (25 mL). After 45 min, the absorbance of the solutions was read at $\lambda = 425$ nm on HITACHI U-2900 spectrophotometer using the reference solution for comparison. Samples were analyzed in 3 replicates. The total content of flavonoids (mg·g^{-1}) was expressed as quercetin QE equivalent according to the following formula:

$$X = \frac{k \times A}{m}, \quad (1)$$

where X are total flavonoids (mg·g^{-1}); A is the absorbance of the solution being studied; k is the convection factor for quercetin and equal to 8.750; m is the sample with the raw material (g) which was the amount of fresh and dry material.

2.4. Total Phenolic Acids Estimation. Total phenolic acids estimation was carried out according to Arnov method [21], which corresponds to the recommendations of the European Pharmacopoeia. To 5.0 g of homogenized raw material placed in a round-bottomed flask 20 mL of methanol was added and the mixture was heated for 30 min at 70°C in a water bath at reflux. The hydrolysate was filtered through a hard filter paper into an Erlenmeyer flask of 100 mL. The filtered medium was returned to the round-bottomed flask with 20 mL of methanol and heated at reflux for 30 min. This process was repeated 3 times. The combined filtrates were taken to the tube with 1 mL of blueberry extract, 1 mL of 0.5 N hydrochloric acid, 1 mL of Arnov reagent, and 1 mL of 1 N sodium hydroxide, made up to 10 mL with distilled water. The absorbance was measured at $\lambda = 490$ nm. The total phenolic acid content, expressed as acid equivalent weight of caffeic acid (CAE) in the fruit, was calculated from the equation obtained from the calibration curve of caffeic acid ($y = 1.7321x + 0.0227$; $R2 = 0.9992$). Samples were analyzed in triplicate.

2.5. Tannin Estimation. The amount of tannin estimation was determined using Pharmacopoeia procedure [20]. The content of tannins was expressed as fresh and dry weight percentage.

2.6. Anthocyanins Estimation by means of Colorimetry. Samples of raw material (1.0 g) were extracted with 50 mL HCl (1 mol·dm^3) and heated in water bath for 1 hour. The obtained extract was hydrolyzed with 20 mL n-butanol, and then two portions of 10 mL n-butanol were added as a solution. Anthocyanin extract was rinsed in 50 mL flask with n-butanol. The absorbance was measured immediately at 533 nm [22]. The percentage of anthocyanins, as delphinidin chloride, was calculated from the expression

$$P = \frac{A \times V \times F}{m}, \quad (2)$$

where P are total anthocyanins (mg·100 g^{-1}); A is absorbance at 533 nm; V is value of butanol phase (50 mL); F is coefficient for delphinidin chloride (2,6); m is mass of sample to be examined (mg).

2.7. Antioxidant Activity. Antioxidant activity (%) was evaluated on a base of the ability to neutralize the DPPH radicals by means of spectrophotometry according to Chen and Ho [23]: to do this, water extracts were prepared from fruits; extracts were then evaporated till dried and lyophilized. Analyses were performed for 20 μg·mL^{-1} concentration. The absorbance measurements were made at $\lambda = 517$ nm wavelength using spectrophotometer HITACHI U-2900.

2.8. Chemicals. All reagents and solvents were of analytical grade chemicals from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, USA) and POCH (Gliwice, Poland).

2.9. Statistical Analysis. Achieved results from laboratory experiments were statistically processed by means of variance analysis method and Tukey's confidence intervals at 5% confidence level.

3. Results

3.1. Principal Physicochemical Parameters of Studied Material. Determinations related to the chemical composition of three wild strawberry cultivars fruits here presented were preceded with the evaluation of general physicochemical parameters,

Table 2: Dry matter (%) content in raw and dried fruits as well as moisture content in dried fruits in successive years of the study.

Cultivars	Raw material							
	Fresh material				Dry material			
	2011	2012	2013	Mean	2011	2012	2013	Mean
"Baron von Solemacher"	35.20cA	36.19cA	37.73cA	**36.37**	11.36aA	11.33aA	11.24aA	**11.31**
"Yellow Wonder"	28.47aA	27.95aA	28.23aA	**28.22**	11.26aA	11.05aA	11.01aA	**11.11**
"Regina"	31.12bA	30.98bA	31.17bA	**31.09**	11.35aA	10.49aA	10.81aA	**10.88**
Mean	**31.60**	**31.71**	**32.38**	**31.89**	**11.32**	**10.96**	**11.02**	**11.10**

Explanatory notes: different letters a, b, c. . . and A, B, C. . . in the same column and line indicate statistically significant differences ($P < 0.05$). In each column and for each cultivar different letters mean significant differences ($P < 0.05$).

Table 3: Content of flavonoids TFL (as quercetin) mg·g^{-1}, phenolic acids TPC (as caffeic acid) mg·g^{-1}, tannins (%), and anthocyanins ACN (as delphinidin mg·100 g^{-1}) in raw material.

Cultivars	Raw material							
	Fresh material				Dry material			
	TFL	TPC	TAN	ACN	TFL	TPC	TAN	ACN
"Baron von Solemacher"	0.593c	2.454b	3.40c	114.00a	1.245b	4.858b	6.09b	300.00b
"Yellow Wonder"	0.471a	1.648a	2.19a	90.00a	1.178a	4.483a	4.83a	214.61a
"Regina"	0.524b	2.840b	2.70b	160.50b	1.210ab	4.987b	5.00a	444.25c
Mean	**0.530**	**2.314**	**2.76**	**121.50**	**1.211**	**4.776**	**5.31**	**319.62**

Explanatory notes: see Table 2.

that is, dry matter content in raw fruits and weight loss after drying-moisture content in dried fruits. Data presented in Table 2 indicate that dry matter content in raw fruits ranged from 27.95% to 37.73%. Among compared cultivars, "Baron von Solemacher" fruits were characterized by the highest concentration of the component (36.37%, on average), while "Yellow Wonder" contained the lowest level of the parameter (28.22%). Significant differences between cultivars in subsequent years of study were observed. Considering the water content in dried material, fruits of studied wild strawberry cultivars slightly differed from each other and statistical analysis did not reveal any significant differences. Regardless of the cultivar, moisture content of air-dried fruits oscillated around 11.10%, on average (Table 2).

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3.2. Flavonoids Contents. Different levels of flavonoids in raw and dried fruits of three wild strawberry cultivars are presented in Figure 1 and Table 3. The highest concentrations of flavonoids was found in fruits of Baron von Solemacher cv. 0.593 mg·g^{-1} (raw material) and 1.245 mg·g^{-1} (dried material). On the other hand, the lowest quantities of analyzed compounds were recorded in fruits of Yellow Wonder cv.

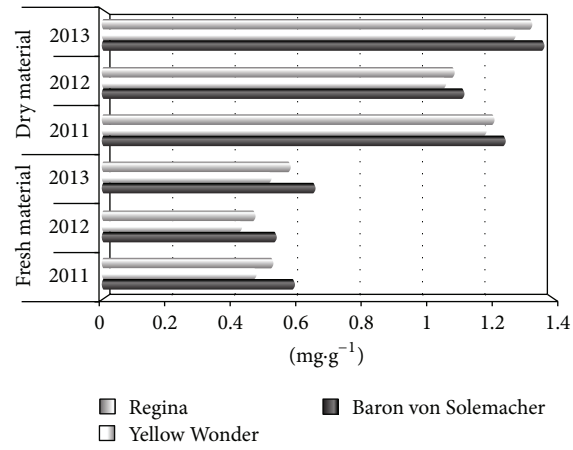

Figure 1: Content of flavonoids (as quercetin) in raw material.

(0.471 and 1.178 mg·g^{-1}, resp., for raw and dried material). Significant differences in flavonoids contents over the years of study for both analyzed types of material were observed. The dried fruits of all studied genotypes were characterized by over twice as high flavonoids amounts as compared to raw fruits.

3.3. Total Phenolic Acids Contents. Analysis of phenolic acids concentration revealed that raw and dried fruits of Regina cv. were the best sources of these compounds 2.840 mg·g^{-1} in raw and 4.987 mg·g^{-1} in dried material (Table 3 and Figure 2).

Slightly less phenolic acids were found in fruits of Baron von Solemacher cv. 2.454 mg·g^{-1} and 4.858 mg·g^{-1} in raw and dried fruits, respectively. Fresh fruits of Yellow Wonder cv.

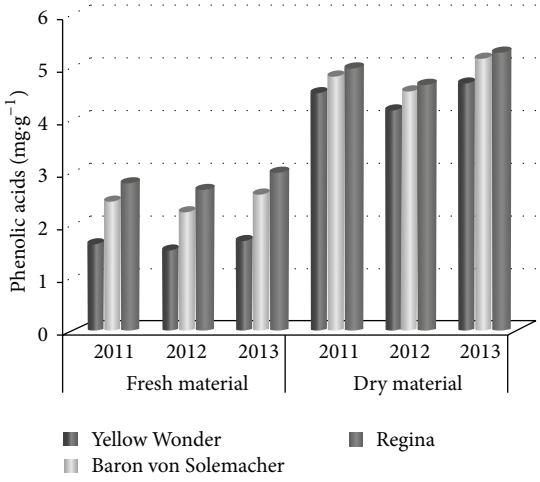

Figure 2: Content of phenolic acids (as caffeic acid) mg·g^{-1} in raw material in successive years of the study.

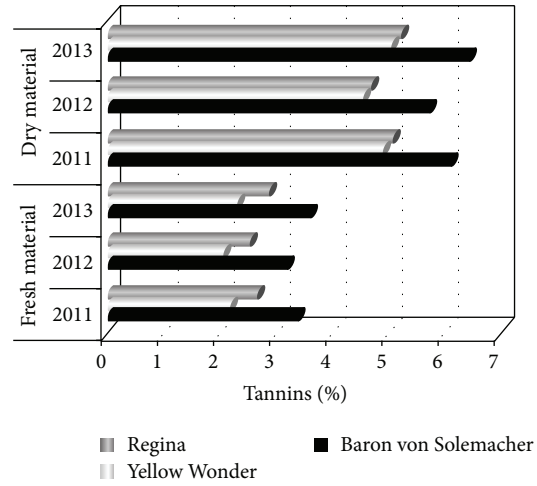

Figure 3: Content of tannins (%) in raw material in successive years of the study.

contained almost twice as low phenolic acids as Regina cv. fruits. However, mean values recorded for dried fruits of all analyzed genotypes slightly differed and statistical analysis confirmed that the differences were significant.

3.4. Total Tannins Contents. Changes in tannins contents for all studied wild strawberry fruits were similar as those for flavonoids. Raw fruits contained 2.76% of tannins, on average, while dried ones contained 5.31% (Table 3 and Figure 3). Significant influence of all examined factors on tannins contents in studied materials was found on a base of determinations performed.

3.5. Total Anthocyanins Contents. Anthocyanins were another group of substances analyzed in wild strawberry fruits (Table 3 and Figure 4). Fruits of Regina cv. were the best source of anthocyanins as similar as phenolic acids. From 144.12 mg·100 g^{-1} to 177.07 mg·100 g^{-1} were recorded in raw fruits of this cultivar, whereas dried fruits contained from 398.54 mg·100 g^{-1} to 460.40 mg·100 g^{-1}. Among compared cultivars, the Yellow Wonder cv. appeared to be the worst since it contained only from 80.97 mg·100 g^{-1} to 99.00 mg·100 g^{-1} (raw material) and from 192.52 mg·100 g^{-1} to 236.90 mg·100 g^{-1} (dried material) of anthocyanins. The lowest levels of analyzed substances for all studied genotypes were recorded in 2012.

3.6. Antioxidant Activity. Significant differences in the ability to neutralize the free DPPH (diphenylpicrylhydrazyl) radical by extracts made of examined wild strawberry fruits were recorded (Table 4).

Extracts prepared from dried fruits revealed definitely highest ability (23.93%) as compared to raw material (13.11%). When comparing studied cultivars, the highest neutralizing capacity was shown by extracts made of Regina cv. fruits 14.27% (raw material) and 24.60% (dried fruits). The free

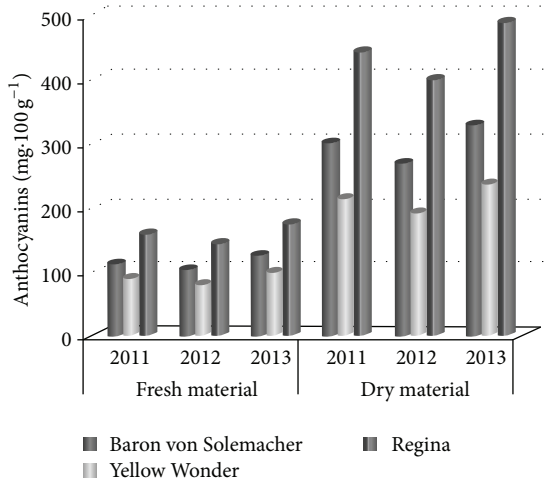

Figure 4: Content of anthocyanins (mg·100 g^{-1}) in raw material.

radicals were worse reduced by extracts prepared from fruits of Yellow Wonder cv. and Baron von Solemacher cv.

4. Discussion

Fragaria vesca has traditionally been a popular delicious fruit for its flavor,taste, fresh use, freezing, and processing. Morphological, biometric, and agronomical characteristics have been widely used to describe wild strawberry cultivars. In recent years, cultivated berries have become very attractive for consumers because of potentially beneficial phytochemicals contained in these fruits. The importance of flavonoids and other phenolics has been suggested to play a preventive role in the development of cancer and heart disease [24]. Considerable data suggests that higher content of flavonoids, phenolic acids, tannins, and anthocyanins in berry fruits contributes to their higher antioxidant activity [17, 25]. Following substances should be counted as the most important bioactive components of wild strawberry:

Table 4: Antioxidant activity (%) expressed as the ability to neutralize the DPPH radical in water extracts made of studied materials in successive years of the study.

Cultivars	Raw material							
	Fresh material				Dry material			
	2011	2012	2013	Mean	2011	2012	2013	Mean
"Baron von Solemacher"	12.65a	11.34a	14.02b	**12.67**	23.84a	21.51b	26.05b	**23.80**
"Yellow Wonder"	12.42a	11.16a	13.63a	**12.40**	23.44a	21.05a	25.70a	**23.40**
"Regina"	14.30b	13.21b	15.30c	**14.27**	24.66b	22.15c	26.98c	**24.60**
Mean	**13.12**	**11.90**	**14.31**	**13.11**	**23.98**	**21.57**	**26.24**	**23.93**

Explanatory notes: see Table 2.

phenolic acids (ellagic, *p*-coumaric, gallic acids), flavonoids (flavonols, quercetin, and kaempferol), proanthocyanidins, and anthocyanins (pelargonidin, cyanidin) [26–28]. Due to these compounds, the fruits have anticarcinogenic, antioxidant, anticoagulant, immunomodulating, anti-inflammatory, blood pressure, and cholesterol regulating features [29, 30]. Measuring such parameters is wildly used to evaluate the potential health benefits of breeding material or various agronomic factors [9]. However, little is known on the phenolic profiles and antioxidant potential of wild strawberry in important local cultivars. In this paper, we use the contents of secondary metabolites for cultivar identification. The differences in the concentrations of secondary compounds determine the nutritional importance of the analyzed cultivars. Furthermore, they provide information on the marketing potential of the 3 cultivars and present an important chemical insight into the popular cultivars grown in this region.

Secondary metabolites content in berry fruits varies among species and cultivars, but it can also be affected by growth conditions including environmental factors and cultivation techniques [10, 11, 31–34]. It has been shown that higher growing temperatures (day and night) increase the flavonols and anthocyanins contents in strawberries [26]. Authors of the present study, on a base of performed research, proved differentiated contents of biologically active substances: flavonoids, phenolic acids, tannins, and anthocyanins in raw and dried fruits depending on wild strawberry cultivar. Furthermore, they analyzed the antioxidant ability of extracts made of studied materials by means of neutralizing the free DPPH radical. Mean content of flavonoids and tannins in fresh fruits of "Baron von Solemacher" cv. was the highest and amounted to 1.245 mg·g^{-1} and 6.09%, respectively. Raw fruits of "Regina" cv. were characterized by the highest average concentrations of phenolic acids and anthocyanins: 4.987 mg·g^{-1} and 444.25 mg·100 g^{-1}, respectively. According to Antal et al. [35], the quantity of anthocyanins in raw berries ranges from 30 mg·100 g^{-1} (*Fragaria moschata*) to 165 mg·100 g^{-1} (*Vaccinium myrtillus*). Huang et al. [14] found higher contents of anthocyanins in berry fruits, which correspond with values achieved in the present study for raw fruits of examined wild strawberry genotypes. In opinion of Olsson et al. [11], the amount of phenolic substances as well as antioxidant activity was different within *Fragaria* x *ananassa* Duch. genus. Antal et al. [35] evaluated fresh fruits and found that strawberries had total phenolics of 3.680 mg·kg fresh weight. As it follows from performed analyses, mean content of phenolic acids for fresh wild strawberry fruits was at the level of 2.314 mg·g^{-1}. The differences in polyphenol content of strawberry fruit from the literature may be due to the different conditions during the growth of plants (climatic conditions, temperature, precipitation, and soil conditions), the length of the growing season, and harvest date. They may also be caused by using various analytical procedures or methods identifying the active ingredients by scientists in various centers. Identification of chromatographic methods usually has a lower content of active substances, because its task is to determine the minimum content and separation and identification of individual compounds in the raw material. Commonly used spectrophotometric procedures are based on similar assumptions, so after taking into account differences methods are comparable to those applied in this paper. During the three years of the research, the highest contents of analyzed biologically active substances were recorded in 2013, while the lowest were recorded in 2012.

In a previous investigation, we found that fresh wild strawberry fruits possess high amounts of bioactive compounds. However, fresh fruits are not available all year round, being harvested in Poland only in June–September. Therefore, it is important to find a proper substitute that could be used when fresh berries are not available. It was decided to prepare dried fruits, to determine the contents of some important bioactive compounds and their antioxidant potential therein, and to compare them with the same parameters in fresh fruits. Dried fruits have a greater nutrient density, greater fiber content, increased shelf life, and significantly greater phenol antioxidant content compared to fresh fruits. The quality of the antioxidants in the processed dried fruit is the same as in the corresponding fresh fruit. Phenols in dried fruit may be important antioxidants [24]. Data presented in Tables 2–4 indicate diverse contents of biologically active compounds in raw and dried materials depending on the cultivar. The highest mean concentrations of analyzed substances in air-dried fruits were similar as for fresh ones. Dried fruits of analyzed genotypes were characterized by over twice as high quantities of biologically active substances and antioxidant activity as fresh fruits. Results from the present study are a confirmation of the results achieved by Vinson et al. [24] related to the secondary metabolites in fresh and dried fruits.

5. Conclusion

As a conclusion, our results clearly demonstrate that considerable variation exists in the phenolic compounds among wild strawberry genotypes. The obtained results allowed the identification of cultivars which were characterized by the highest content of investigated biologically active substances and the highest ability to free radicals elimination. Fruits of these cultivars were characterized by the highest prohealth properties. The results from the performed experiments also have practical application during the breeding program preparation in order to obtain new cultivars of this species. Therefore, dried fruits are good source of important bioactive compounds and more dried fruits should be recommended to be added to the diet by dieticians and nutritionists.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 607252, 11 pages
http://dx.doi.org/10.1155/2015/607252

Research Article

Quality Assessment of Ojeok-San, a Traditional Herbal Formula, Using High-Performance Liquid Chromatography Combined with Chemometric Analysis

Jung-Hoon Kim,[1,2] Chang-Seob Seo,[1] Seong-Sil Kim,[1] and Hyeun-Kyoo Shin[1]

[1] *Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine, Daejeon 305-811, Republic of Korea*
[2] *Division of Pharmacology, School of Korean Medicine, Pusan National University, Yangsan, Gyeongnam 626-870, Republic of Korea*

Correspondence should be addressed to Hyeun-Kyoo Shin; hkshin@kiom.re.kr

Received 22 October 2014; Revised 16 March 2015; Accepted 17 March 2015

Academic Editor: Shixin Deng

Ojeok-san (OJS) is a traditional herbal formula consisting of 17 herbal medicines that has been used to treat various disorders. In this study, quantitative analytical methods were developed using high-performance liquid chromatography equipped with a photodiode array detector to determine 19 marker compounds in OJS preparations, which was then combined with chemometric analysis. The method developed was validated in terms of its precision and accuracy. The intra- and interday precision of the marker compounds were <3.0% of the relative standard deviation (RSD) and the recovery of the marker compounds was 92.74%–104.16% with RSD values <3.0%. The results of our quantitative analysis show that the quantities of the 19 marker compounds varied between a laboratory water extract and commercial OJS granules. The chemometric analysis used, principal component analysis (PCA) and hierarchical clustering analysis (HCA), also showed that the OJS water extract produced using a laboratory method clearly differed from the commercial OJS granules; therefore, an equalized production process is required for quality control of OJS preparations. Our results suggest that the HPLC analytical methods developed are suitable for the quantification and quality assessment of OJS preparations when combined with chemometric analysis involving PCA and HCA.

1. Introduction

Ojeok-san (OJS) is a traditional herbal formula used in Korean medicine that consists of 17 compositional herbal medicines: Atractylodis rhizoma, Ephedrae herba, Citri Unshius pericarpium, Magnoliae cortex, Platycodonis radix, Aurantii Fructus Immaturus, Angelicae gigantis radix, Zingiberis rhizoma, Paeoniae radix, Poria sclerotium, Angelicae dahuricae radix, Cnidii rhizoma, Pinelliae tuber, Cinnamomi cortex, Glycyrrhizae radix et rhizoma, *Zingiberis* rhizoma recens, and Allii fistulosi bulbus. Traditionally, OJS has been used to treat disorders such as fever, anhidrosis, headache, whole body pain, contracture of the nape and neck, vomiting, abdominal and heart pain, and menstrual irregularities [1].

Recent studies have reported on the therapeutic effects of OJS against lumbago and inferior limb pain [2], primary dysmenorrheal [3], clastogenicity [4], and airway inflammation and pulmonary fibrosis [5]. Since a combination of multiple components is considered necessary to exhibit the therapeutic effects of the herbal formula, simultaneous determination of the compositional constituents has been developed for qualitative and quantitative analysis. Several previous studies have analyzed the chemical constituents of OJS using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with pulsed amperometric detection (PAD) or diode array detection (DAD) [6, 7].

Cluster analysis is a data analysis method used to assign similar objects belonging to the same group and is used in

a variety of practical applications like bioinformatics, using chemometric analyses, such as principal component analysis (PCA) and hierarchical clustering analysis (HCA) [8, 9].

PCA is an unsupervised pattern recognition technique and is a useful tool for visualizing similarities or differences in multivariate data [10]. PCA can represent objects or variables on a graph and is used to study the proximity of objects to classify them and to detect atypical objects [11]. HCA is a procedure that has a pyramid-like structure and is a very useful and widely adopted technique in information processing [12]. HCA determines similarities between samples by measuring the distance between all possible sample pairs in a high-dimensional space and any similarities between the samples are represented on two-dimensional diagrams [13]. The HPLC analytical method combined with chemometric analysis has been widely accepted for the quality control of herbal medicines, as it can be part of a powerful strategy to differentiate the source, location, or species in herbal medicines [14–16].

Recently, herbal formulas have been manufactured by pharmaceutical companies in diverse dosage forms, such as powder, granules, and tablets, as these are more convenient and easier to take than traditional decoction forms. However, the compositional herbal ratio or the origin of herbal components of a herbal formula may differ between different companies, and so the formula produced by each company may contain a variety of chemical constituents [17–19]. Such chemical inequalities cannot warrant equivalent therapeutic effects between different herbal formula preparations and may lead to a loss of innate characteristics of a given herbal formula.

Therefore, in this study, we developed analytical methods for the quantification of 19 marker compounds in a laboratory-produced water extract and in commercial granules of OJS using HPLC–PDA. In addition, chemometric analysis data were combined with the quantitative results and employed to assess the quality of OJS preparations via the Pearson correlation coefficient and PCA and HCA data.

2. Materials and Methods

2.1. Chemicals and Reagents. The HPLC-grade acetonitrile and water used were purchased from JT Baker Inc. (Phillipsburg, NJ, USA) and the guaranteed reagent grade acetic acid used was obtained from Junsei (Chuo-ku, Tokyo, Japan). The gallic acid (**1**), chlorogenic acid (**3**), ferulic acid (**6**), benzoic acid (**8**), neohesperidin (**12**), and cinnamic acid (**15**) used were obtained from Sigma-Aldrich (St. Louis, MO, USA). The protocatechuic acid (**2**) and nodakenin (**9**) used were purchased from ChromaDex (Irvine, CA, USA) and NPC BioTech (Geumsan, Chungnam, Korea), respectively. The albiflorin (**4**), paeoniflorin (**5**), liquiritin (**7**), naringin (**11**), cinnamaldehyde (**17**), and glycyrrhizin (**19**) used were purchased from Wako Pure Chemical Industries (Chuo-ku, Osaka, Japan). The hesperidin (**10**), ononin (**13**), oxypeucedanin hydrate (**14**), byakangelicin (**16**), and benzoylpaeoniflorin (**18**) used were purchased from Chengdu Biopurify Phytochemicals (Chengdu, Sichuan, China).

The purity of the standard compounds was ≥98%; their chemical structures are shown in Figure 1. Compositional herbal medicines of OJS were purchased from the herbal medicine company, Kwangmyungdang Medicinal Herbs (Ulsan, Gyeongbuk, Korea). A voucher specimen (2012-KE04-1–17) was deposited in the Herbal Medicine Formulation Research Group of the Korea Institute of Oriental Medicine. Commercial OJS samples denoted as "OJS02–OJS10" were purchased from nine pharmaceutical companies located in Korea. The compositional herbal ratio was shown in Table 1.

2.2. Preparation of the OJS Water Extract and Commercial Granules. Dried herbal drugs consisting of OJS were mixed and extracted using a 10-fold volume of distilled water (w/v) at 100°C for 2 h under pressure (1 kgf/cm^2) using an electric extractor (COSMOS-660, KyungSeo Machine Co., Incheon, Korea). The extracted decoction was filtered through a standard sieve (number 270, 53 μm, Chunggyesangongsa, Seoul, Korea) and freeze-dried to make OJS water extract powder denoted as "OJS01."

Powdered OJS01 (200 mg) and commercial OJS granules (OJS02–OJS10, 500 mg) were dissolved in 10 mL of distilled water and the solutions were filtered through a 0.2 μm syringe filter (SmartPor, Woongki Science, Seoul, Korea) before being injected into the HPLC system.

2.3. Chromatographic Conditions. The HPLC system used was a Shimadzu LC-20A (Kyoto, Japan) chromatograph equipped with a solvent delivery unit (LC-20AT), an autosampler (SIL-20AC), a column oven (CTO-20A), a degasser (DGU-20A$_3$), and a photodiode array detector (SPD-M20A). Separation was conducted on a Gemini C_{18} column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA). The column temperature was set at 40°C. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). The composition of the mobile phase was 6%–20% (B) for 0–20 min, 20%–25% (B) for 25–30 min, 25%–40% (B) for 30–40 min, 40%–46% (B) for 40–50 min, and 46%–87% (B) for 50–55 min, held for 5 min and then reequilibrated to 6% (B) until the end of the analysis. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The detection wavelengths of all standards and samples were in the UV at 230, 250, 260, 270, 275, 280, 290, 310, 325, and 335 nm.

2.4. Method Validation

2.4.1. Linearity. The 19 standard compounds were accurately weighed and dissolved in methanol to prepare stock solutions at a concentration of 1000 μg/mL. Stock solutions of the marker compounds were serially diluted to construct calibration curves. The diluted concentrations of marker compounds were plotted against the peak area on the calibration curves and the linearity was measured from the correlation coefficient.

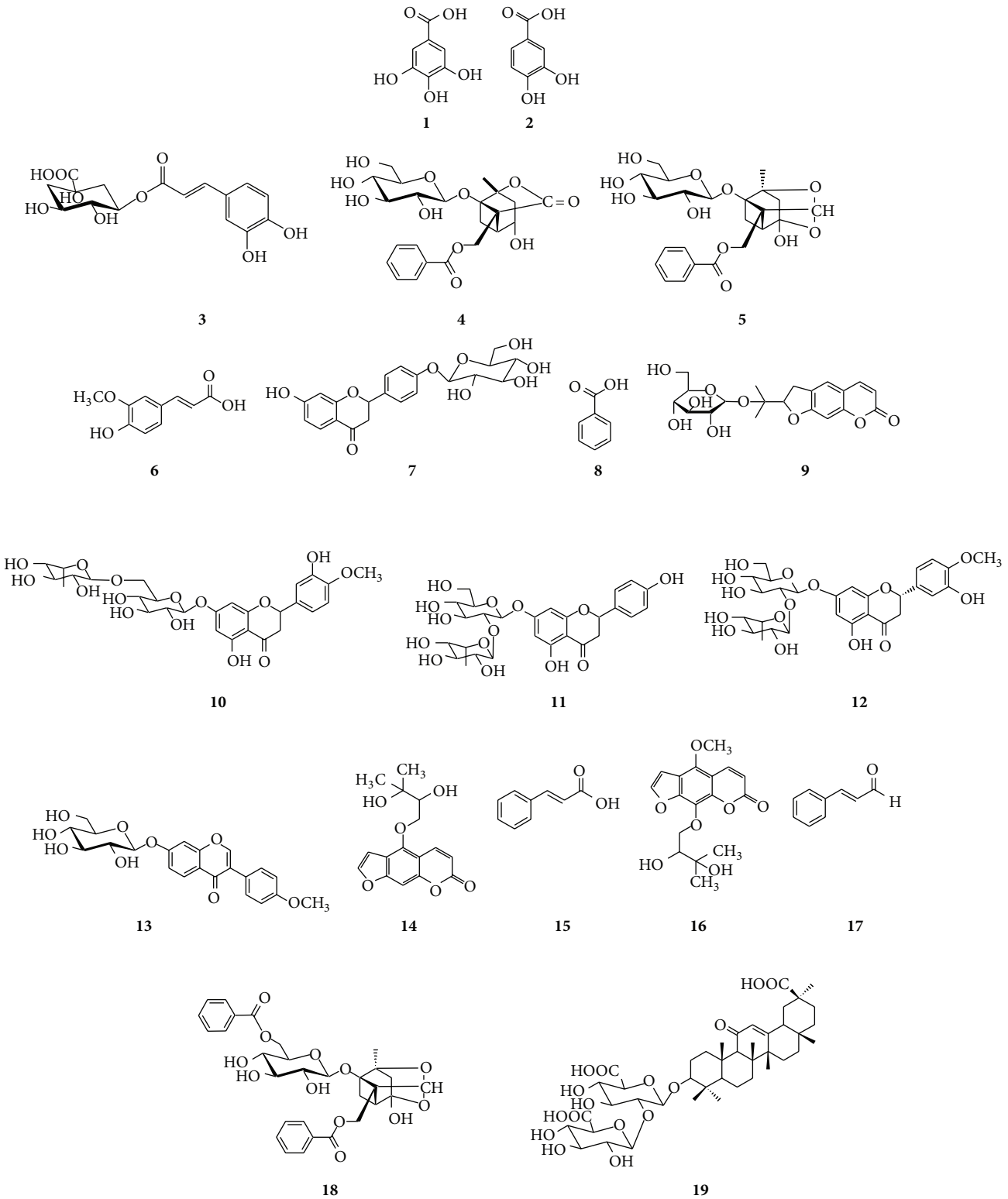

Figure 1: Chemical structures of 19 marker compounds in Ojeok-san (OJS). (**1**) Gallic acid, (**2**) protocatechuic acid, (**3**) chlorogenic acid, (**4**) albiflorin, (**5**) paeoniflorin, (**6**) ferulic acid, (**7**) liquiritin, (**8**) benzoic acid, (**9**) nodakenin, (**10**) hesperidin, (**11**) naringin, (**12**) neohesperidin, (**13**) ononin, (**14**) oxypeucedanin hydrate, (**15**) cinnamic acid, (**16**) byakangelicin, (**17**) cinnamaldehyde, (**18**) benzoylpaeoniflorin, and (**19**) glycyrrhizin.

Table 1: Compositional ratio of herbal medicine consisting of Ojeok-san (OJS) samples.

Herbal medicine	OJS01[a]	OJS02[b]	OJS03	OJS04	OJS05	OJS06	OJS07	OJS08	OJS09	OJS10
Atractylodis rhizoma	0.133	0.212	0.215	—	0.212	0.443	0.212	0.532	0.214	0.443
Ephedrae herba	0.067	0.045	0.046	—	0.045	0.223	0.045	0.268	0.045	0.223
Citri Unshius pericarpium	0.067	0.091	0.090	—	0.091	0.223	0.091	0.268	0.090	0.223
Magnoliae cortex	0.053	0.018	0.018	—	0.018	0.223	0.018	0.268	0.018	0.223
Platycodonis radix	0.053	0.095	0.097	—	0.095	0.223	0.095	0.268	0.097	0.223
Aurantii Fructus Immaturus	0.053	0.069	0.070	—	0.069	—	0.069	—	0.070	—
Angelicae gigantis radix	0.053	0.082	0.084	—	0.082	0.223	0.082	0.268	0.083	0.223
Zingiberis rhizoma	0.053	0.049	0.050	—	0.049	0.223	0.049	0.268	0.050	0.223
Paeoniae radix	0.053	0.060	0.061	—	0.060	0.223	0.060	0.268	0.061	0.223
Poria sclerotium	0.053	0.004	0.004	—	0.004	0.223	0.004	0.268	0.004	0.223
Cnidii rhizoma	0.047	0.069	0.067	—	0.069	0.223	0.069	0.268	0.066	0.223
Angelicaedahuricae radix	0.047	0.071	0.069	—	0.071	0.223	0.071	0.268	0.069	0.223
Pinelliae tuber	0.047	0.051	0.050	—	0.051	0.223	0.051	0.268	0.050	0.223
Cinnamomi cortex	0.047	0.011	0.011	—	0.011	—	0.011	0.000	0.011	—
Glycyrrhizaeradix et rhizoma	0.040	0.045	0.046	—	0.045	0.223	0.045	0.268	0.045	0.223
Zingiberis rhizoma crudus	0.067	0.027	0.028	—	0.027	—	0.027	—	0.027	—
Ponciri Fructus Immaturus	—	—	—	—	—	0.223	—	0.268	—	0.223
Zizyphi Fructus	—	—	—	—	—	0.223	—	0.268	—	0.223
Cinnamomi ramulus	—	—	—	—	—	0.223	—	0.268	—	0.223
Cyperi rhizoma	—	—	—	—	—	—	—	0.160	—	0.133
Allii fistulosi bulbus	0.067	—	—	—	—	—	—	—	—	—
Single dose	1	1	1	—	1	1	1	1	1	1

[a]OJS01, Ojeok-san water extract from the laboratory and [b]OJS02–OJS10 = Ojeok-san granules from Korean manufacturers.

2.4.2. LOD and LOQ. Blank samples were analyzed in triplicate and the area of the noise peak was calculated as the response. The LOD and LOQ were calculated as LOD = 3.3 × SD/S and LOQ = 10 × SD/S, where SD is the standard deviation of the response and S is the slope of the calibration curve.

2.4.3. Precision. The precision was calculated by analyzing sample extracts containing low and high concentrations of the marker compounds. The precision was represented by the relative standard deviation (RSD), which was calculated using the equation RSD = (standard deviation/mean) × 100. The precision was measured three times in a single day (intraday precision) and over three consecutive days (interday precision).

2.4.4. Recovery. The accuracy of the method used was evaluated through the recovery test. Both low and high concentrations of the marker compounds were added to the samples. The recovery was calculated as follows: recovery (%) = ((detected concentration − initial concentration)/spiked concentration) × 100.

2.5. Chemometric Analysis. The relationship between OJS samples was evaluated using the Pearson coefficient of the amounts of the marker compounds. To cluster the OJS sample, PCA and HCA were performed based on the rows (OJS samples) and columns (the amounts of the 19 marker compounds). The evaluation of the Pearson coefficient and the clustering analysis (PCA and HCA) were carried out using the open-source software package R (v. 3.0.2).

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions. The mobile phase modifier, gradient ratio, and UV detection wavelength were considered as the main factors for optimizing the conditions for the HPLC analysis of the OJS water extract. A C_{18} column was employed for the simultaneous determination of the 19 marker compounds in the OJS water extract, as it has been the most frequently used technique in the chemical analysis of herbal medicines [20, 21]. Two different modifiers, 1% acetic acid and 0.1% formic acid, were compared to find the optimal conditions for the separation of the 19 marker compounds. Peak resolution and shape of the marker compounds were considered better indicators when 0.1% formic acid was used as a modifier.

Various ratios of the components of the mobile phase (A : B) were tested using gradient elution, and the optimal separation was observed at the following gradient conditions: 6%–20% (B) for 0–20 min, 20%–25% (B) for 25–30 min, 25%–40% (B) for 30–40 min, 40%–46% (B) for 40–50 min, and 46%–87% (B) for 50–55 min, held for 5 min and then reequilibrated to 6% (B).

The UV wavelength in the range 190–400 nm was scanned to find the maximum absorption for each marker

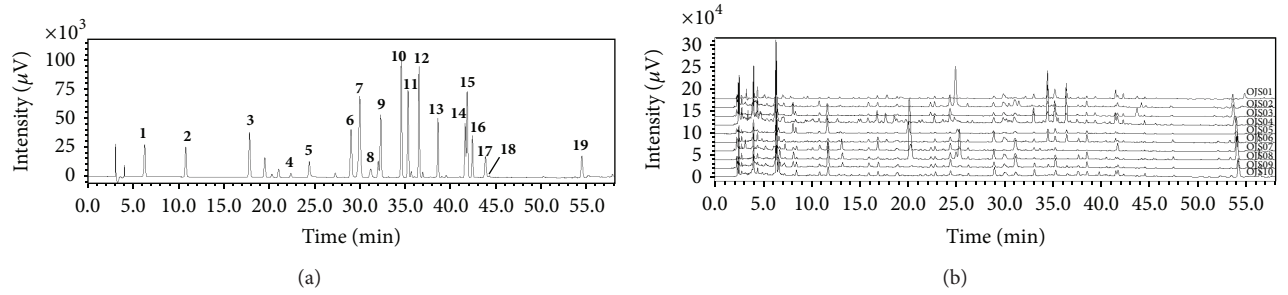

Figure 2: Chromatograms of (a) the standard marker compounds and (b) OJS samples at a detection wavelength of UV 254 nm. (**1**) Gallic acid, (**2**) protocatechuic acid, (**3**) chlorogenic acid, (**4**) albiflorin, (**5**) paeoniflorin, (**6**) ferulic acid, (**7**) liquiritin, (**8**) benzoic acid, (**9**) nodakenin, (**10**) hesperidin, (**11**) naringin, (**12**) neohesperidin, (**13**) ononin, (**14**) oxypeucedanin hydrate, (**15**) cinnamic acid, (**16**) byakangelicin, (**17**) cinnamaldehyde, (**18**) benzoylpaeoniflorin, and (**19**) glycyrrhizin. OJS01, Ojeok-san water extract from the laboratory; OJS02–OJS10, Ojeok-san granules from Korean manufacturers.

compound. For albiflorin, paeoniflorin, benzoic acid, and benzoylpaeoniflorin, this occurred at 230 nm; for ononin and glycyrrhizin at 250 nm; for protocatechuic acid at 260 nm; for gallic acid and byakangelicin at 270 nm; for liquiritin and cinnamic acid at 275 nm; for hesperidin, naringin, and neohesperidin at 280 nm; for cinnamaldehyde at 290 nm; for oxypeucedanin hydrate at 310 nm; for chlorogenic acid and ferulic acid at 325 nm; and for nodakenin at 335 nm. For the conditions described above, the 19 marker compounds were reasonably separated on C_{18} column for quantitative analysis (Figure 2).

3.2. Method Validation

3.2.1. Linear Regression, LOD, and LOQ. The linearity of the calibration curve was measured using the correlation coefficient (r^2), which ranged in value from 0.9993 to 1.0000 for each compound. The LOD and LOQ values were 0.004–0.090 μg/mL and 0.012–0.272 μg/mL, respectively (Table 2).

3.2.2. Precision and Recovery. The intra- and interday precision, which were represented by the RSD values, were RSD < 3.0% for the two concentration levels (Table 3). The recoveries of the 19 marker compounds were in the range 92.74%–104.16%, with RSD < 4.0% at different spiked concentrations (Table 4). These results indicate that the developed analytical method was precise, accurate, and reliable for the analysis of the 19 marker compounds in the OJS samples.

3.3. Quantification of the Marker Compounds in the OJS Samples.

The method we established was successfully applied to determine the 19 reference compounds in the OJS water extract (OJS01) and commercial OJS granules (OJS02–OJS10). There was wide variation observed in the contents of the marker compounds in the 10 OJS samples. While OJS01 contained the 19 marker compounds, the commercial OJS granules showed lack of one or more of the following compounds: protocatechuic acid, chlorogenic acid, ferulic acid, nodakenin, hesperidin, neohesperidin, and cinnamaldehyde.

Moreover, variation in the content of these compounds was apparent between the OJS samples: 2.8–16.6-fold for gallic acid, 1.1–3.5-fold for protocatechuic acid, 1.4–37.0-fold for chlorogenic acid, 2.0–64.0-fold for albiflorin, 2.1–8.7-fold for paeoniflorin, 1.1-fold for ferulic acid, 5.9–57.1-fold for liquiritin, 1.1–7.8-fold for benzoic acid, 5.0–224.5-fold for nodakenin, 5.5–624.9-fold for hesperidin, 1.6–8.5-fold for naringin, 2.4–73.0-fold for neohesperidin, 3.5–13.8-fold for ononin, 3.8–71.8-fold for oxypeucedanin hydrate, 3.7–21.3-fold for cinnamic acid, 5.0–124.0-fold for byakangelicin, 1.5-fold for cinnamaldehyde, 3.8–112.6-fold for benzoylpaeoniflorin, and 3.1–8.6-fold for glycyrrhizin (Table 5).

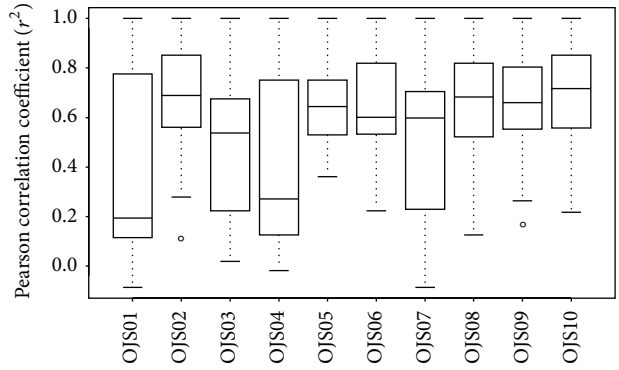

Figure 3: Pearson correlation coefficient (r^2) between the OJS samples. OJS01, Ojeok-san water extract from the laboratory; OJS02–OJS10, Ojeok-san granules from Korean manufacturers.

This result implies that the water extract and commercial granules of OJS were not chemically equivalent because of the variation in the content of the marker compounds.

3.4. Evaluation of Correlation between the OJS Samples Using Chemometric Analysis.

Similarities between the OJS samples were assessed using the Pearson correlation coefficient (r^2), which is a measurement of the distance between two samples and shows the degree of their relationship: a stronger correlation is observed when r^2 is closer to a value of 1 [22]. The average value of r^2 for OJS01 was the lowest, followed by OJS04, while the values of the other OJS samples were in the range $0.5 < r^2 < 0.8$ (Figure 3). This means that OJS01 and

Table 2: Regression, correlation coefficient (r^2), LOD, and LOQ of the marker compounds of OJS.

Compound	UV wavelength	Regression equation		Linear range (μg/mL)	r^2	LOD (μg/mL)	LOQ (μg/mL)
		Slope	Intercept				
Gallic acid	270 nm	39,349	4,315	0.63–5.00	0.9995	0.015	0.047
Protocatechuic acid	260 nm	42,285	2,576	0.31–5.00	0.9994	0.014	0.043
Chlorogenic acid	325 nm	32,676	9,500	1.56–25.00	0.9994	0.019	0.056
Albiflorin	230 nm	10,703	2,218	1.56–25.00	0.9998	0.057	0.172
Paeoniflorin	230 nm	15,956	−377	4.69–75.00	1.0000	0.038	0.115
Ferulic acid	325 nm	44,533	17,050	1.56–25.00	0.9993	0.014	0.041
Liquiritin	275 nm	24,585	12,410	4.69–75.00	0.9999	0.025	0.075
Benzoic acid	230 nm	38,560	11,910	1.56–25.00	0.9998	0.016	0.048
Nodakenin	335 nm	34,254	17,529	4.69–75.00	0.9999	0.018	0.054
Hesperidin	280 nm	18,406	25,320	10.94–175.00	0.9999	0.033	0.100
Naringin	280 nm	15,468	20,566	10.94–175.00	0.9999	0.039	0.119
Neohesperidin	280 nm	25,094	24,132	7.81–125.00	0.9999	0.024	0.073
Ononin	250 nm	58,807	3,317	0.31–5.00	0.9994	0.010	0.031
Oxypeucedanin hydrate	310 nm	16,087	2,865	1.56–25.00	0.9993	0.038	0.114
Cinnamic acid	275 nm	93,234	10,584	0.63–10.00	0.9998	0.007	0.020
Byakangelicin	270 nm	23,738	5,392	1.56–25.00	0.9994	0.026	0.077
Cinnamaldehyde	290 nm	156,619	8,846	0.33–5.25	0.9996	0.004	0.012
Benzoylpaeoniflorin	230 nm	28,272	274	0.16–2.50	0.9997	0.021	0.065
Glycyrrhizin	250 nm	6,765	1,864	1.56–25.00	0.9993	0.090	0.272

OJS04 were weakly correlated with the other OJS granules, which showed a mild correlation between samples [23].

Clustering is a partitioning process of objects set into disjoint clusters: objects in the same cluster are similar, while objects belonging to different clusters differ considerably according to their attributes [24], to which PCA and HCA can then be applied.

The 10 OJS samples were distributed on a PCA plot using their PC1 and PC2 scores, as these had higher eigenvalues and, thus, contained the chemically relevant variance [25]. OJS01 and OJS04 had a negative PC1 score, while the other samples had a positive PC1 score, and these were further divided by their PC2 score. The laboratory OJS water extract was differentiated from the commercial OJS granules, except for OJS04, by its PC1 score, which was the most influential factor for clustering the samples. Moreover, the distribution of the commercial OJS samples, especially OJS03 and OJS07, was not located close to each other but spread wide by their PC2 score. Therefore, this was a lower influential factor on the clustering samples after the PC1 score. The marker compounds contributing to the distribution of OJS samples were mainly cinnamic acid, cinnamaldehyde, albiflorin, and benzoylpaeoniflorin, which are denoted by the red-colored arrows in the PCA plot in Figure 4.

HCA is a method used to measure the distance between objects and find the underlying structure. It uses an iterative procedure that either associates or dissociates a group object by object to classify objects [26]. New clusters are produced by measuring the smallest increase in the sum of the squared within-cluster distances between all the possible clusters, and these are represented by dendrograms [27]. The 10 OJS samples were classified using Ward's method employing the Euclidean distance as a measurement for the HCA. OJS01 showed an exclusively close correlation with OJS04 and formed a separate cluster from the other commercial samples. These were segregated at a height around a value of 11. Under a height value around 5, the commercial OJS samples were further divided into two groups, namely OJS03 and OJS07, and OJS02, OJS05, OJS06, and OJS08–OJS10, which is similar to the results from the PCA analysis (Figure 5).

Taking the results of the quantification and chemometric analyses together, the OJS water extract (OJS01) produced in the laboratory showed little correlation with the commercially manufactured OJS granules from a chemical perspective. This result demonstrates that the low correlation between the OJS samples, particularly the laboratory-produced water extract and the commercial granules, can presumably be ascribed to the different ratios of the compositional herbal medicines, herbal resources, or extraction procedures of the OJS preparations between different pharmaceutical companies.

Table 3: Intra- and interday precision of the marker compounds of OJS.

Compound	Spiked concentration (μg/mL)	Intraday ($n = 3$)			Interday ($n = 3$)		
		Detected concentration (μg/mL)	RSD[a] (%)	Accuracy (%)	Detected concentration (μg/mL)	RSD (%)	Accuracy (%)
Gallic acid	1.00	1.00	1.68	100.41	1.00	1.68	100.41
	2.00	2.00	0.37	100.18	2.00	0.48	100.12
Protocatechuic acid	1.00	1.02	0.89	101.59	1.00	1.79	100.11
	2.00	2.00	0.45	99.97	2.01	0.43	100.31
Chlorogenic acid	2.00	1.96	0.27	98.04	1.96	0.38	97.98
	4.00	4.02	0.06	100.49	4.02	0.09	100.51
Albiflorin	2.00	1.99	1.09	99.56	1.97	2.57	98.61
	4.00	4.00	0.27	100.11	4.02	0.45	100.52
Paeoniflorin	10.00	10.22	1.36	102.20	10.22	1.37	102.20
	20.00	19.89	0.35	99.45	19.89	0.35	99.45
Ferulic acid	2.00	2.01	0.56	100.53	1.99	1.10	99.62
	4.00	4.00	0.26	100.09	4.00	0.27	100.10
Liquiritin	5.00	5.22	1.13	104.46	5.24	1.50	104.80
	10.00	9.89	0.30	98.88	9.88	0.40	98.80
Benzoic acid	3.00	2.88	0.67	95.97	2.87	1.22	95.67
	6.00	6.06	0.16	101.01	6.06	0.29	101.08
Nodakenin	5.00	5.01	0.42	100.12	5.03	0.28	100.51
	10.00	10.00	0.11	99.97	9.99	0.16	99.93
Hesperidin	20.00	20.59	0.39	102.97	20.60	0.41	102.98
	40.00	39.70	0.10	99.26	39.70	0.11	99.25
Naringin	20.00	20.83	0.30	104.15	20.83	0.31	104.15
	40.00	39.58	0.08	98.96	39.58	0.08	98.96
Neohesperidin	15.00	14.11	0.15	94.07	14.10	0.23	94.02
	30.00	30.44	0.03	101.48	30.46	0.02	101.53
Ononin	1.00	0.98	0.26	97.60	0.98	0.24	97.60
	2.00	2.01	0.06	100.60	2.01	0.06	100.60
Oxypeucedanin hydrate	1.00	0.98	1.99	97.91	0.97	2.65	97.35
	2.00	2.01	0.49	100.52	2.01	0.64	100.66
Cinnamic acid	1.00	0.99	0.09	99.26	0.99	0.50	99.02
	2.00	2.00	0.02	100.19	2.00	0.12	100.24
Byakangelicin	1.00	0.99	1.53	99.25	0.99	1.87	99.01
	2.00	2.01	0.22	100.49	2.01	0.36	100.59
Cinnamaldehyde	1.05	1.02	0.63	97.43	1.03	0.93	97.64
	2.10	2.11	0.31	100.27	2.11	0.34	100.29
Benzoylpaeoniflorin	1.00	1.01	1.53	100.87	1.00	1.29	99.81
	2.00	2.00	0.32	100.05	2.00	0.45	99.97
Glycyrrhizin	3.00	2.93	0.38	97.53	2.92	0.68	97.35
	6.00	6.04	0.09	100.62	6.04	0.16	100.66

[a]RSD (%) = (SD/mean) × 100.

Therefore, verification of the herbal resources, using an identical combination ratio, or using a valid extraction process, is required to produce chemically equalized OJS preparations that can guarantee an equivalent therapeutic efficacy.

4. Conclusions

The analytical method developed using an HPLC-PDA with a reversed-phase C_{18} column was precise, accurate, and reliable and was successfully applied to the simultaneous determination and quantification of 19 marker compounds for the quality assessment of OJS samples. The content of the marker compounds varied between the OJS samples. Moreover, a laboratory-produced OJS water extract was not closely related to the commercial OJS granules, which also showed a wide distribution in the results of chemometric analyses, such as the Pearson correlation coefficient, PCA, and HCA. Our results suggest that HPLC–PDA combined with chemometric analysis can be a useful strategy for

Table 4: Recovery of the marker compounds of OJS ($n = 3$).

Compound	Initial concentration (μg/mL)	Spiked concentration (μg/mL)	Detected concentration (μg/mL)	Recovery (%)	RSD (%)[a]
Gallic acid	1.90	1.00	2.88	98.05	2.28
		2.00	3.82	96.15	3.65
Protocatechuic acid	0.39	1.00	1.41	102.22	1.75
		2.00	2.47	104.16	0.88
Chlorogenic acid	6.52	2.00	8.37	92.74	1.21
		4.00	10.36	95.89	1.08
Albiflorin	3.62	2.00	5.57	97.19	1.41
		4.00	7.61	99.59	2.80
Paeoniflorin	15.89	10.00	25.84	99.54	1.86
		20.00	34.91	95.12	0.78
Ferulic acid	3.75	2.00	5.65	95.33	1.09
		4.00	7.53	94.58	0.24
Liquiritin	16.01	5.00	20.98	99.43	1.74
		10.00	25.16	91.55	0.43
Benzoic acid	6.72	3.00	9.59	95.74	1.91
		6.00	12.84	101.90	1.70
Nodakenin	8.98	5.00	13.74	95.28	1.03
		10.00	18.40	94.27	0.77
Hesperidin	61.70	20.00	81.66	99.77	0.28
		40.00	99.80	95.26	0.56
Naringin	62.30	20.00	82.34	100.19	0.72
		40.00	99.63	93.32	0.38
Neohesperidin	36.31	15.00	50.24	92.90	0.18
		30.00	66.90	101.97	0.07
Ononin	0.56	1.00	1.50	94.75	0.05
		2.00	2.52	98.29	0.35
Oxypeucedanin hydrate	7.18	1.00	8.11	93.19	2.25
		2.00	9.14	97.97	2.43
Cinnamic acid	0.94	1.00	1.92	97.97	0.84
		2.00	2.93	99.43	0.25
Byakangelicin	4.95	1.00	5.94	99.43	1.40
		2.00	6.96	100.58	1.67
Cinnamaldehyde	1.66	1.05	2.63	92.64	1.97
		2.10	3.66	95.06	1.17
Benzoylpaeoniflorin	0.38	1.00	1.39	100.55	1.19
		2.00	2.40	100.85	1.95
Glycyrrhizin	23.08	3.00	26.00	97.50	0.47
		6.00	29.19	101.91	0.83

[a]RSD (%) = (SD/mean) × 100.

Table 5: The average content of the marker compounds in OJS samples ($n = 3$).

Compound	Content (mg/g)									
	OJS01[a]	OJS02[b]	OJS03	OJS04	OJS05	OJS06	OJS07	OJS08	OJS09	OJS10
Gallic acid	0.096 ± 0.001	0.609 ± 0.023	0.978 ± 0.054	0.531 ± 0.005	0.273 ± 0.003	0.372 ± 0.004	0.371 ± 0.013	0.372 ± 0.023	1.592 ± 0.068	0.379 ± 0.006
Protocatechuic acid	0.019 ± 0.000	0.063 ± 0.001	ND[c]	0.032 ± 0.000	0.022 ± 0.002	0.019 ± 0.000	ND	0.018 ± 0.001	0.035 ± 0.001	0.036 ± 0.002
Chlorogenic acid	0.326 ± 0.002	0.009 ± 0.002	ND	0.333 ± 0.014	0.013 ± 0.000	0.082 ± 0.006	ND	0.028 ± 0.001	0.023 ± 0.002	0.036 ± 0.014
Albiflorin	0.184 ± 0.008	1.635 ± 0.020	5.950 ± 0.066	0.249 ± 0.010	0.243 ± 0.006	0.093 ± 0.011	5.060 ± 0.071	0.938 ± 0.017	1.072 ± 0.015	0.701 ± 0.040
Paeoniflorin	0.796 ± 0.006	3.363 ± 0.030	2.457 ± 0.046	2.294 ± 0.030	0.385 ± 0.017	1.529 ± 0.034	2.190 ± 0.048	1.679 ± 0.005	2.522 ± 0.052	1.539 ± 0.010
Ferulic acid	0.187 ± 0.000	ND	ND	0.172 ± 0.004	ND	ND	ND	ND	ND	ND
Liquiritin	0.800 ± 0.003	0.325 ± 0.008	0.259 ± 0.011	0.237 ± 0.009	0.083 ± 0.000	0.214 ± 0.005	0.014 ± 0.002	0.247 ± 0.002	0.236 ± 0.003	0.150 ± 0.003
Benzoic acid	0.337 ± 0.003	0.950 ± 0.012	0.449 ± 0.003	0.371 ± 0.007	0.244 ± 0.001	0.275 ± 0.001	1.902 ± 0.103	0.998 ± 0.005	1.612 ± 0.005	0.476 ± 0.003
Nodakenin	0.449 ± 0.002	ND	0.037 ± 0.000	0.002 ± 0.001	0.010 ± 0.000	ND	0.057 ± 0.001	0.045 ± 0.000	0.030 ± 0.001	ND
Hesperidin	3.086 ± 0.003	0.437 ± 0.013	0.348 ± 0.004	4.999 ± 0.010	0.376 ± 0.001	0.875 ± 0.006	ND	0.044 ± 0.001	0.214 ± 0.002	0.008 ± 0.002
Naringin	3.115 ± 0.002	0.578 ± 0.017	1.084 ± 0.007	2.983 ± 0.001	0.631 ± 0.001	0.783 ± 0.002	0.366 ± 0.004	0.580 ± 0.001	1.276 ± 0.004	0.750 ± 0.003
Neohesperidin	1.816 ± 0.001	0.277 ± 0.001	0.198 ± 0.001	3.139 ± 0.003	0.235 ± 0.000	0.592 ± 0.001	ND	ND	0.105 ± 0.000	0.043 ± 0.001
Ononin	0.028 ± 0.000	0.038 ± 0.000	0.022 ± 0.000	0.018 ± 0.000	0.014 ± 0.000	0.049 ± 0.000	0.004 ± 0.000	0.055 ± 0.000	0.033 ± 0.000	0.028 ± 0.000
Oxypeucedanin hydrate	0.359 ± 0.001	0.021 ± 0.001	0.052 ± 0.000	0.165 ± 0.000	0.028 ± 0.001	0.057 ± 0.000	0.005 ± 0.000	0.028 ± 0.001	0.019 ± 0.000	0.080 ± 0.001
Cinnamic acid	0.047 ± 0.000	0.033 ± 0.000	0.022 ± 0.000	0.055 ± 0.001	0.003 ± 0.000	0.011 ± 0.001	0.064 ± 0.002	0.013 ± 0.001	0.023 ± 0.000	0.041 ± 0.000
Byakangelicin	0.248 ± 0.002	0.013 ± 0.000	0.046 ± 0.000	0.071 ± 0.001	0.010 ± 0.001	0.030 ± 0.000	0.002 ± 0.000	0.016 ± 0.000	0.015 ± 0.000	0.026 ± 0.000
Cinnamaldehyde	0.083 ± 0.001	ND	0.122 ± 0.002	ND	ND	ND	ND	ND	ND	ND
Benzoylpaeoniflorin	0.019 ± 0.001	0.563 ± 0.006	0.049 ± 0.001	0.058 ± 0.004	0.005 ± 0.000	0.049 ± 0.001	0.048 ± 0.002	0.107 ± 0.001	0.052 ± 0.000	0.038 ± 0.002
Glycyrrhizin	1.155 ± 0.005	1.101 ± 0.032	0.959 ± 0.034	0.762 ± 0.044	0.248 ± 0.023	2.025 ± 0.015	1.179 ± 0.038	2.124 ± 0.028	0.790 ± 0.049	1.445 ± 0.037

The average content is represented as mean ± SD.

[a]OJS01, Ojeok-san water extract from the laboratory, [b]OJS02–OJS10 = Ojeok-san granules from Korean manufacturers, and [c]ND, not detected.

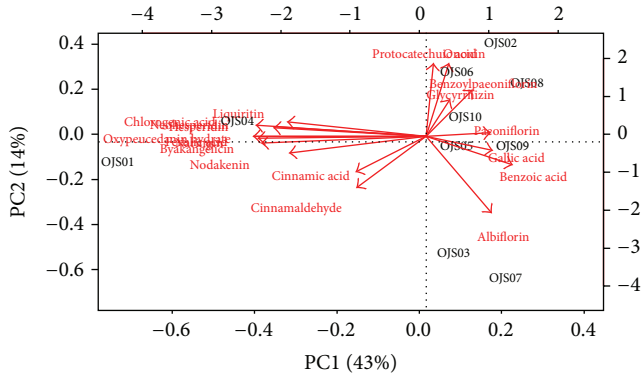

FIGURE 4: Biplot of the principal components (PC1 versus PC2) of the variables (the contents of the 19 marker compounds) with the objectives (OJS samples). The effect of the marker compounds on the distribution of OJS samples is shown by the red-colored arrows. PC1 and PC2 contributed to 43% and 14% of total variance, respectively. OJS01, Ojeok-san water extract from the laboratory; OJS02–OJS10, Ojeok-san granules from Korean manufacturers.

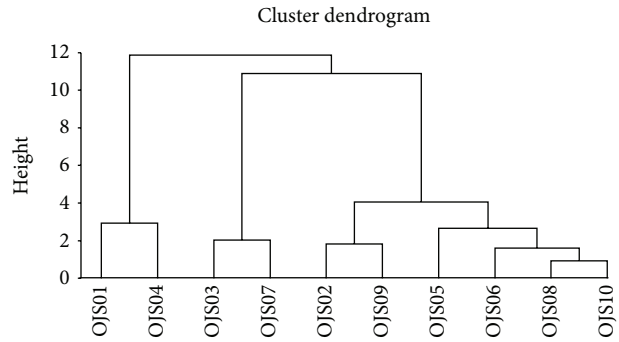

FIGURE 5: Dendrogram of the hierarchical clustering of OJS samples. OJS01, Ojeok-san water extract from the laboratory; OJS02–OJS10, Ojeok-san granules from Korean manufacturers.

the quality evaluation of OJS samples from different origins. It is necessary to produce chemically equalized OJS preparations for better quality samples.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

This study was supported by a grant from the Korea Institute of Oriental Medicine (no. K14030).

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 673547, 8 pages
http://dx.doi.org/10.1155/2015/673547

Research Article

Physicochemical Characteristics and Composition of Three Morphotypes of *Cyperus esculentus* Tubers and Tuber Oils

Souleymane Bado,[1] Patrice Bazongo,[2] Gouyahali Son,[3] Moe Thida Kyaw,[4] Brian Peter Forster,[1] Stephan Nielen,[1] Anne Mette Lykke,[5] Amadé Ouédraogo,[2] and Imaël Henri Nestor Bassolé[2]

[1]*Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria*
[2]*Life and Earth Sciences Training and Research Unit, University of Ouagadougou, 03 BP 7021, Ouagadougou, Burkina Faso*
[3]*Département Mécanisation, Institut de Recherche en Sciences Appliquées et Technologies, 03 BP 7047, Ouagadougou 03, Burkina Faso*
[4]*Department of Medical Research (Lower Myanmar), No. 5 Ziwaka Road, Dagon Township, Yangon 11191, Myanmar*
[5]*Department of Biosciences, Aarhus University, Vejlsoevej 25, 8600 Silkeborg, Denmark*

Correspondence should be addressed to Imaël Henri Nestor Bassolé; hbassole@hotmail.com

Received 27 November 2014; Revised 9 April 2015; Accepted 23 April 2015

Academic Editor: Jian Yang

Tuber characteristics and nutrient composition of three morphotypes of *Cyperus esculentus* tubers and tuber oils were determined. The mean value for length and width of the tuber and one thousand dried tuber weights ranged from 0.98 to 1.31 cm, 0.90 to 1.19 cm, and 598 to 1044 g, respectively. Tubers displayed high level of starch (30.54–33.21 g 100 g^{-1}), lipid (24.91–28.94 g 100 g^{-1}), and sucrose (17.98–20.39 g 100 g^{-1}). The yellow tubers had significantly higher content in lipid compared to black ones. Levels of ascorbic acid, tocopherol, and β-carotene of the three morphotypes differed significantly. Yellow ones (morphotypes 1 and 2) were the richest in tocopherol and the poorest in β-carotene. Saturated fatty acid content of morphotype 2 was significantly lower than that of morphotypes 1 and 3. Morphotype 3 had the significantly lowest PUFA content compared to morphotypes 1 and 2. Morphotype 1 was found to be richer in Ca, Cu, and Mn contents. Al, Mg, P, S, and Si were most abundant in morphotype 2. Morphotype 3 had the highest content of Cl, K, and Zn.

1. Introduction

Cyperaceae is a family of monocotyledonous graminoid flowering plants known as sedges, which superficially resemble grasses or rushes. About 5,500 species have been described in the family [1] including *Cyperus esculentus*. *Cyperus esculentus* provides edible tubers commonly called tigernut, chufa sedge, nut grass, yellow nutsedge, tigernut sedge, or earth almond. Tigernut is a perennial crop cultivated particularly in tropical and subtropical areas worldwide and extensively in Africa, Asia, and some European countries for their sweetish tubers. In Africa, tigernut is mostly cultivated in the west, Ivory Coast, Ghana, Mali, Niger, Nigeria, Senegal and Togo where they are used primarily uncooked as a side dish [2]. The tubers are used fresh as a vegetable or dried as a sweet snack. They are also grinded into flour and used as a thickener, for bread and cakes or mixed with water as drink. The tubers are often considered as "health" food because they have excellent nutritional properties and prevent heart diseases and thrombosis. Tigernut is known to activate blood circulation, to reduce risk of colon cancer and diabetes, and to favor weight loss [3]. Tigernut is also known to have aphrodisiac, carminative, diuretic, emmenagogue, stimulant, and tonic effects and even some medicinal uses such as treatment of flatulence, indigestion, diarrhea, dysentery, and excessive thirst [4]. Tigernut is used as livestock food and is in southern USA ranked among the top 10 most important waterfowl foods [5]. Tigernut flour is a rich source of

carbohydrate, oil, and some useful mineral elements such as iron and calcium which are necessary for body growth and development [6, 7]. Three varieties have been reported on the basis of their color, namely, yellow, black, and brown varieties [8]. Tigernut was reported to be rich in carbohydrates, dietary fiber, lipids, and oleic acid [3, 9]. Despite its great potentialities the tigernut remains an underutilized plant [7]. Most of the studies focused on the yellow variety while very little information exists on the physical characteristics of tigernut tubers. A better understanding of morphological parameters of the tigernut tubers as well as their link to the nutrition composition will help to identify valuable varieties and promote their use. So, this crop could contribute to the poverty alleviation among vulnerable populations, particularly rural women, in Western Africa. The aim of this study was to determine the physical traits as well as the chemical characteristics of the tubers from the three morphotypes of tigernut grown in Burkina Faso.

2. Material and Methods

2.1. Plant Material. Tubers of *Cyperus esculentus* L. were sampled in January and February 2007–2009, in 5 villages located in western and southwestern Burkina Faso: Loropéni (10°18′N, 3°32′W), Mangodara (9°54′N, 4°21′W), Ouéléni (10°51′N, 5°21′W), Tangora (10°38′N, 4°45′W), and Tiéfora (10°38′N, 4°33′W). Five kilograms of tubers was collected in each village, immediately hand-sorted to eliminate damaged ones, and taken to the laboratory. Prior to any analysis, the samples were washed with distilled water, drained, and air-dried. Each village sample was split into two parts; one part was finely ground with a Moulinex grinder robot (GT550, Zurich, Switzerland). Both parts were packing in an airtight container and stored at −18°C until analysis.

2.2. Analytical Methods

2.2.1. Physical Analysis. To determine the mean length and width of the tubers, 100 tubers were per village randomly picked and their two linear dimensions were measured using a Vernier caliper with an accuracy of 0.01 mm (Canon Instruments, Japan). The thousand dried tubers weight (TSW) was obtained by counting 1000 dried tubers and weighted on an electronic balance to 0.001 g accuracy (Ohaus, USA). The variation in tubers size and color was used to classify the tigernut into different morphotypes.

2.2.2. Chemical Analysis. The official methods of the Association of Official Analytical Chemists [10] were used to determine moisture, protein, crude oil, and ash contents of the tubers. Moisture (g water 100 g^{-1} sample) was determined by drying a 3 g ground sample at 105°C to constant weight. Nitrogen content was determined by using the Kjeldahl method and multiplied by a factor of 6.25 to determine the crude protein content (g protein 100 g^{-1} sample). Crude fat (g fat 100 g^{-1} sample) was obtained by exhaustively extracting 5.0 g of each sample in a Soxhlet apparatus using petroleum ether (boiling point range 40–60°C) as the extractant. Mineralization was performed on 3 g samples by combustion in a muffle furnace at 550°C for 8 h (g ash 100 g^{-1} sample) (AOAC 920.39C). Carbohydrate content was estimated by difference of mean values: 100 − (sum of percentages of ash, protein, and lipids) [11].

2.2.3. Starch and Sugar Analysis. AOAC method 996.11 was used to determine starch content of *Cyperus esculentus* tuber flours. The assay consisted of using thermostable alpha-amylase and amyloglucosidase to enzymatically hydrolyze starch into glucose that was then quantified with a spectrophotometer (μQuant, Bioteck Instruments Inc, USA). Glucose, sucrose fructose, and maltose were analyzed by HPLC according to the AOAC Official Method 982.14 [12]. Samples for HPLC sugars analysis were prepared by homogenizing 0.3 g of *Cyperus esculentus* flour in 3 mL distilled water and 7 mL 95% alcohol and shaken before being centrifuged at 10 000 rpm for 20 min. The clear supernatant was filtered through 0.45 μm filter and degasified before analysis by HPLC. Filtered solution (20 μL) was injected into HPLC 1100 Series (Agilent, Waldbronn, Germany) equipped with a G1362A refractive index detector. Sugars were separated using a commercially packed with Zobax-NH_2 column (250 × 4.6 mm (Dupont, Wilmington, DE, USA)) with a particle size of 5 μm and thermostatized at 30°C. The filtered and degasified mixture of acetonitrile/water (80/20) was used as mobile phase at a flow rate of 1 mL/min for 30 min run time [13]. The sugars peaks were identified by comparing their retention times with individual standard sucrose, maltose, glucose, and fructose approximately 99% pure (Sigma-Aldrich, Steinheim, Germany) and the chromatograms analyzed using the Agilent Technologies Chemstation Software.

2.2.4. Vitamin Analysis. Vitamin C was determined in tubers as previously described [14, 15]. An aliquot of 25 g of tigernut was added to 25 mL of a solution containing 45 g/L metaphosphoric acid and 7.2 g/L of DL-1,4-dithiotreitol (DTT). The mixture was homogenized and centrifuged at 22,100 g for 15 min at 4°C. The supernatant was vacuum-filtered through Whatman no. 1 filter. Prior to HPLC analysis, the vacuum-filtered samples (10 mL) were passed through a Millipore 0.45 μm membrane. Then, 20 μL was injected into a HPLC system fitted with a reversed-phase column, C18 Spherisorb ODS2 (5 μm) stainless-steel column (4.6 mm × 250 cm). The mobile phase was a 0.01% sulphuric acid solution adjusted to a pH of 2.6, at a flow rate of 1 mL/min at room temperature. Detection was performed at 245 nm with 486 Absorbance Detector (Waters, Milford, MA). Vitamin C was quantified through a calibration curve built with ACS grade ascorbic acid (>99% pure, Sigma-Aldrich, Steinheim, Germany) pure standards in the range of 0.2–50 μg/mL.

To determine vitamin E (α-tocopherol) and β-carotene, approximately 5 g of ground samples were extracted with 50.0 mL of hexane. The mixture was then vortexed for 5 min and filtered using 0.2 μm pore size PTFE membrane. The filtered hexane fraction was directly injected into RP-HPLC system for β-carotene and vitamin E analysis [16]. The RP-HPLC system (Shimadzu) consisted of an autosampler and column oven equipped with Inertsil ODS-3V (250 × 4.6 mm, 5 μm) reversed-phase column. For β-carotene

analysis, mobile phase was acetonitrile (6 : 4, v/v, containing 0.05: BHA as antioxidant) (eluent A) and MeOH (eluent B). The following gradient was used: initial condition was 70% (A) and 30% (B) for 5 min, followed by 80% (A) and 20% (B) for 5 min, at a flow rate of 1.5 mL/min. Elution was monitored using a photodiode-array detector at 472 nm [17]. For vitamin E content, methanol mobile phase was used at a flow rate of 1.0 mL/min. The α-tocopherol was detected by a Shimadzu SPD-10A (UV/VIS) detector (292 nm wavelength). Standards of β-carotene (≥97.0% purity, Sigma-Aldrich, Steinheim, Germany) and DL-α-tocopherol (≥96% purity, Sigma-Aldrich, Steinheim, Germany) ranging from 0.5 to 6.0 μg/mL and from 0.02 to 1.0 μg/mL were used for calibration.

2.2.5. Tuber Oil Fatty Acids Analysis. Fatty acid methyl esters were determined according to International Union of Pure and Applied Chemistry (IUPAC) method II.D.19 [18]. On hundred milligrams of extracted oils was saponified in a volumetric flask, with 1.2 mL of 0.5 M KOH in MeOH by heating and stirring under reflux for 5 min. After saponification oils were esterified by adding 1.2 mL 20% borontrifluoride through condenser and boiled for 2 min and then the flask was moved from the magnetic stirrer and fatty acid methyl esters were extracted by adding 1 mL of n-hexane. Saturated NaCl solution was added until the n-hexane is in the neck of volumetric flask, mixed carefully, flipped once or twice, and let settle for about 30 min. After separation the n-hexane phase was transferred to a vial for fatty acid methyl esters analysis. Gas chromatography (GC) of fatty acid methyl esters was performed using a Perkin Elmer GC-autosystem XL with a programmable temperature vaporizer (PTV) split-injector and a flame ionization detector (FID). Helium was used as carrier gas. The column temperature was initially maintained at 100°C for 2 min and then raised by 5°C/min to 225°C and finally held at 225°C for 16 min. The injection volume was 0.2 μL with a 1 : 100 split. The PTV injector was initially maintained at 50°C and immediately after the injection raised to 270°C. The FID was kept at 250°C. The capillary column employed was CP Sil 88 (Chrompak, Varian Instruments, Walnut Creek, CA; 50.0 m × 0.25 mm and 0.2 μm film thickness). The peaks were identified by comparing retention times with authentic fatty acid methyl esters. Quantification was based on the area under each fatty acid peak as compared to the total area of all fatty acid peaks.

2.2.6. Mineral Composition of Powered Tubers. The elements, Mg, P, Cr, Fe, Mn, Cu, Zn, Sr, Ca, K, and Cd, in digests were measured using an atomic absorption spectrophotometer (Analyst 800, Perkin-Elmer) and/or a coupled plasma mass Spectrophotometer (ELAN DRCII Axial Field Technology, Perkin-Elmer). About 0.2 g of powered tuber was digested with 3 mL of HNO_3 (65%) and 0.5 mL of H_2O_2 (30%) in a closed vessel microwave digestion system (MLS-ETHOS plus) and diluted to 50 mL with Millipore water. Digestion conditions for the microwave system were applied as follows: 2 min for 250 W, 2 min for 0 W, 6 min for 250 W, 5 min for 400 W, 8 min for 550 W, and vent for 8 min. A blank digest was carried out in the same way. Al, Si, S, and Cl were analysis by polarized Energy Dispersive X Ray Fluorescent (EDXRF), Spectro X-LAB 2000. Prior to analysis, 4 g of ground dried samples triplicate was pelleted by 5 tons using SpectroPess (Chemplex Industries, Inc.) and then pellets were analyzed using different excitation conditions with an EDXRF spectrometer [19]. Standard Reference Material 1568a rice flour was obtained from National Institute of Standards and Technology, Gaithersburg, USA, and was used as food reference material to evaluate the analytical methods.

2.3. Statistical Analysis. All samples were tested at least in duplicate in each analytical technique. The values of different parameters were expressed as the mean ± standard deviation. Comparison of means was performed by one-way analysis of variance (ANOVA) followed by Wilcoxon's multiple comparison tests. Principal component analysis (PCA) was performed to compare the physical and chemical data of 3 morphotypes of *Cyperus esculentus* tubers. PCA was carried out using the 33 physical and chemical variables which differed significantly between morphotypes. Principal component analysis (PCA) is used in exploratory analysis. It gives graphical representations of intersample and intervariable relationships and provides a way to reduce the complexity of the data. Statistical significance was set at the 5% level of probability using JMP In 5.1 software (SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1. Morphological Variants. Tubers from five collection sites were grouped into three morphological variants on the basis of their color (yellow or black) and size (big or small tuber). Then three variants were identified: (1) yellow and big (morphotype 1), yellow and small (morphotype 2), and black and big (morphotype 3) (Table 1). Thus, tuber samples from Mangodara and Tiéfora were classified as morphotype 1, those from Loropéni and Ouéléni as morphotype 2, and those from Tangora as morphotype 3 (Figure 1).

3.2. Physical Characteristics. The tuber characteristics of the three morphotypes of *Cyperus esculentus* are shown in Table 1. The moisture content was not significantly different ($p > 0.05$) among the three morphotypes. Lengths were ranged from 0.98 ± 0.06 to 1.31 ± 0.06 cm. Morphotype 2 tubers were significantly shorter than those of morphotypes 1 and 3. Morphotypes 1 and 3 had slightly bigger tubers than approximate average length (0.63 to 1.21 cm) found for tigernuts from other countries [5]. The width of the tuber and one thousand dried tuber weights varied from 0.90 ± 0.08 to 1.19 ± 0.05 cm and from 598.00 ± 115.00 to 1044.00 ± 394.60 g, respectively. The tuber width of morphotype 2 was significantly lower than that of morphotype 3. Both morphotypes 1 and 3 had higher one-thousand-tuber weight than morphotype 2. The one-thousand-tuber weight seems to be more influenced by tuber width than length. One thousand weights of investigated tubers were far higher than those obtained for brown tubers by Coşkuner et al. [5] that showed how genetic diverse is *C. esculentus* cultivated around the world.

(a) (b) (c)

Figure 1: Morphotypes of *Cyperus esculentus* grown in Burkina Faso. (a) Morphotype 1: big size and yellow color. (b) Morphotype 2: small size and yellow color. (c) Morphotype 3: big size and black color.

Table 1: Physical characteristics and proximate composition of *Cyperus esculentus* morphotypes tubers.

Parameters	Morphotype 1	Morphotype 2	Morphotype 3
Mean length (cm)	1.24 ± 0.05^{a}	0.98 ± 0.06^{b}	1.31 ± 0.06^{a}
Mean width (cm)	$0.97 \pm 0.05^{a,b}$	0.90 ± 0.08^{b}	1.19 ± 0.05^{a}
1000 dried tubers (g)	814.3 ± 184.1^{a}	598.00 ± 115.00^{b}	1044.00 ± 394.60^{a}
Moisture (g 100 g^{-1})	5.19 ± 0.18^{a}	4.56 ± 0.22^{a}	4.99 ± 0.78^{a}
Crude oil (g 100 g^{-1})	26.14 ± 0.71^{b}	28.94 ± 0.37^{a}	24.91 ± 0.94^{c}
Protein (g 100 g^{-1})	$3.47 \pm 0.71^{a,b}$	4.33 ± 0.6^{a}	3.3 ± 0.26^{b}
Ash (g 100 g^{-1})	$1.81 \pm 0.24^{a,b}$	1.69 ± 0.21^{b}	2.21 ± 0.39^{a}
Carbohydrates (g 100 g^{-1})	$68.24 \pm 1.28^{a,b}$	64.73 ± 1.21^{b}	69.21 ± 1.30^{a}

Values are means ± standard deviation for $n = 3$. Data in the same row followed by different letters are significantly different ($p < 0.05$).

3.3. Proximate Composition. Crude oil contents of the three morphotypes varied from 24.91 ± 0.94 to 28.94 ± 0.37 g 100 g^{-1} of dry weight (DW). Crude oil content was higher in morphotypes 2 followed by morphotype 1, with morphotype 3 as the lowest (Table 1).

Crude oil content of the three morphotypes reported in this paper is lower than those reported for black and brown tubers from Cameroon [20]. However, the lipid content values are similar to those of white tubers [21] and higher than the content of tigernut genotype from Spain reported by Alegría-Torán and Farré-Rovira [22]. The data reported indicate that the lipid content of tigernut is influenced by genetic material and geographical location. Protein levels in the three morphotypes ranged from 3.3±0.26 to 4.33±0.6 g 100 g^{-1}. The morphotype 2 protein content was significantly higher than morphotype 3. The levels of protein are not related to either colour or size. The protein content for the three morphotypes from Burkina Faso was very low compared to tubers from Cameroon [20], Nigeria [23], and Turkey [5]. The ash content of the three morphotypes was ranged between 1.81 ± 0.24 and 2.21 ± 0.39 g 100 g^{-1}. Morphotype 3 had significantly higher ash content than morphotype 2. No significant difference was found between morphotype 1 and the two other ones. The ash content of the three morphotypes is lower than those reported for black, brown, and yellow tubers [5, 20, 21]. Morphotypes 1 and 3 had similar carbohydrate content which was higher than that of morphotype 2. Tubers from Burkina Faso are richer in carbohydrates than those from Nigeria [7] and Spain [22].

Table 2: Carbohydrate composition (g 100 g^{-1}) of *Cyperus esculentus* morphotypes tubers.

Parameters	Morpho. 1	Morpho. 2	Morpho. 3
Starch	30.54 ± 2.75^{a}	33.21 ± 1.1^{a}	30.54 ± 0.5^{a}
Sucrose	18.99 ± 0.56^{b}	17.98 ± 1.03^{b}	20.39 ± 1.15^{a}
Fructose	3.02 ± 0.37^{a}	3.59 ± 0.72^{a}	1.6 ± 0.69^{a}
Glucose	6.79 ± 1.34^{a}	6.33 ± 0.97^{a}	0 ± 0^{b}

Values are means ± standard deviation for $n = 3$. Data in the same row followed by different letters are significantly different ($p < 0.05$).

3.4. Starch and Other Carbohydrate Contents. Starch, sucrose, fructose, and glucose contents of three morphotypes are reported in Table 2.

The starch content of the three morphotypes ranged from 30.54 ± 2.75 to 33.21 ± 1.1 g 100 g^{-1}. It appeared that starch content was not significantly different among morphotypes. However, the values for the three morphotypes were slightly higher than those reported by Coşkuner et al. [5] and similar to data of Linssen et al. [24]. The tigernuts from Burkina Faso displayed 1.6 ± 0.69 to 3.59 ± 0.72 g 100 g^{-1} of fructose with no significant difference among morphotypes. The sucrose and glucose contents of the three morphotypes ranged from 17.98 ± 1.03 to 20.39 ± 1.15 g 100 g^{-1} and from 0 to 6.79 ± 1.34 g 100 g^{-1}, respectively. Morphotype 3 had significantly higher sucrose than morphotype 1 and morphotype 2, while glucose content was not detected. The sucrose content was within the range previously reported [5, 25]. Karacali [26]

Table 3: Vitamin contents of *Cyperus esculentus* morphotypes tubers.

Parameters	Morph. 1	Morph. 2	Morph. 3
Vitamin C (mg 100 g^{-1})	5.48 ± 1.05^{c}	26.78 ± 2.51^{a}	8.33 ± 1.83^{b}
Vitamin E (μg 100 g^{-1})	209.71 ± 1.30^{b}	270.56 ± 1.74^{a}	149.86 ± 1.94^{c}
β-Carotene (μg 100 g^{-1})	7.3 ± 0.57^{b}	6.13 ± 0.62^{c}	10.05 ± 1.79^{a}

Values are means ± standard deviation for $n = 3$. Data in the same row followed by different letters are significantly different ($p < 0.05$).

reported that the amount and composition of sugars vary according to fruit species, varieties, and ecological conditions, and technical and cultural practices affect the flavour. In addition, irrigation, harvest time, and storage conditions also affect the sugar composition of almond kernel [27–29]. Regarding the taste of sugars, sucrose is sweeter than glucose, and fructose is sweeter than sucrose [30]. Balta et al. [31] reported a positive correlation between glucose and fructose contents and the sweet taste of almond. Yellow morphotypes (morphotypes 1 and 2) had higher glucose and fructose contents than black ones; they should be sweeter.

3.5. Vitamin Contents. The ascorbic acid, tocopherol, and β-carotene contents of the three morphotypes are shown in Table 3. Vitamins contents of the three morphotypes differed significantly. The ascorbic acid levels varied from 5.48 ± 1.05 to 26.78 ± 2.51 mg 100 g^{-1} and were within the usual range for tubers and lower than that of nuts [32, 33]. The highest content of ascorbic acid was recorded with morphotype 2, followed by morphotypes 3 and 1.

Tocopherol content of three morphotypes ranged from 149.86 ± 1.94 to 270.56 ± 8.33 μg 100 g^{-1}. The morphotype 2 tocopherol content was significantly higher than that of morphotype 1, which was also significantly higher than that of morphotype 3. The tocopherol content obtained in this study is lower than that reported in tigernut oil from Ghana [9].

β-Carotene content of three morphotypes varied from 6.13 ± 0.62 to 10.05 ± 1.79 μg 100 g^{-1}. Morphotypes 2 and 3 had the lowest and the highest content, respectively. Burmeister et al. [34] reported higher β-carotene content compared to Burkinabe tubers.

3.6. Fatty Acid Composition. Oils of the three morphotype tubers contained high amounts of monounsaturated fatty acids (MUFAs) (65.91 ± 1.75–67.75 ± 1.41%), followed by saturated fatty acids (SUFAs) (20.65 ± 0.38–22.03 ± 1.11%) and polyunsaturated fatty acids (PUFAs) (10.2 ± 0.36–12.53 ± 0.73%) (Table 4). The SUFA content of morphotype 2 was significantly lower than that of morphotypes 1 and 3. Morphotype 3 had significantly lowest PUFA content compared to morphotypes 1 and 2. The MUFA content was not significantly different among the morphotypes. The SUFA, MUFA, and PUFA proportions were similar to those previously reported [9]. However the tigernut studied here had better

Table 4: Fatty acid composition (% total fatty acids) of total lipid from *Cyperus esculentus* morphotypes tubers' oil.

Fatty acids	Morphotype 1	Morphotype 2	Morphotype 3
Myristic acid	0.16 ± 0.03^{b}	0.14 ± 0^{b}	0.4 ± 0.24^{a}
Pentadecanoic acid	0.03 ± 0.03^{a}	0 ± 0^{a}	0.05 ± 0.05^{a}
Palmitic acid	15.81 ± 0.95^{a}	15.83 ± 0.32^{a}	15.22 ± 0.55^{a}
Stearic acid	4.73 ± 0.33^{b}	3.89 ± 0.15^{c}	5.36 ± 0.26^{a}
Arachidic acid	0.57 ± 0.03^{b}	0.55 ± 0.02^{b}	0.68 ± 0.01^{a}
Behenic acid	0.1 ± 0^{a}	0.09 ± 0.02^{a}	0.1 ± 0.01^{a}
Lignoceric acid	0.17 ± 0.01^{b}	0.15 ± 0.03^{b}	0.24 ± 0.02^{a}
Palmitoleic acid	0.40 ± 0.05^{a}	0.37 ± 0.04^{a}	0.53 ± 0.02^{a}
cis-7-Hexadecenoic acid	0.35 ± 0.05^{a}	0.33 ± 0.03^{a}	0.44 ± 0.2^{a}
Heptadecenoic acid	0 ± 0^{a}	0.03 ± 0.03^{a}	0.03 ± 0.03^{a}
Oleic acid	64.25 ± 1.99^{a}	65.42 ± 1.17^{a}	65.76 ± 1.8^{a}
Vaccenic acid	0.99 ± 0.14^{a}	0.99 ± 0.12^{a}	0.93 ± 0.11^{a}
Eicosenoic acid	0.25 ± 0.05^{a}	0.28 ± 0.06^{a}	0.37 ± 0.16^{a}
Cetoleic acid	0.05 ± 0.05^{a}	0.03 ± 0.03^{a}	0.09 ± 0.1^{a}
Nervonic Acid	0 ± 0^{a}	0.05 ± 0.05^{a}	0.05 ± 0.05^{a}
Linoleic acid	12.39 ± 0.72^{a}	12.07 ± 0.23^{a}	10.04 ± 0.37^{b}
Linolenic acid	0.14 ± 0.02^{b}	0.14 ± 0.01^{b}	0.17 ± 0.01^{a}
Total saturated	21.56 ± 0.71^{a}	20.65 ± 0.38^{b}	22.03 ± 1.11^{a}
Total monounsaturated	65.91 ± 1.75^{a}	67.15 ± 1.21^{a}	67.75 ± 1.41^{a}
Total n-6 PUFA	12.39 ± 0.72^{a}	12.07 ± 0.23^{a}	10.04 ± 0.37^{b}
Total n-3 PUFA	0.14 ± 0.02^{b}	0.14 ± 0.01^{b}	0.17 ± 0.01^{a}
Total PUFA	12.53 ± 0.73^{a}	12.21 ± 0.23^{a}	10.2 ± 0.36^{b}

Values are means ± standard deviation for $n = 3$. Data in the same row followed by different letters are significantly different ($p < 0.05$).

SUFA and PUFA content than those reported by Sánchez-Zapata et al. [3]. A total of seventeen fatty acids have been identified in each morphotype. Among the fatty acids, oleic acid (64.25 ± 1.99–65.76 ± 1.8%), palmitic acid (15.22 ± 0.55–15.83 ± 0.32%), linoleic acid (10.04 ± 0.37–12.39 ± 0.72%), and stearic acid (3.89 ± 0.15–5.36 ± 0.26%) were the most abundant fatty acids, in three morphotypes, as previously reported [5, 35].

3.7. Mineral Contents. The three *Cyperus esculentus* morphotypes tubers appeared to be important sources of mineral (Table 5). The most abundant minerals were K, P, Si, Cl, S, and Mg and their content was significantly different at $p < 0.05$ except for S and Mg. Some contaminants such Cr, Sr, and Cd were detected at low amounts.

Morphotype 1 was found to be richer in Ca, Cu, and Mn contents. Al, Mg, P, S, and Si were most abundant in morphotype 2. Morphotype 3 had the highest content of Cl, K, and Zn. The mineral compositions of the three morphotypes in the present study are different from those recorded with accessions from Niger, Nigeria, and

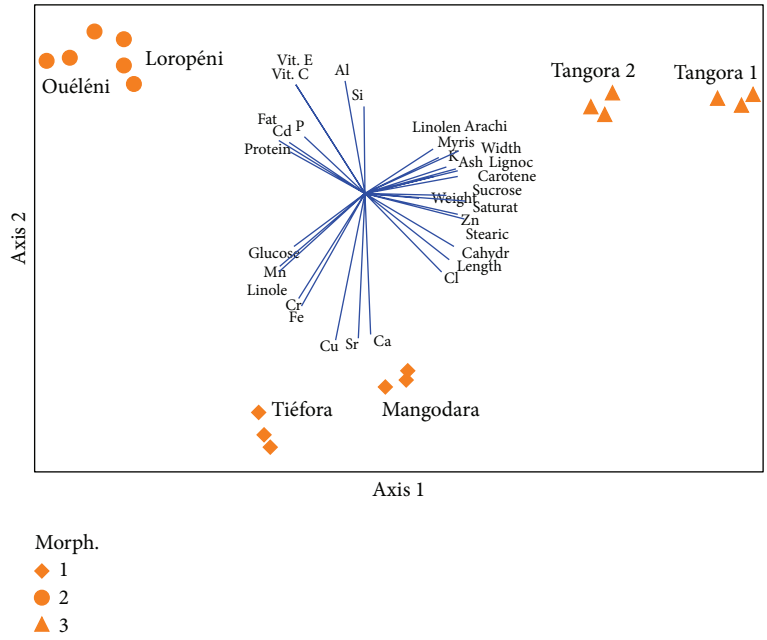

Figure 2: Ordination of the Burkina Faso *Cyperus esculentus* tubers showing three morphotypes. Myris = myristic acid, Stearic = stearic acid, Arachi = arachidic acid, Lignoc = lignoceric acid, Saturat = total saturated acids, Linole = linoleic acid, Linolen = linolenic acid, Cahydr = carbohydrates, Vit. C = vitamin C, and Vit. E = vitamin E.

Table 5: Mineral composition (mg 100 g^{-1}) of *Cyperus esculentus* morphotypes tubers.

Parameters	Morph. 1	Morph. 2	Morph. 3
Al	34.35 ± 2.55^{b}	43.37 ± 6.13^{a}	39.86 ± 0.74^{a}
Ca	32.27 ± 5.66^{a}	19.09 ± 3.22^{b}	22.13 ± 1.64^{b}
Cd	$0.01 \pm 0.01^{a,b}$	0.02 ± 0.01^{a}	0.01 ± 0^{b}
Cl	167 ± 0.53^{a}	155.4 ± 2.83^{b}	167.2 ± 5.51^{a}
Cr	1.65 ± 0.05^{a}	1.12 ± 0.31^{b}	0.18 ± 0.01^{c}
Cu	0.71 ± 0.03^{a}	0.48 ± 0.05^{b}	0.43 ± 0.01^{c}
Fe	11.44 ± 0.48^{a}	8.25 ± 1^{b}	3.57 ± 0.17^{c}
K	608.3 ± 97.84^{b}	556.9 ± 80.4^{b}	845.8 ± 7.94^{a}
Mg	100.5 ± 1.79^{a}	107.3 ± 8.2^{a}	102.2 ± 2.86^{a}
Mn	1.55 ± 0.44^{a}	1.44 ± 0.15^{a}	0.38 ± 0.02^{b}
P	$229.6 \pm 51.54^{a,b}$	283.7 ± 14.96^{a}	236.4 ± 16.53^{b}
S	164.3 ± 18.23^{a}	194.1 ± 61.62^{a}	148.8 ± 8.39^{a}
Si	181.6 ± 50.35^{b}	242.5 ± 35.89^{a}	$220.3 \pm 47.32^{a,b}$
Sr	0.36 ± 0.09^{a}	0.17 ± 0.02^{b}	0.19 ± 0.02^{b}
Zn	2.34 ± 0.31^{b}	1.88 ± 0.22^{b}	2.7 ± 0.03^{a}

Values are means ± standard deviation for $n = 3$. Data in the same row followed by different letters are significantly different ($p < 0.05$).

Turkey [22, 35, 36]. Field observations of the soil type where the tigernuts are mainly growing showed that morphotype 1 is grown on more sandy soil, whereas morphotypes 2 and 3 are cultivated on soil with, respectively, more reddish and brownish clay. Therefore different mineral content can be due to differences in soil composition which can influence mineral uptake and storage in the tuber.

3.8. Principal Component Analysis. Principal component analysis (PCA) is used in exploratory analysis, which gives an overview of multivariate data [37]. A PCA using 33 physical and chemical variables showed clear differences among the three morphological types of tubers and gives a good overview of the characteristics of each type (Figure 2).

The first three principal components accounted for 82.27% of the total variation among the accessions. Most of the variation was explained by the first principal component (52%), followed by the second (22%) and the third (9%) (Table 6).

Loadings of the variables on the first two principal components show that the first component had high positive loadings from length, width, carbohydrate, sucrose, β-carotene, stearic acid, stearic acid, linoleic acid, total saturated fatty acids, and Zn and high negative loadings from lipids, linolenic acid, and Mn. The second component had high negative loadings from Cu, Sr, and Ca. Morphotype 1 had negative loadings in PC2 and was characterized by high Ca, Sr, Cu, and Fe contents whereas morphotype 2 showed positive scores in PC2 and had high protein, lipid, vitamin C, vitamin E, and P contents. Morphotype 3 was located in the positive side of PC1 and was characterized by the highest content of ash, β-carotene, and myristic, arachidic, and linolenic acids.

4. Conclusion

We present in this study the physical and chemical variability of tigernuts (*Cyperus esculentus*) cultivated in Burkina Faso. The data revealed that three *Cyperus esculentus* morphotypes are important source of macronutrients (starch, fat, and

Table 6: Eigenvectors and percent explained variation by the first six principal components of physical and chemical data parameters of 3 morphotypes of *Cyperus esculentus* tubers.

Variable	Eigenvectors					
	PC1	PC2	PC3	PC4	PC5	PC6
Length	0.201	−0.158	−0.059	0.210	−0.061	0.133
Width	0.217	0.060	−0.044	0.260	−0.092	−0.045
Weight	0.128	−0.012	0.092	0.067	−0.561	0.313
Crude oil	−0.205	0.126	0.207	−0.036	0.067	0.013
Protein	−0.178	0.099	−0.369	−0.073	−0.111	0.050
Ash	0.164	0.042	−0.013	0.147	0.127	0.625
Carbohydrates	0.212	−0.126	0.148	0.053	0.121	0.039
Sucrose	0.195	−0.005	−0.036	0.044	0.092	−0.399
Glucose	−0.170	−0.125	−0.342	−0.206	0.015	0.095
Vitamin C	−0.165	0.259	0.079	0.011	0.166	0.008
Carotene	0.221	0.041	−0.082	−0.185	0.214	0.007
Vitamin E	−0.165	0.259	0.079	0.011	0.166	0.008
P	−0.144	0.135	−0.277	0.407	−0.045	0.009
Cr	−0.158	−0.251	0.197	0.088	0.126	−0.017
Fe	−0.151	−0.268	0.185	0.008	0.123	−0.018
Mn	−0.202	−0.175	−0.067	0.176	0.150	−0.006
Cu	−0.070	−0.349	−0.040	−0.144	0.021	0.065
Zn	0.222	−0.050	0.223	0.048	−0.077	−0.073
Sr	−0.016	−0.344	−0.172	0.194	0.101	0.010
Cd	−0.180	0.121	−0.311	−0.218	0.003	0.061
Ca	0.015	−0.335	−0.135	0.256	0.135	0.007
K	0.194	0.063	−0.191	0.336	−0.051	−0.122
Al	−0.046	0.269	0.068	0.331	0.093	−0.281
Si	−0.003	0.207	0.149	0.132	0.382	0.453
Cl	0.182	−0.186	−0.104	−0.191	0.210	−0.002
Myristic acid	0.177	0.087	−0.156	−0.050	0.450	−0.043
Stearic acid	0.237	−0.062	0.073	−0.038	0.043	−0.042
Arachidic acid	0.224	0.100	−0.016	−0.192	−0.079	−0.007
Lignoc	0.220	0.054	−0.133	−0.252	−0.005	0.017
Saturat	0.239	−0.019	0.015	−0.065	0.117	−0.040
Linole	−0.206	−0.187	−0.067	0.056	0.006	0.010
Linolen	0.164	0.105	−0.414	0.093	0.024	0.025
Eigenvalue	16.69	6.97	2.67	1.81	1.71	0.79
Individual %	52.14	21.78	8.34	5.66	5.33	2.46
Cumulative %	52.14	73.93	82.27	87.92	93.26	95.72

sucrose) and minerals (potassium, phosphorus, silicon, chlorine, sulfur, and magnesium). Some interesting differences were noticed such as the content of carbohydrates, starch, saturated fatty acids, and polyunsaturated fatty acids. The yellow morphotypes showed the highest content of fructose, glucose, and crude oil. Black morphotype was richer in carbohydrates with high content in sucrose whereas the yellow are source of fructose and glucose. These data revealed genetic variability among cultivated tigernuts from Burkina Faso and from others grown worldwide. Thus, tigernuts from Burkina Faso displayed particular composition which could be of great interest for nutritional quality and food processing.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge the support by the government of Burkina Faso, DANIDA (Project no. 10-002AU), and FAO/IAEA, Plant Breeding and Genetics Laboratory.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 535387, 7 pages
http://dx.doi.org/10.1155/2015/535387

Research Article

Analysis of Reaction between α-Lipoic Acid and 2-Chloro-1-methylquinolinium Tetrafluoroborate Used as a Precolumn Derivatization Technique in Chromatographic Determination of α-Lipoic Acid

Magdalena Godlewska,[1] Angelika Odachowska,[1] Monika Turkowicz,[2] and Joanna Karpinska[1]

[1]*Faculty of Chemistry, University of Bialystok, Hurtowa 1, 15-399 Bialystok, Poland*
[2]*Białystok Provincial Sanitary and Epidemiological Station, Department of Food Products, Food-Contact Articles and Nutrition Research, Legionowa 8, 15-099 Bialystok, Poland*

Correspondence should be addressed to Joanna Karpinska; joasia@uwb.edu.pl

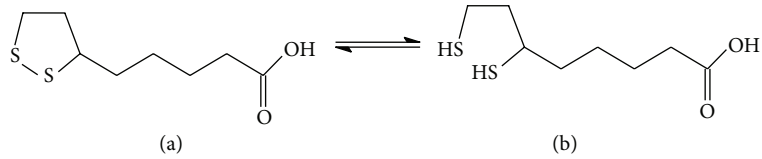
Received 29 October 2014; Revised 5 January 2015; Accepted 20 January 2015

Academic Editor: Shao-Nong Chen

The present study offers results of analysis concerning the course of reaction between reduced α-lipoic acid (LA) and 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT). In water environments, the reaction between CMQT and hydrophilic thiols proceeds very rapidly and the resultant products are stable. For the described analysis, optimum reaction conditions, such as concentration of the reducing agent, environment pH, and concentration of the reagent were carefully selected. The spectrophotometric assay was carried out measuring absorbance at $\lambda = 348$ nm (i.e., the spectral band of the obtained reaction product). Furthermore, the calibration curve of lipoic acid was registered. It was concluded that the Lambert-Beer law was observed within the range 1–10 μmol L^{-1}. Later, the reaction between LA and CMQT was used as precolumn derivatization in a chromatographic determination of the lipoic acid in the range 2.5–50 μmol L^{-1}. Practical applicability of the designed methods was evaluated by determining lipoic acid in *Revitanerv* pharmaceutical preparation which contains 300 mg LA in a single capsule. The error of the determination did not exceed 0.5% in relation to the declared value.

1. Introduction

Lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid, Figure 1(a)), along with its reduced form (dihydrolipoic acid, DHLA, Figure 1(b)), is an important cofactor of mitochondrial enzymes and a natural antioxidant. It is present in both eukaryotic and prokaryotic microorganisms [1] and in all plant and animal cells [2]. In oxidative decarboxylation of pyruvate, α-ketoglutarate, branched-chain α-keto acids and glycine it acts as a catalyst [3, 4]. During the above processes, lipoic acid undergoes reduction to dihydrolipoic acid. The value of standard redox potential of the dihydrolipoic/lipoic acid couple is equal to −320 mV [3, 5]. Owing to such a high redox potential, the couple serves as an antioxidant for antioxidants [6]. LA is also involved in reduction of such compounds as tocopherol radical, oxidized vitamin C, glutathione, and Q_{10} coenzyme [6]. Lipoic acid is both water- and fat-soluble. For this reason, it is present in blood plasma, cytoplasm, and cell membranes [5, 6]. This property makes it an intermediary agent between lipophilic (tocopherol, Q_{10} coenzyme) and hydrophilic (glutathione) antioxidants [3]. Intracellularly, lipoic acid, together with other antioxidants, acts as a free radical scavenger [3, 6–8]. The acid forms chelate bonds with metal ions [3, 9, 10]. It has been observed that lipoic acid supplementation has beneficial effects in treatment of conditions related to oxidative stress (e.g., atherosclerosis)



(a) (b)

Figure 1: Lipoic acid (LA) (a) and its reduced form dihydrolipoic acid (DHLA) (b).

[6, 11], diabetes [5, 12], cataract, neurodegenerative diseases [13], liver diseases [13], and acquired immunodeficiency diseases [14]. Also, application of lipoic acid in geriatrics has yielded promising results [3, 4, 15]. Notably, the acid and its derivatives have proven to exhibit advantageous effects in treatment of cancers [13, 16].

For humans, the principal source of lipoic acid is food, but the content of the acid in food products varies. In particular, the products of animal origin, especially red meat, contain between 0.25 and 2.36 μg LA g^{-1} [3], whereas foods of plant origin, for example, fresh potatoes, contain between 1.5 and 4.2 μg LA g^{-1} of the substance [17].

Determination of lipoic acid content in clinical samples (i.e., blood, plasma, tissues) or in food products is extremely useful for evaluation of the acid's function in human metabolism. A number of analytic techniques have been developed for that purpose, including mainly gas chromatography (GC) [17, 18], high-performance liquid chromatography (HPLC) with electrochemical detection [19, 20], mass spectrometry [20–22], fluorescent [23, 24] or UV spectroscopy [25, 26], and capillary electrophoresis (CE) [27]. Due to the presence of polar sulfide and carboxyl groups in the LA molecule, the determination with GC technique requires transformation of lipoic acid into nonpolar derivatives, such as S,S-dibenzyl-methyl or S,S-diethoxycarbonyl methyl esters, after its previous reduction with sodium borohydride [17]. Recently, 4-bromomethyl-6,7-dimethoxycoumarin was used as derivatisation reagent for UV and MALDI-TOF detection of lipoic acid [22].

As for the analysis of lipoic acid content by means of high-performance liquid chromatography, employing electrochemical detectors, as well as mass, UV, and fluorescent spectrometers, is carried out. The usage of an electrochemical detector allows for simultaneous denotation of different chemical forms of lipoic acid, including the oxidized and reduced ones. Using spectrophotometric or fluorescent detection, on the other hand, can be quite problematic, because the molecule of the acid does not contain any chromophoric or fluorophoric groups. Therefore, such an analysis requires a prior reaction which would bound the LA molecule with appropriate signaling groups.

The presented study focuses on the course and analytical application of the reaction between reduced form of lipoic acid, dihydrolipoic acid (DHLA) and 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT, Figure 2). The described technique was used for the first time to determine LA content in a pharmaceutical preparation.

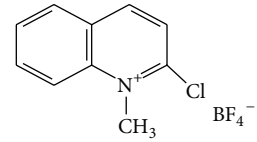


Figure 2: Formulae of 2-chloro-1-methylquinolinium tetrafluoroborate.

2. Materials and Methods

2.1. Laboratory Equipment

2.1.1. Hitachi U-2800A UV/VIS Spectrophotometer. All spectrophotometric determinations were done using a Hitachi U-2800A spectrophotometer (Japan). The following working settings of the apparatus were applied: scan speed 1200 nm/min and spectral bandwidth (1.5 nm).

The chromatographic system (Thermo Separation) consisted of a 3D Spectra System UV 3000, a low-gradient pump P2000, a vacuum membrane degasser SCM Thermo Separation, and a Rheodyne loop injector (20 μL) and was used for analysis of the lipoic acid derivative solutions. ChromQuest Chromatography Data system software for Windows NT was applied for acquisition and storage of data. The analysis was performed with the use of a Supelco Supelcosil LC-8 HPLC column with the following dimensions: 15 mm length × 4.6 I.D. and 5 μm particle size. Mixture of $5 \cdot 10^{-2}$ mol L^{-1} pH 3 disodium hydrogen phosphate and acetonitrile in the molar ratio of 35 : 65 was used as mobile phase. The mobile phase flow rate was equal to 1 mL min^{-1}, and the chromatograms were monitored at 348 nm.

2.2. Reagents. 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared directly in the laboratory. For this purpose, the following procedure was employed [28]. In a 50 mL conical flask 1 g of 2-chloro-quinoline and 1 g of 3-methoxytetrafluoroborate were mixed with 1.2 mL of nitromethane. The mixture was stirred until the solid components dissolved, and 4 mL of diethyl ether was added. When the white crystals precipitated, they were drained using a water aspirator and washed twice with 2 mL of diethyl ether. Next, the obtained substance was dried in a desiccator in the presence of $CaCl_2$. Thus prepared compound was used to prepare a 10^{-2} mol L^{-1} stock solution by dissolving its weighted measure in MiliQ water. In a similar vein, working solutions with desired concentrations were later obtained by diluting the stock solution with MiliQ water.

Lipoic acid (LA) was produced by Sigma-Aldrich, USA. The stock solution of lipoic acid with the concentration of 10^{-2} mol L^{-1} was obtained by dissolving a proper weighted measure of the substance in methanol. Working solutions were prepared by appropriately diluting the stock solution with MiliQ water.

Sodium borohydride ($NaBH_4$) was produced by POCh, Poland. Its stock solution (0.3 mol L^{-1}) was prepared by weighing a 0.114 g measure of $NaBH_4$ and dissolving it in 10 mL of MiliQ water.

EDTA/NaOH 0.9% NaCl 5 mmol L^{-1} buffer solution was prepared by weighing a 0.731 g measure of EDTA, 0.1 g measure of NaOH, and 0.450 g measure of NaCl and dissolving them in 500 mL of MiliQ water. Solutions with required pH values were obtained by adding appropriate volumes of 1 mol L^{-1} NaOH or HCl.

6% solution of sodium bicarbonate was prepared by weighing a 3 g measure of $NaHCO_3$ and dissolving it in 50 mL of MiliQ water.

Stock solution of disodium hydrogen phosphate with the concentration of 5×10^{-2} mol L^{-1} and pH 3.00 was prepared by weighing a 3.549 g measure of the solid substance and dissolving it in a 500 mL flask in MiliQ water. The solution's pH 3.00 was achieved by adding 3 mol L^{-1} H_3PO_4.

Methanol and acetonitrile (HPLC grade) was produced by Merck, Germany.

Revitanerv Pharmaceutical Preparation (BLAUFARMA, Poland). A single capsule contained microcapsuled α-lipoic acid (300 mg), borage seed oil (40% content of γ-linolenic acid), gelatin, vitamin E, niacin, pantothenic acid, vitamin B6, vitamin B2, magnesium stearate, silicon dioxide, titanium dioxide, vitamin B1, and selenium.

2.3. Experimental Procedures

2.3.1. Spectrophotometric Determination of Lipoic Acid by Means of a Derivatization Reaction. 100 μmol L^{-1} lipoic acid solution was introduced into a number of 10 mL test tubes in 0.1, 0.25, 0.5, 0.75, and 1 mL volumes, respectively. After 1 mL of 5 mmol EDTA/NaOH buffer with pH of 9.5 was added into each test tubes. Next 66.8 μL of $NaBH_4$ was introduced into each tube to reach final concentration at 0.075 mol L^{-1}. The samples were carefully stirred and put in a water bath for 6 minutes at a temperature of 60°C. After the heating stopped, the samples were cooled down to the room temperature. The surplus of unreacted reducing agent was removed by adding 66.8 μL of hydrochloric acid (c = 0.5 mol L^{-1}), later neutralizing the samples with 85 μL 6% $NaHCO_3$. Eventually, the solutions were transferred to 10 mL volumetric flasks.

Next, the volume of 50 μL of CMQT solution with the concentration of 10^{-2} mol L^{-1} was added to prepared DHLA solution. The mixtures were stirred, filled up with MiliQ water to the mark, and carefully stirred once again. Measurements of absorbance were conducted at λ = 348 nm 24 minutes after the addition of CMQT, using as a reference the mixture of the same reagents not containing lipoic acid.

2.3.2. Direct Spectrophotometric Technique for Determination of Lipoic Acid. The direct spectrophotometric method for determination of lipoic acid relied on measuring the absorbance of the oxidized form of the acid at λ = 210 nm. The procedure was as follows: first, a series of standard LA solutions with concentrations varying between 50 μmol L^{-1} and 500 μmol L^{-1} were prepared. The solutions were obtained by diluting the adequate volumes of the stock solution (C_{LA} = 10^{-2} mol L^{-1}) with MiliQ water in 10 mL volumetric flasks. Subsequently, the absorbance measurements were conducted at the wavelength of 210 nm (MiliQ water was used as a reference).

2.3.3. Determination of Lipoic Acid in the Pharmaceutical Preparation. The contents of a single capsule were dissolved in a small amount of methanol. The sample was stirred for 20 minutes, and the resultant suspension was filtered. In the next step, the filtrate was transferred to a 100 mL volumetric flask which was then filled up to the mark with methanol. In this way, a stock solution with the concentration of $1.45 \cdot 10^{-2}$ mol L^{-1} was obtained. During the analysis, a working solution was used, obtained by a hundredfold dilution of the stock solution. Further procedure was as follows: 1 mL of a buffer solution with pH 9.5 was mixed with 412 μL of the working solution obtained from the capsule and 66.8 μL of $NaBH_4$ solution (0.3 mol L^{-1}). The ensuing preparation was heated for 6 minutes at 60°C. After cooling down, the amounts of 66.8 μL of HCl (0.5 mol L^{-1}) and 85 μL of $NaHCO_3$ (6%) were added. Next, a 50 μL volume of CMPI solution (10^{-2} mol L^{-1}) was introduced; the flask was filled up to the mark with water and carefully stirred. Absorbance of the reaction mixture was measured 24 minutes after stirring at λ = 348 nm, using the same mixture of reagents without lipoic acid as reference.

2.3.4. Determination of Lipoic Acid in the Pharmaceutical Preparation Using a Direct Spectrophotometric Technique. The contents of a single capsule were dissolved in a small amount of methanol and the sample was stirred for about 20 minutes. The mixture was later filtered and the filtrate was transferred into a 100 mL volumetric flask which was subsequently filled up to the mark with methanol. Thus obtained solution was tenfold diluted by transferring its 1 mL volume into a 10 mL volumetric flask and filling up the flask to the mark with MiliQ water. Afterwards, 412 μL of this solution was again transferred into a 10 mL flask. The flask was filled up to the mark with MiliQ water and its contents were stirred. Eventually, the absorbance of the ensuing solution was registered at λ = 210 nm using water as a reference.

3. Results

3.1. Preliminary Analysis. Lipoic acid does not contain any chromophoric groups. In the spectrum of an oxidized LA molecule there is a weak band at 330 nm and a more intense nonspecific band at 210 nm (Figure 3).

In the spectrum of the reduced form of LA, the only nonspecific band occurs at 200 nm. As for CMQT, its spectral

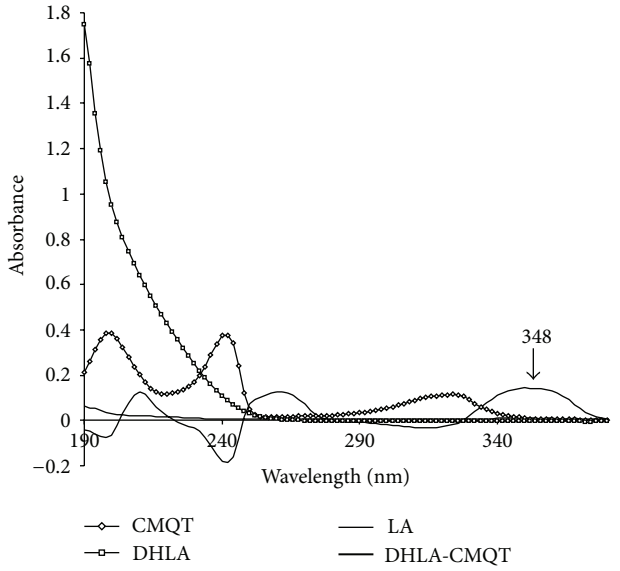


Figure 3: Spectra of lipoic acid (LA), dihydrolipoic acid (DHLA), 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), and derivatization product (DHLA-CMQT) (concentration of all reagents and compounds = 10^{-5} mol L^{-1}; all spectra with exception of derivatization product were recorded against bidistilled water as a blank; spectrum of derivatisation product were recorded against mixture of reagents without DHLA).

characteristic is more complex, as can be seen in Figure 3. Specifically, its spectrum features 3 bands: at 200, 240, and 325 nm. The used derivatizing agent selectively reacts with thiol groups, the reaction proceeds rapidly and in a quantitative manner, and the resultant bonding is stable under the conditions of spectrophotometric analysis. The UV spectrum of the CMQT-DHLA reaction product is markedly different from the spectrum of the reagent. In particular, the intensity of the bands at 200 and 240 nm is increased, and new bands appear at 260 and 348 nm, whereas the band at 325 nm diminishes. Due to the intensity of absorbance and practically no interference from the unreacted surplus CMQT, all the measurements described below were carried out at λ = 348 nm.

3.2. Optimization of the Reaction Conditions. It was established that the intensity of the band at 348 nm was related with the following factors: pH of the lipoic acid reduction environment, temperature and heating time during the reduction, pH of the DHLA-CMQT reaction environment, and $NaBH_4$: LA molar ratio and excess of the used reagent in relation to the amount of reduced lipoic acid. Accordingly, optimization of the reaction conditions was carried out. Selecting different concentrations of the reagents and pH of the environment, the concentration of the analyzed factor was adjusted, keeping the other determinants constant. For the analysis, lipoic acid with the concentration of 10 μmol L^{-1} was used. The exact procedure followed was described above in the equipment and reagents section. Table 1 contains the resolved parameters of the investigated process. Essentially, it was ascertained that lipoic acid reacted with CMQT

Table 1: Features of the developed methodology.

Parameter	Studied range	Selected value
Molar ratio LA : $NaBH_4$	1 : 25–1 : 100	1 : 50
pH of reduction	7–10	9,5
Temperature of reduction/time of heating	60°C 0–15 min	6 min
pH of medium of reaction DHLA-CMQT	4.5–10	6
Excess of CMQT in ratio to DHLA	1 : 1–1 : 5	1 : 5

in the molar ratio DHLA : CMQT = 1 : 2. For quantitative denotation of lipoic acid in the S-quinolinic derivative form at least a fivefold excess of the reagent should be used.

3.3. Spectrophotometric Determination of Lipoic Acid in the Pharmaceutical Preparation. Based on the obtained data the stability constant of the DHLA-CMQT complex was calculated by mole ratio method as $\beta = 4.739 \times 10^{18}$. The obtained value shows that the stability of the reaction product makes it eligible for quantitative determination of lipoic acid. Accordingly, ensuring the optimum environment for the DHLA-CMQT bonding, the calibration curve was registered. It was observed that the Lambert-Beer law was fulfilled in the concentration range between 1.0 and 10 μmol L^{-1}. The calibration curve was constructed by linear least-square analysis. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on parameters of calibration curves (LOD = $a + 3S_{y/x}$, where a-intercept calibration line; $S_{y/x} = \sqrt{\sum_i (y_i - \hat{y}_i)^2/(n-2)}$, $\hat{y}_i$-value for a given value of x readily calculated from the regression equation, LOQ = $a + 10S_{y/x}$ [29]). The obtained value of the molar absorption coefficient equaled $1.5 \cdot 10^4$ mol$^{-1} \cdot$ cm$^{-1} \cdot$ L shows the good sensitivity of the determination. Statistical evaluation of the designed spectrophotometric method of LA determination is presented in Table 2. At the same time, a direct method of determination of lipoic acid involving the absorbance measurement of the oxidized form of the acid at 210 nm was devised. It turned out that the direct absorbance measurement allows for determination of lipoic acid in a higher concentration range (i.e. 50–750 μmol L^{-1}) with higher LOD and LOQ values. The results are provided in Table 2.

For the sake of practical assessment, the discussed spectrophotometric methods were used to determine lipoic acid in the *Revitanerv* preparation whose declared LA content equals 300 mg per capsule. Table 3 provides the relevant figures. The data gathered in Table 3 proved the practical usefulness of the developed derivatization procedure. The obtained error of determination is low which testified the good accuracy of the elaborated spectrophotometric method based on derivatisation reaction and good agreement between both spectrophotometric methods is observed.

3.4. Application of the DHLA-CMQT Derivatization for Chromatographic Determination of Lipoic Acid. During the investigation of the DHLA-CMQT reaction it was observed that

Table 2: Validation parameters of elaborated methods.

Parameter	Direct spectrophotometric method	Spectrophotometric method based on derivatization reaction	HPLC method based on DHLA-CMQT product as precolumn derivatization reaction
Linearity range/μmol L^{-1}	50–750	1.0–10	2.5–50
Equation of calibration curve/x-concentration in mol L^{-1}	$y = 1.6 \cdot 10^3 x + 1.6 \cdot 10^{-3}$	$y = 1.5 \cdot 10^4 x + 0.4 \cdot 10^{-3}$	$y = 4.0 \cdot 10^7 x - 110.75$
Correlation coefficient	0.999	0.999	0.997
LOD/μmol L^{-1}	1.120	0.258	0.214
LOQ/μmol L^{-1}	37	0.784	0.647
RSD/%	0.5	3.0	2.06
SD	0.018	$7 \cdot 10^{-3}$	0.014

Table 3: Assay results of the commercial lipoic acid dosage forms; $n = 5$.

	Declared value/mg	Determined/mg	Average error/%
Direct spectrophotometric method	300 mg/capsule	298 ± 3.26	±0.46
Spectrophotometric method based on derivatization reaction		300.66 ± 2.87	±0.23
HPLC method based on DHLA-CMQT product as precolumn derivatization reaction		301.44 ± 2.63	±0.48

the obtained product was stable. Therefore, it was decided to employ the studied reaction as precolumn derivatization of lipoic acid in a liquid chromatography technique. RP-8 column was used for the purpose, to which 20 μL of the postreaction mixture was transferred. A number of solvents and their mixtures were analyzed (trichloroacetic acid c = 0.05 mol L^{-1} : acetonitrile = 95 : 5, methanol : water = 50 : 50, methanol : trichloroacetic acid c = 0.05 mol L^{-1} = 10 : 90, acetonitrile : water = 80 : 20, acetonitrile : Na_2HPO_4 c = 0.05 mol L^{-1} pH 3, the molar ratio varied in the range 80 : 20–20 : 80). The most satisfactory separation was achieved in the case of acetonitrile : Na_2HPO_4 c = 0.05 mol L^{-1} pH 3 mixed in the volumetric ratio of 35 : 65. Also, it was noted that the retention time of the S-quinolinic derivative of dihydrolipoic acid in the given conditions equaled 3.222 ± 0.001 minutes. Ensuring the optimum conditions of derivatization and preserving the previously established chromatographic process configuration a series of chromatograms were registered for LA solutions with variable concentrations. It was observed that linearity was achieved for the LA concentration range from 2.5 μmol L^{-1} to 50 μmol L^{-1}. Statistical evaluation of the devised chromatographic method is presented in Table 2.

It is worth emphasizing that our results are comparable with those obtained by capillary liquid chromatography coupled with ultraviolet detection [22]. The range of detection of proposed HPLC-UV method is 2.5–50 μmol L^{-1} with the LOD = 0.88 pg/injection and LOQ = 2.67 pg/injection which are on the same level as those recently published [22] but worse than in method with fluorescence detection [17, 23, 24]. The proposed procedure was compared with other methods proposed for LA analysis (Table 4).

The comparison shows that the proposed method is superior in terms of its sensitivity and precision. Additionally, it is worth emphasizing that such good parameters were obtained using conventional analytical chromatographic system with DAD detector.

Practical usability of the method was verified by determining the content of lipoic acid in the *Revitanerv* preparation. The results are provided in Table 3. The obtained results testified the good accuracy of the proposed procedure. The error of assay does not exceed 0.5% and good agreement with declared contents of LA is observed.

4. Conclusion

The presented study discusses findings related to the investigation of lipoic acid derivatization with the use of 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT). Among others, the experiments allowed concluding that obtaining S-quinolinic derivatives of lipoic acid requires prior reduction of the acid. The product of the reduction is characterized by sufficient stability which renders the reaction applicable for use in spectrophotometric and chromatographic determination of lipoic acid. It is true that introducing derivatization to the determination procedure requires additional effort. The undertaking, however, brings tangible profits. The spectrophotometric method which employs derivatization makes it possible to determine lipoic acid in a lower concentration

Table 4: Comparison of the proposed method with elaborated methods of analyzing LA.

Method	Derivatisation agent	Range of determination	LOD	LOQ	Reference
HPLC-FL	N-(1-Pyrene)iodoacetamide N-(1-Pyrenemethyl)iodoacetamide	0.75–120 μmol L^{-1}	<3.1 fmol	Not given	[23]
	Ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfonate (SBD-F)	0.94–60 μmol L^{-1}	0.13 pmol	0.44 pmol	[24]
Capillary LC-UV	4-Bromomethyl-6,7-dimethoxycoumarin	0.1–20 μmol L^{-1}	0.03 μmol L^{-1}	Not given	[21]
	4-Bromomethyl-6,7-dimethoxycoumarin	0.1–40 μmol L^{-1}	5 fmol	Not given	[22]
HPLC-UV	Derivatisation with 1-benzyl-2-chloropyridinium bromide	0.2–50 μmol L^{-1}	0.1 μmol L^{-1}	0.20 μmol L^{-1}	[26]
	Without derivatisation	48.5–2422.7 μmol L^{-1}	21.32 μmol L^{-1}	81.40 μmol L^{-1}	[25]
Proposed HPLC-UV method	2-Chloro-1-methylquinolinum tetrafluoroborate	2.5–50 μmol L^{-1}	0.21 μmol L^{-1}	0.65 μmol L^{-1}	

range in comparison to the direct absorbance measurement techniques. Also, it is marked by lower limits of detection and quantitation. Taking advantage of the analyzed reaction as a precolumn derivatization technique signals a possibility of employing the described process for quantitative determination of lipoic acid in complex biological samples. The presented results showed that the method based on CMQT-DHLA reaction is characterised by sufficient sensitivity which allows using it for direct determination of LA in biological samples [20] without preconcentration of the analyte. The application of reaction-CMQT-LA in chromatographic analysis enables direct assaying of LA in the presence of others low-molecular-mass thiols and their disulfides in biological samples [26, 28].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by Grant no. 2011/01/N/NZ9/01768 from Polish National Centre of Science.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 259757, 9 pages
http://dx.doi.org/10.1155/2015/259757

Research Article

Detection of Gelatin Adulteration in Traditional Chinese Medicine: Analysis of Deer-Horn Glue by Rapid-Resolution Liquid Chromatography-Triple Quadrupole Mass Spectrometry

Jia Chen, Xian-Long Cheng, Feng Wei, Qian-Qian Zhang, Ming-Hua Li, and Shuang-Cheng Ma

National Institute for Food and Drug Control, State Food and Drug Administration, 2 Tiantan Xili, Beijing 100050, China

Correspondence should be addressed to Xian-Long Cheng; lncxl@sina.com and Feng Wei; weifeng@nifdc.org.cn

Received 29 September 2014; Revised 3 February 2015; Accepted 27 February 2015

Academic Editor: Shao-Nong Chen

Simultaneous identification of donkey-hide gelatin and bovine-hide gelatin in deer-horn glue was established by rapid-resolution liquid chromatography-triple quadrupole mass spectrometry. Water containing 1% NH_4HCO_3 was used for sample dissolution and trypsin was used for hydrolysis of the gelatins. After separation by a SB-C18 reversed-phase analytical column, collagen marker peptides were detected by mass spectrometry in positive electrospray ionization mode with multiple reaction monitoring. The method was specific, precise and reliable, and suitable for detection of adulterants derived from donkey-hide gelatin and bovine-hide gelatin in deer-horn glue.

1. Introduction

Deer-horn glue (*Cervi Cornus Colla*) is a traditional Chinese medicine (TCM) that has been widely used in China for about 2000 years. It is a solid glue prepared from deer horn by decoction and concentration [1]. It is viewed as a nutritious, high-quality TCM, as indicated in "Shennong's Herbal," and is predominantly used for treating kidney disorders and Qi deficiency. It is claimed that long-time consumption of deer-horn glue will nourish yin, replenish blood, and prolong life. Because of the high market price and an inability to satisfy demand, adulteration is common and the most widely practiced approach is to substitute and/or replace the authentic material with donkey- and bovine-hide gelatin.

It has long been difficult to control the quality of deer-horn glue because of the absence of appropriate quality assessment methods. The polymerase chain reaction method has been used in DNA analysis for collagen identification [2, 3], but the method is not suitable for gelatin identification because of the breakdown of gelatin DNA during sample processing. Literature research has revealed that proteomic methods have been proposed as alternative tools for the assessment of collagen species in gelatins [4] and mass spectrometry has been successfully applied to elucidate differences among homological gelatins [5]. In our work, the focus of research has been on method specificity for differentiation of homological gelatins. In our previous work [6, 7], for instance, tryptic peptides of gelatins were measured by ultrahigh performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS), and principal component analysis was used to classify donkey-hide gelatin, bovine-hide gelatin, and deer-horn glue. Thereafter, gelatins were analyzed by doubly charged selected ion monitoring (DCSIM) with tandem mass spectrometry (MS/MS) to aid in the identification of the gelatins. The possibility of detecting the target peptides in such gelatins with rapid-resolution liquid chromatography (RRLC) coupled to electrospray ionization- (ESI-) ion trap (IT) MS would be a useful development.

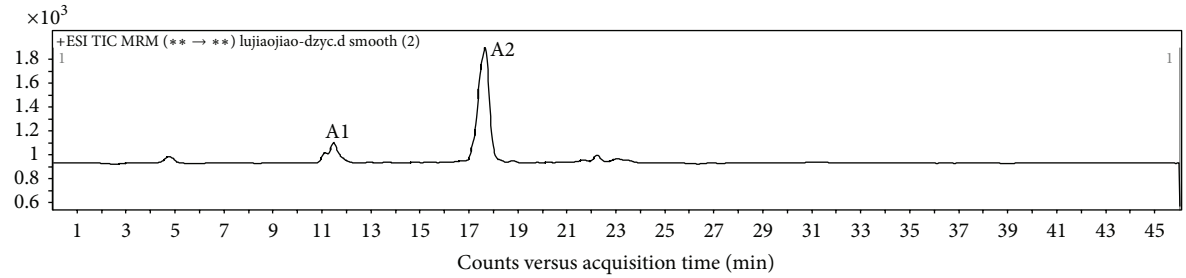
Generally, HPLC-QQQ MS/MS is a sensitive analytical method available for detection of the adulterants. As shown recently, high-pressure liquid chromatography-mass

Table 1: Gelatin sources.

Sample	Standard gelatin	Source	Lot number by NIFDC
1	Deer-horn glue	*Cervus elaphus* Linnaeus	121694-201301
2	Donkey-hide gelatin	*Equus asinus* L.	121274-201202
3	Bovine-hide gelatin	*Bos taurusdomesticus* Gmelin	121695-201301

Table 2: Precursor and product ions for the gelatin species and operating parameters for fragmentation voltage and collision-activated dissociation voltage.

Number	Precursor m/z	Product ion m/z	Originated from	Retention time min	Fragment voltage eV	Collision energy eV
A1	732.8	817.9/961.9	Deer-horn glue	11.2080	175	30
A2	765.4	554.0/733.0	Deer-horn glue	17.1209	135	15
B1	641.3	783.3/726.2	Bovine-hide gelatin	7.4309	135	37
B2	790.9	912.4/841.3	Bovine-hide gelatin	12.5446	175	32
B3	747.3	903.3/847.1	Bovine-hide gelatin	13.4004	155	26
B4	604.8	569.8/910.1	Bovine-hide gelatin	15.2002	135	25
C1	618.8	721.9/778.9	Donkey-hide gelatin	7.7407	135	23
C2	539.8	612.4/923.8	Donkey-hide gelatin	10.1043	135	15
C3	765.9	823.1/991.0	Donkey-hide gelatin	18.8379	155	45



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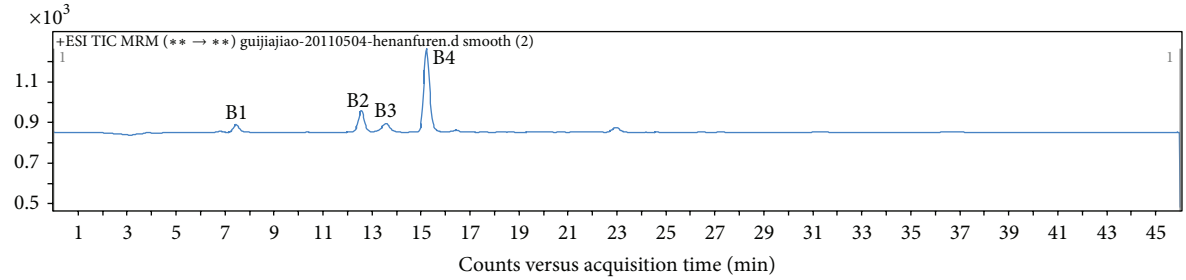

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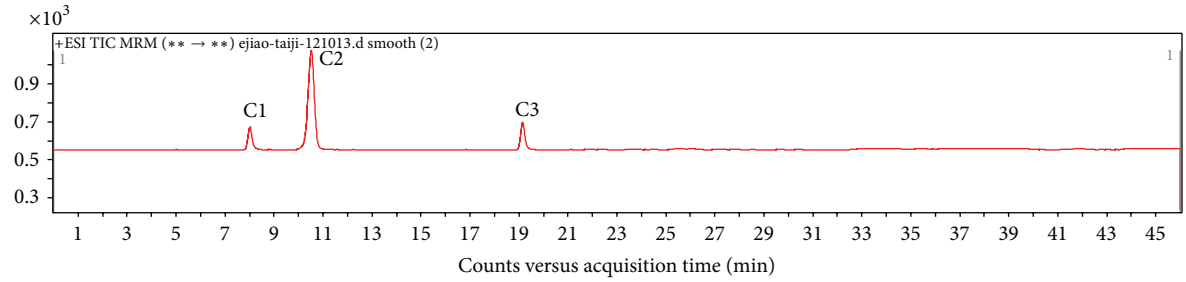

(c)

Figure 1: (a) Characteristic selected ion chromatograms for deer-horn glue. (b) Characteristic selected ion chromatograms for bovine-hide gelatin. (c) Characteristic selected ion chromatograms for donkey-hide gelatin.

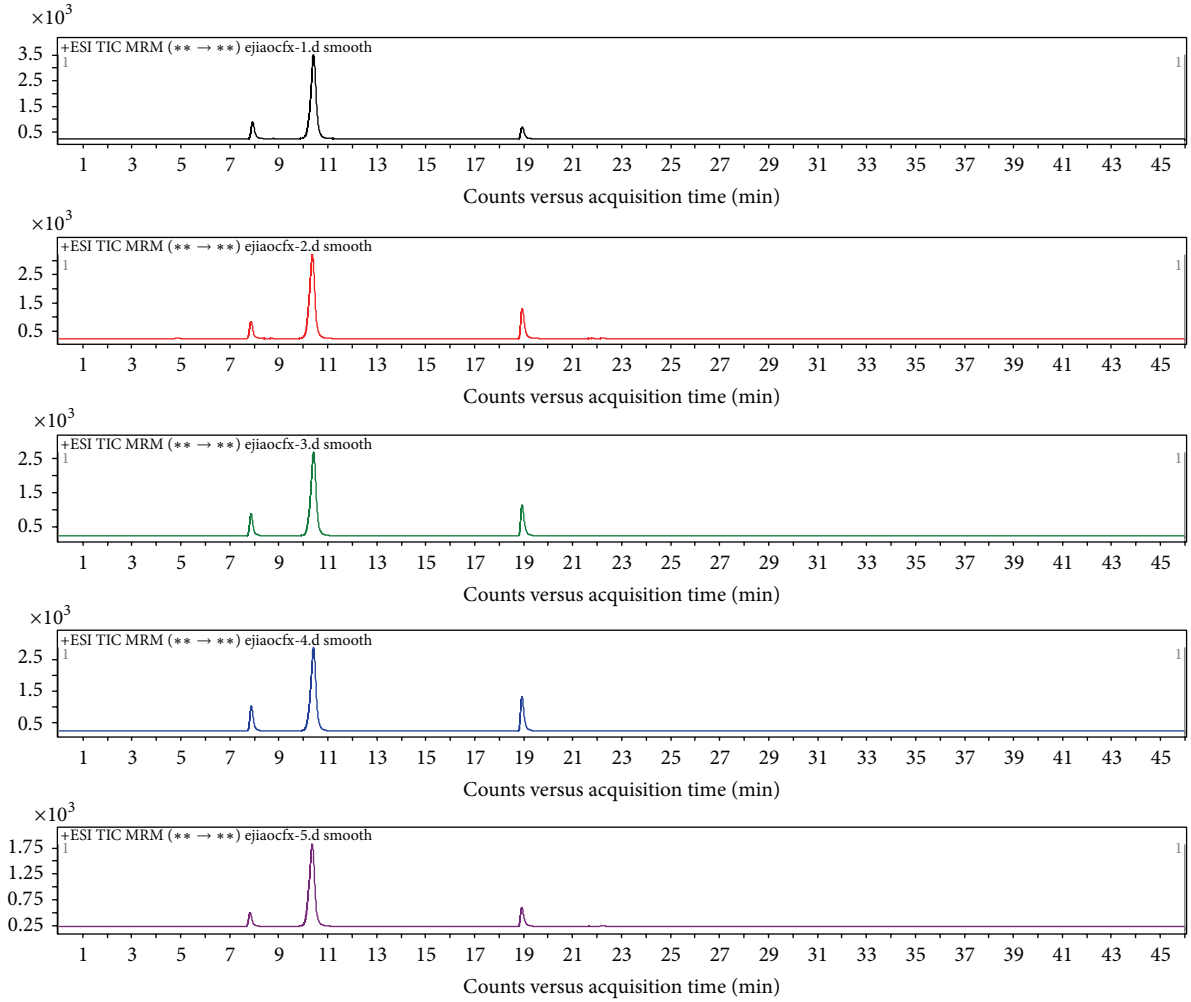

Figure 2: Characteristic selected ion chromatograms obtained for the tryptic digests of five donkey-hide gelatins.

spectrometry (HPLC-MS) is a widely used technique for qualitative and quantitative analyses, combining the efficient separation capability of HPLC with the powerful structural capability of MS [8–19]. In addition, the MS method offers the potential for high sensitivity and selectivity through multiple reaction monitoring (MRM) without the need for baseline chromatographic separation of the target analytes [20–22].

In the present work, RRLC-QQQ-MS with MRM has been used for characterization of deer-horn glue and detection of gelatin adulteration. A fully validated method has been developed, permitting measurement of the collagen marker peptides in commercial samples of deer-horn glue adulterated with donkey-hide and bovine-hide gelatins.

2. Experimental

2.1. Materials and Reagents. Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) and HPLC-grade acetonitrile (MeCN) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrahigh-purity water was prepared using a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Trypsin (sequencing grade) was obtained from Promega (Madison, WI, USA). Syringe filters (0.22 μm) were purchased from Millipore (Billerica, MA, USA). All other chemicals used were of analytical grade. All samples were collected by the National Institute for Food and Drug Control.

2.2. Sample Preparation. First, 100 mg of the gelatin was dissolved in 50 mL of a 1% NH_4HCO_3 solution (pH 8.0). Then 10 μL of trypsin solution (1 mg/mL in 1% NH_4HCO_3, pH 8.0) was added to 100 μL of the gelatin solution. The mixture was incubated at 37°C for 12 h. All gelatin samples were prepared in this way. The sources of the gelatin samples are shown in Table 1.

2.3. Chromatographic Separation and Mass Spectrometry. The RRLC analysis was performed using an Agilent 1200 LC system (Agilent, MA, USA). Chromatographic separation was performed on an Agilent Zorbax SB-C18 reversed-phase analytical column (100 mm × 2.1 mm; 1.8 μm particle size) at a column temperature of 45°C. The sample injection volume was 5 μL. The mobile phase consisted of 0.1% formic acid

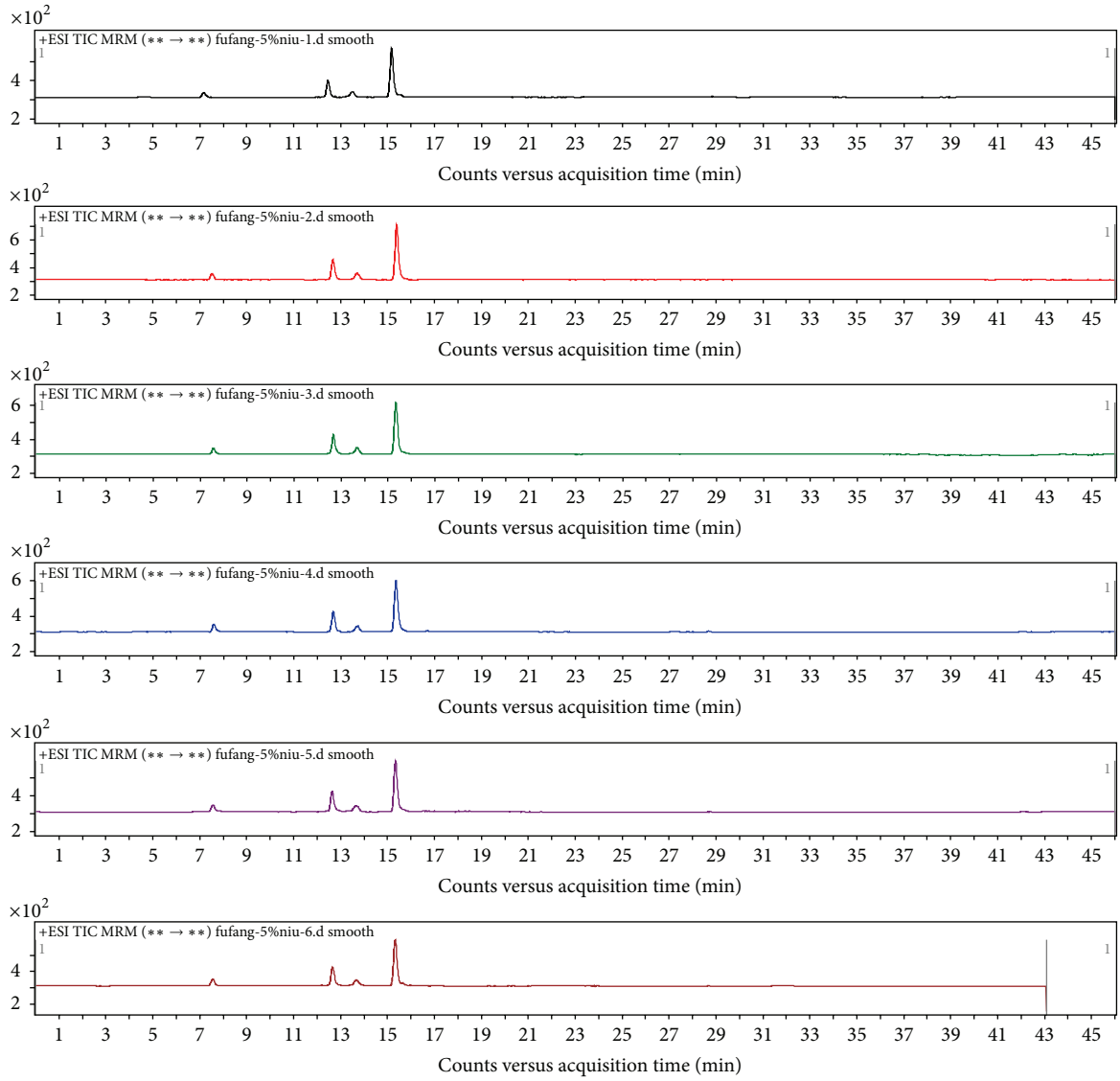

Figure 3: Characteristic selected ion chromatograms obtained for the tryptic digests of six bovine-hide gelatins.

in water (eluent A) and acetonitrile (eluent B). Gradient elution was performed as follows: 0–25 min eluent B 5% → 20%; 25–40 min eluent B 20% → 50%. The flow rate was 0.3 mL·min^{-1}.

Mass spectrometry experiments were performed with an ESI source in positive ion mode. The vaporizer temperature was maintained at 350°C. The temperature of the drying gas was set at 350°C. The flow rate of the drying gas and the pressure of the nebulizer gas were set at 6 L/min and 60 psi, respectively. In MRM scan mode, the precursor and product ions should be set. The intensity of the precursor ion should be higher after optimizing the fragmentation voltage and the intensity of the product ion should also be higher after collision energy (CE) optimization. After optimization, the voltages for fragmentation and the CE were recorded (Table 2). An Agilent ChemStation was used for instrument (Agilent 6410B series triple quadrupole MS system) control and data processing. This included definitive identification of metabolites using retention times and fragmentation transition matching. Chromatographic separation was achieved using identical conditions to those described above for IT-MS experiments [6, 7]. Gradient elution was performed as follows: 0–25 min eluent B 5% → 20%; 25–40 min eluent B 20% → 50%. The flow rate was 0.3 mL·min^{-1}.

3. Results and Discussion

Method validation was performed according to the guidelines of the Chinese Pharmacopoeia (2010 edition) for TCM. The key performance parameters evaluated were selectivity, signal linearity, sensitivity, and repeatability.

3.1. Selectivity. The specificity of the method was investigated using deer-horn glue as a blank sample, while donkey- and

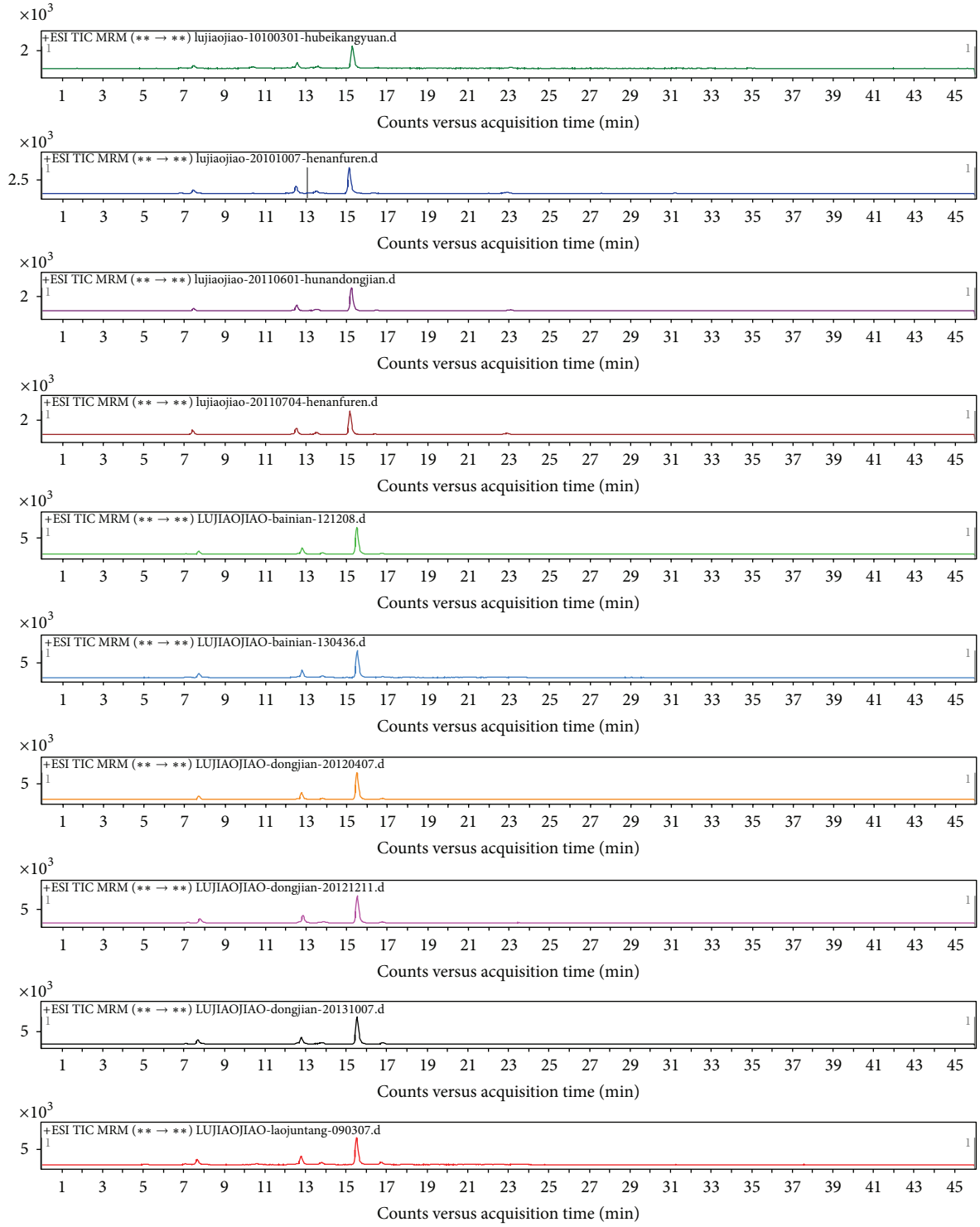

Figure 4: Representative MRM chromatograms for bovine-hide gelatins in tested samples.

bovine-hide gelatin serving as positive control samples. In previous work, the gelatins were characterized using DCSI-MS/MS. In this study, doubly charged ions at *m/z* 641.3, 747.5, 790.9, and 604.8, which are the species-specific peptides of bovine-hide gelatin, were selected for monitoring. Also, the fragments of these monitored ions resulted in the following additional characteristic molecular ion pairs: *m/z* 641.3 → 783.3, 641.3 → 726.2, 747.5 → 903.3, 747.5 → 847.1, 790.9 → 912.4, 790.9 → 841.3, 604.8 → 569.8, and 604.8 → 910.1. Doubly charged ions at 539.8, 618.8, and 765.9, which are species-specific peptides of donkey-hide gelatin, were selected for monitoring and yielded the following

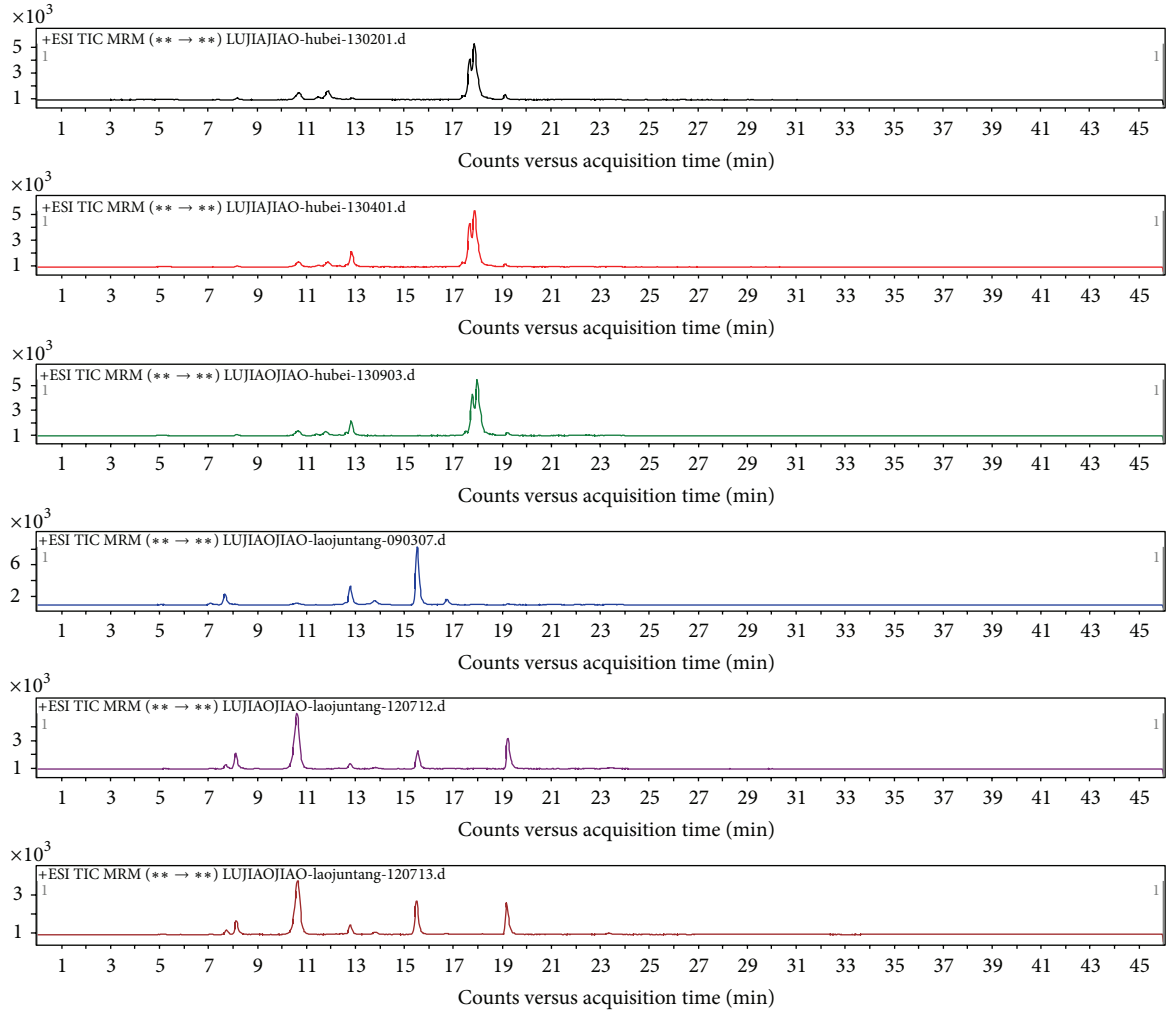

Figure 5: Representative MRM chromatograms for donkey-hide gelatins in tested samples.

molecular ion transition pairs: 539.8 → 612.4, 539.8 → 923.8, 618.8 → 721.9, 618.8 → 778.9, 765.9 → 823.1, and 765.9 → 991.0. The chromatographic peaks were verified by checking the retention times and fragments of the peaks. As a result, chromatographic peaks for deer-horn glue were different to those of donkey-hide gelatin and bovine-hide gelatin. This meant that the mass spectra for the peptides in deer-horn glue were not subject to interference, as shown in Figure 1.

3.2. Signal Linearity

3.2.1. Calibration Curves for Bovine-Hide Gelatin. A matrix solution of deer-horn gelatin standard was prepared by dissolving 100.0 mg of standard in 50 mL of a 1% NH_4HCO_3 solution (pH 8.0). Next, 100.6 mg of the bovine-hide gelatin standard was dissolved in 50 mL of a 1% NH_4HCO_3 solution (pH 8.0). Increasing aliquots (0.1, 0.5, 1.0, 1.5, and 5.0 mL) of the bovine-hide gelatin standard solutions were dissolved in 10 mL of the differing matrix solutions. Then, 100 μL of the gelatin standard solution was taken and 10 μL of trypsin solution (1 mg/mL in 1% NH_4HCO_3, pH 8.0) was added. The mixtures were incubated at 37°C for 12 h.

3.2.2. Calibration Curves for Donkey-Hide Gelatin. For sample preparation, 119.6 mg of the donkey-hide gelatin standard was dissolved in 50 mL of a 1% NH_4HCO_3 solution (pH 8.0). This solution was then subjected to the same method as outlined in Section 3.2.1.

The regression equations, correlation coefficients, and test ranges for calibration are shown in Table 3. The results showed that there was an excellent correlation between the ratio of peak area response and concentration for each compound within the test ranges examined.

3.3. Sensitivity. The limit of detection (LOD), defined as the peak signal with a signal to noise ratio = 3/1, was determined based on injections (2 μL) of low level standard solutions. The results demonstrated that the method was very sensitive with LODs of 10×10^{-6} g/mL and 20×10^{-6} g/mL for the peptides in the bovine- and donkey-hide gelatin samples, respectively.

Table 3: Signal linearity curves for two analytes.

Analytes	Linear equations	Range (μg/mL)	R^2
Bovine-hide gelatin	$Y = 3715X + 321.1$	20.12–1006	0.957
Donkey-hide gelatin	$Y = 32485X - 1130$	23.92–1196	0.995

Table 4: Results for commercial samples of deer-horn glue.

Number	Sample	Number	Origin	Donkey-hide gelatin	Bovine-hide gelatin	Deer-horn glue
1	Deer-horn glue	001	Henan Province	–	+	+
2	Deer-horn glue	002	Henan Province	–	+	–
3	Deer-horn glue	003	Shandong Province	–	+	–
4	Deer-horn glue	004	Henan Province	–	+	+
5	Deer-horn glue	005	Hubei Province	–	+	–
6	Deer-horn glue	006	Hunan Province	–	+	+
7	Deer-horn glue	007	Hebei Province	+	–	+
8	Deer-horn glue	008	Hebei Province	+	–	+
9	Deer-horn glue	009	Hunan Province	–	+	+
10	Deer-horn glue	010	Henan Province	–	+	+
11	Deer-horn glue	011	Henan Province	–	+	–
12	Deer-horn glue	012	Hunan Province	–	+	+
13	Deer-horn glue	013	Inner Mongolia Autonomous Region	–	+	+
14	Deer-horn glue	014	Shandong Province	–	+	+
15	Deer-horn glue	015	Shandong Province	–	–	+
16	Deer-horn glue	016	Shandong Province	–	–	+
17	Deer-horn glue	017	Beijing Municipality	–	–	+
18	Deer-horn glue	018	Beijing Municipality	–	–	+
19	Deer-horn glue	019	Beijing Municipality	–	–	+
20	Deer-horn glue	020	Hubei Province	–	–	+
21	Deer-horn glue	021	Hubei Province	–	–	+
22	Deer-horn glue	022	Hubei Province	–	–	+
23	Deer-horn glue	023	Henan Province	–	–	+
24	Deer-horn glue	024	Henan Province	–	–	+
25	Deer-horn glue	025	Henan Province	–	–	+
26	Deer-horn glue	026	Shandong Province	–	–	+
27	Deer-horn glue	027	Shandong Province	–	–	+
28	Deer-horn glue	028	Beijing Municipality	–	–	+
29	Deer-horn glue	029	Beijing Municipality	–	–	+
30	Deer-horn glue	121694-201301	Standard gelatin from NIFDC	–	–	+
31	Donkey-hide gelatin	121274-201202	Standard gelatin from NIFDC	+	–	–
32	Bovine-hide gelatin	121695-201301	Standard gelatin from NIFDC	–	+	–

3.4. Repeatability. Five replicate samples were prepared by the above method and the selected ion chromatograms, shown in Figures 2 and 3, confirm that the method provided reproducible detection of the collagen marker peptides.

3.5. Species Identification by RRLC-QQQ-MS. The complex peptide pools obtained by tryptic digestion of the gelatins were subjected to LC-MS/MS and the characteristic molecular ion peaks for the bovine- and donkey-hide gelatins were detected as ion pairs listed in Table 2. Typical MRM chromatograms are shown in Figures 4 and 5. Commercial samples were positively identified after matching specific peptides in these samples with the corresponding reference samples. In 29 commercial samples of deer-horn glue analyzed, 12 tested positive for bovine-hide gelatin and 2 tested positive for donkey-hide gelatin, as indicated in Table 4. Overall, the proposed method provides a new and efficient route for unambiguous measurement of collagen marker peptides of bovine- and donkey-hide gelatins.

4. Conclusions

The RRLC-MS method with MRM provides an excellent qualitative tool for quality assessment of deer-horn glue because of its high sensitivity and specificity. As shown, collagen marker peptides associated with donkey-hide gelatin and bovine-hide gelatin and presented as adulterants in deer-horn glue, were readily detected. Furthermore, according to the signal linearity, we can estimate the amount of adulteration roughly and provide a specified limitation for adulteration. In survey analysis, almost 50% of commercial samples were found to have been adulterated by the addition of donkey- and/or bovine-hide gelatin, which were more than 3% of adulterants in samples according to the signal linearity.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This study was supported in part by grants from the Important Program of Ministry of Science and Technology of the People's Republic of China (no. 2014ZX09304-307-002) and the National Natural Science Foundation of China (nos. 81202909 and 81274025).

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 534795, 6 pages
http://dx.doi.org/10.1155/2015/534795

Research Article

A Virtual Instrument System for Determining Sugar Degree of Honey

Qijun Wu[1,2] and Xun Gong[3]

[1]*School of Chemical Engineering, Guizhou Institute of Technology, Guiyang 550003, China*
[2]*School of Chemical Engineering, Guizhou University of Engineering Science, Bijie 551700, China*
[3]*School of Light Industry, Guizhou Institute of Technology, Guiyang 550003, China*

Correspondence should be addressed to Xun Gong; gongxunplmm@163.com

Received 11 March 2015; Revised 10 May 2015; Accepted 11 May 2015

Academic Editor: Guido Crisponi

This study established a LabVIEW-based virtual instrument system to measure optical activity through the communication of conventional optical instrument with computer via RS232 port. This system realized the functions for automatic acquisition, real-time display, data processing, results playback, and so forth. Therefore, it improved accuracy of the measurement results by avoiding the artificial operation, cumbersome data processing, and the artificial error in optical activity measurement. The system was applied to the analysis of the batch inspection on the sugar degree of honey. The results obtained were satisfying. Moreover, it showed advantages such as friendly man-machine dialogue, simple operation, and easily expanded functions.

1. Introduction

LabVIEW is a G language-based virtual instrument (VI) software development tool compiled by National Instruments Corporation. It is mainly used for data collection, analysis, measurement, instrumentation, process monitoring, and so forth. LabVIEW integrates a large number of interface templates for graphic generation. Moreover, it owns abundant numerical analysis controls, advanced acquisition controls, signal analysis controls, perfect simulation debugging tools, and a variety of hardware device driving functions, including RS-232, GPIB, VXI, VISA, and DAQ. It can be used to rapidly develop the virtual systems of data acquisition and analysis control in short development cycle. LabVIEW has been widely used in many fields worldwide including aviation, aerospace, communication, automobile, semiconductor, and biomedicine [1, 2].

Polarimeter is an optical instrument for measuring the optical activity of material. It is commonly used to measure the optical activity of optically active substance. Through measuring the optical activity of the sample, it has access to determine the concentration and purity of a substance and analyze the configuration of organic matters. The optical rotation of a honey depends on the concentrations of the various sugars present in the honey, and this can be used for differentiation between nectar honeys (laevorotatory; negative values of optical rotation) and honeydew honeys (dextrorotatory; positive values of optical rotation). The rotation properties are very important for honey characterisation, in order to distinguish and assess the declared botanical origin of the honey [3, 4].

WZZ-2B automatic polarimeter is a test instrument commonly used in China currently. The optical activity is measured based on the photoelectric automatic balance principle and digitally displayed on a light-emitting diode (LED). However, this instrument does not have the functions of data acquisition and intelligent data processing. In the actual application, the user needs to further analyze and process the data measured to obtain the final results. According to the characteristics of automatic polarimeter, this study developed a LabVIEW-based VI system for measuring the optical activity of materials by connecting the instrument with the

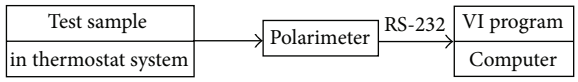

Figure 1: The structure diagram of the system.

computer through the serial port. This system realized the automatic acquisition, processing, and result generation and intelligently completed the test task.

2. The Hardware Centrifugation of the System

The VI system is composed of computer, automatic polarimeter, CH1015 super thermostat bath, and LabVIEW-based program. The polarimeter tube is refitted to provide constant temperature for the tested sample. A polarized light signal after the sample tube is detected by PhotoMultiplier Tube (PMT) and converted into electronic signals, which could be transported into computer through serial port (RS-232). The structural diagram of the VI system is shown as in Figure 1.

3. The Programming of the VI System

The VI program system is constituted by some SubVIs including data acquisition and sugar content analysis, viewing recorded data and exiting the program [5–7]. It mainly used to complete the real-time data display, storage, data processing, and result playback tasks.

3.1. Programming for Data Acquisition. Figure 2 shows the main operation panel of the VI system. The right-hand part of this figure is used to display experimental data and choose the test tasks in real time, while the left-hand part is used to record the field information, such as experimental personnel, experimental samples, and reaction time. By clicking the button of "sugar content analysis," researchers enter into the data processing SubVI to process the experimental data in real time. By clicking the button of "result playback," it has access to playback the data results processed. By clicking the "stop" button, the operation of the VI system is terminated. The SubVIs in the programming call the Event Structure, which can realize the interactions between the operations of users on front panel and program execution.

Figure 3 is a program chart of the data acquisition of the system. The program of data acquisition is realized according to the data protocol sent by the serial port of the polarimeter [5, 7]. The initialization, writing, reading, and closing of the serial port, and so forth, were realized using the VISA nodes in LabVIEW. Before the communication of serial port, it is needed to set port parameters for the nodes of *VISA Configure Serial Port*. Using the *String To Byte Array* node, the data read by *VISA Read* node from the instrument cache are converted into integer array. Using nodes such as *Array Subset* and *Index Array*, the specified optical activity of each frame in the instrument can be read out.

The concurrent structure of *Timed Loop* and *While Loop* is used to accurately display the current measurement time. *Remainder* function is employed to report the accurate time at full minute and highlight the current acquisition time. Time-reporting and alarming sounds are realized through *Beep.vi*.

The Case Structure on the right part of the *While Loop* in Figure 3 is applied to optional save measuring data. File storage path is realized by "file path" subprogram. To avoid the file name repetition, a *List Folder* node is called. In case of indexing out the same file name, the file name is automatically added with a specific character string. *Write To Spreadsheet File.vi* function realizes the simultaneous saving of optical activity data and concentration data collected. Users can click the acquisition button on the operation panel of the VI to collect data. By clicking save data button, the data measured are saved.

Data acquisition of the program cannot be preceded with other events simultaneously. Other events are only allowed to be proceeding when data acquisition is ended. This function is realized by setting the attributes of the event buttons to avoid the conflict of the events called [7].

3.2. Programming for Sugar Content Analysis of Honey. After data acquisition, users can enter into the sugar content analysis operation panel. The program for the sugar degree analysis of honey mainly applied to measure the contents of glucose, fructose, and sucrose to detect honey quality. Such detection functions to determine whether or not the honey is adulterated or whether or not the honey is pure. The principle is indicated as follows [8, 9]: the total gross (A) of the reducing sugars (glucose and fructose) in the honey is firstly measured using potassium ferricyanide method. The contents of glucose, fructose, and sucrose are set as x, y, and z, respectively. Subsequently, according to the linear relation of optical activity and concentration, the optical activities of the honey solutions diluted by distilled water and hydrolyzed by sulfuric acid were measured. Honey after acid hydrolysis of sucrose can only complete hydrolysis into the same amount of glucose and fructose. In data processing, the measured data is multiplied by the dilution ratio to obtain the optical activity of original honey. The rotation $[\alpha]_D^{20}$ for glucose, fructose, and sucrose are 52.5, −91.9, and 66.6. Therefore, there are $A = x + y$, $a_1 = 52.5x - 91.9y + 66.6z$, and $a_2 = 52.5(x + z) - 91.9(y + z)$. By combining these equations, it has access to the sugar degrees of glucose, fructose, and sucrose, respectively.

Figure 4 shows the operation panel of the sugar degree analysis of honey. By clicking the button of "parameters for reducing sugar," the SubVI panel for setting the parameters of reducing sugar is popped. After inputting the parameter of the reducing sugar and clicking the "returning" button, the reducing sugar of operation panel as in Figure 4 is returned. By inputting the optical activity of the honey solutions diluted by distilled water and that of the solution hydrolyzed by sulfuric acid and clicking "calculation," the three sugar degrees and total sugar contents of the all honey samples were listed in the table, as shown by the lower part of Figure 4.

The *Formula Node* inside a *For Loop* in the programming is used to realize the calculation on the sugar degree of the honey. *Array Size* is used to index the sample number that connects to the count port of the *For Loop*; that is, the sugar

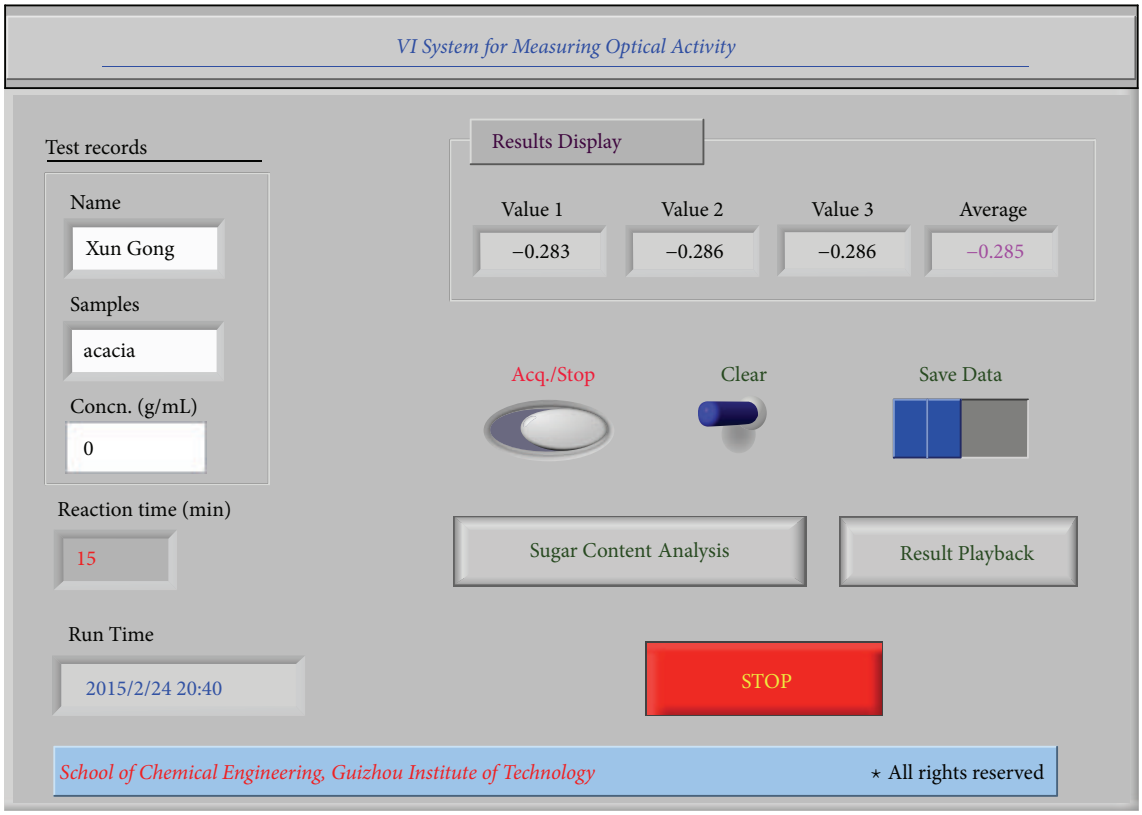

Figure 2: The main operation panel of the VI.

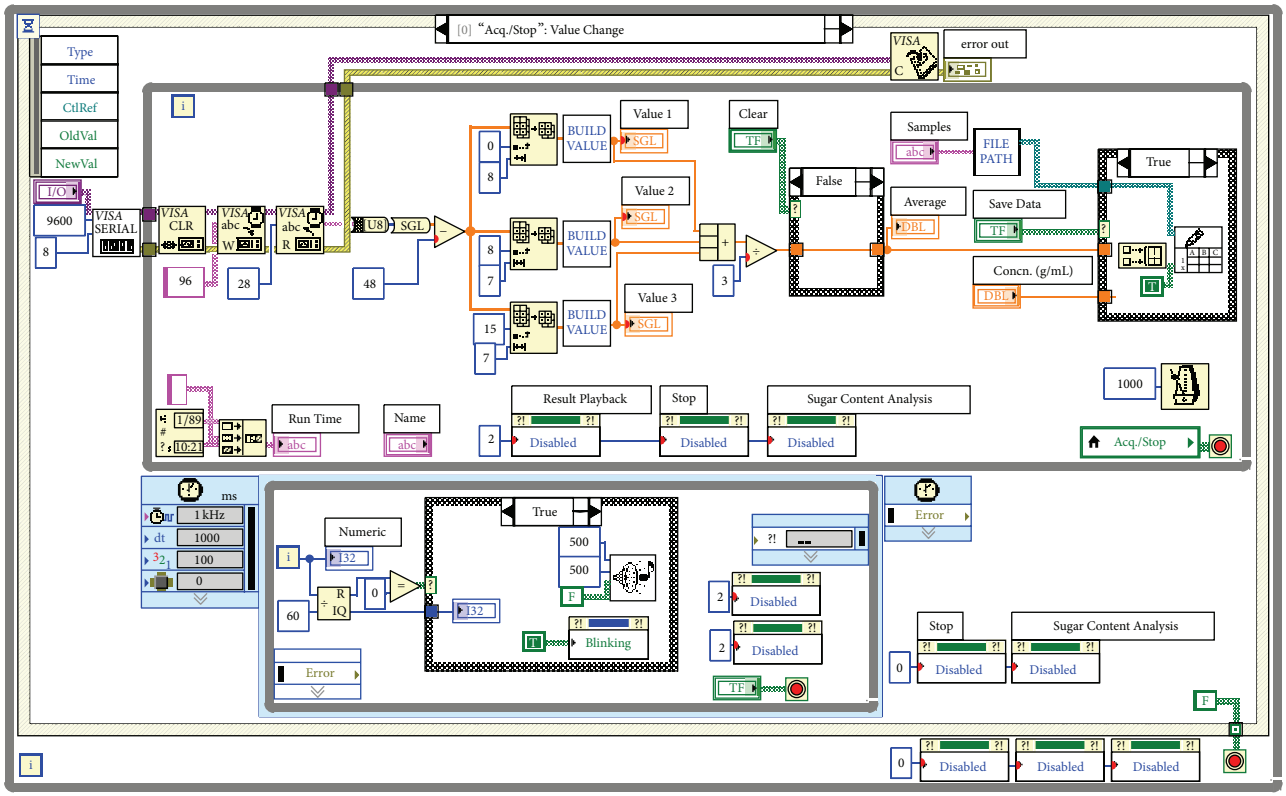

Figure 3: Program for the data acquisition.

Figure 4: Panel for the sugar degree analysis.

degree of multiple different honey samples can be calculated separately. By calling *Express Table*, building the *Property Node of Express Table*, and selecting attributes for *Row Header Strings* and *Column Header Strings*, it is accessible to write the column and row heading for the listbox and record the honey sample and corresponding sugar degree in detail. Figure 5 shows the program chart.

3.3. Programming for Datalog Playback. LabVIEW can record the current data of all the controls in the front panel of the program. Each time of data record gives rise to a record in the forms of the combinations of arbitrary types of data in the datalog file. Before the datalog on the front panel, it is required to select the menu command *Operate* → *Log at Completion* on the front panel of the data recorded. The reading of datalog file is realized by calling the data recorded VI, which is treated as the SubVI here. By right-clicking on the SubVI icons of this block diagram, a menu is popped. By selecting the *Enable Database Access* on the menu, it has access to the state of viewing the datalog. The data that needed to be read can be played back using the *Unbundle By Name* node. Figure 6 shows the playback panel for data processing results. By selecting the number of the record that should be viewed, the datalog under this record number is displayed in real time. In case that there is no data under this record number, the Boolean LED is the highlight. By clicking "return," the users can return to the main operation panel.

4. Results and Discussion

Owning to the high nutritional and medicinal value, honey has been widely applied to Chinese patent drugs and foods. Honey shows a complex composition, with more than 20 kinds of detectable ingredients. The main ingredients include fructose, glucose, and sucrose, accounting for about 70% of the honey in content. In addition, the three substances are optically active. Except for them, there are few other optically active substances in honey. Chinese honey standard stipulates that the reducing sugar content and sucrose content should take a proportion of above 65% and below 5%, respectively. For the exported honey, it is even required that the fructose should account for above 50% of the reducing sugar. The reason of these stipulations lies in that, on one hand, whether or not the honey is mixed with sucrose or starch substances is detectable. On the other hand, it can determine whether or not the honey contains the honey yielded by the bees fed by sucrose. Therefore, the natural characteristics and ingredients of pure honey can be reflected by measuring various sugars in the honey.

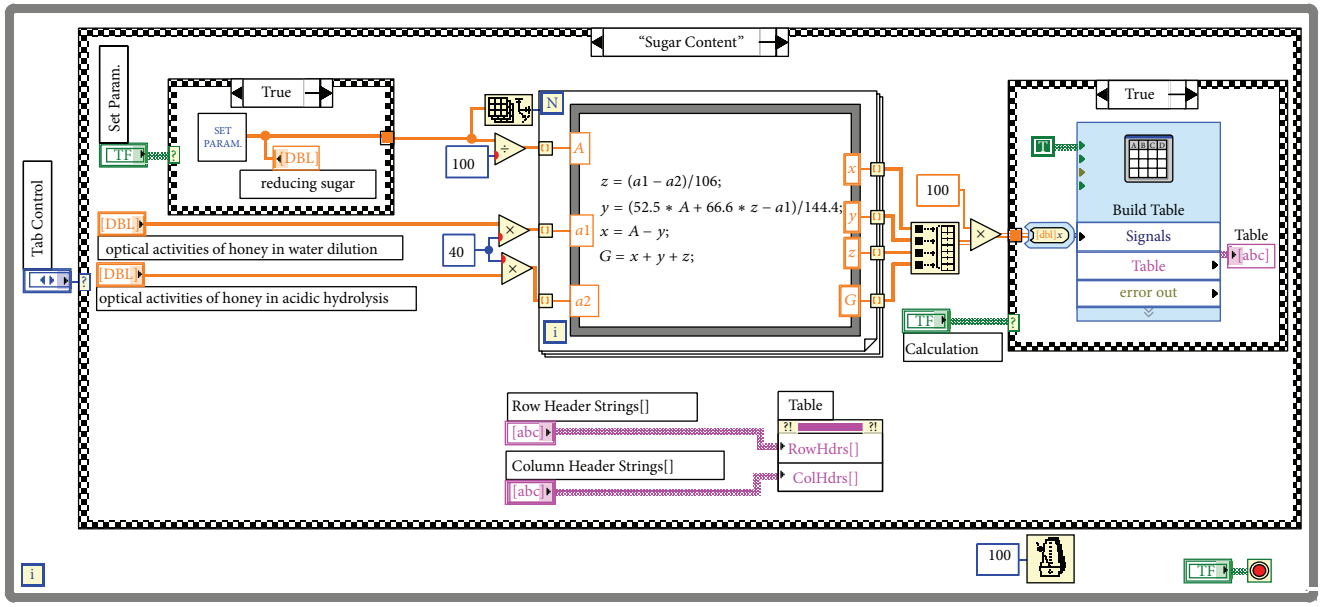

Figure 5: Program for the sugar degree analysis.

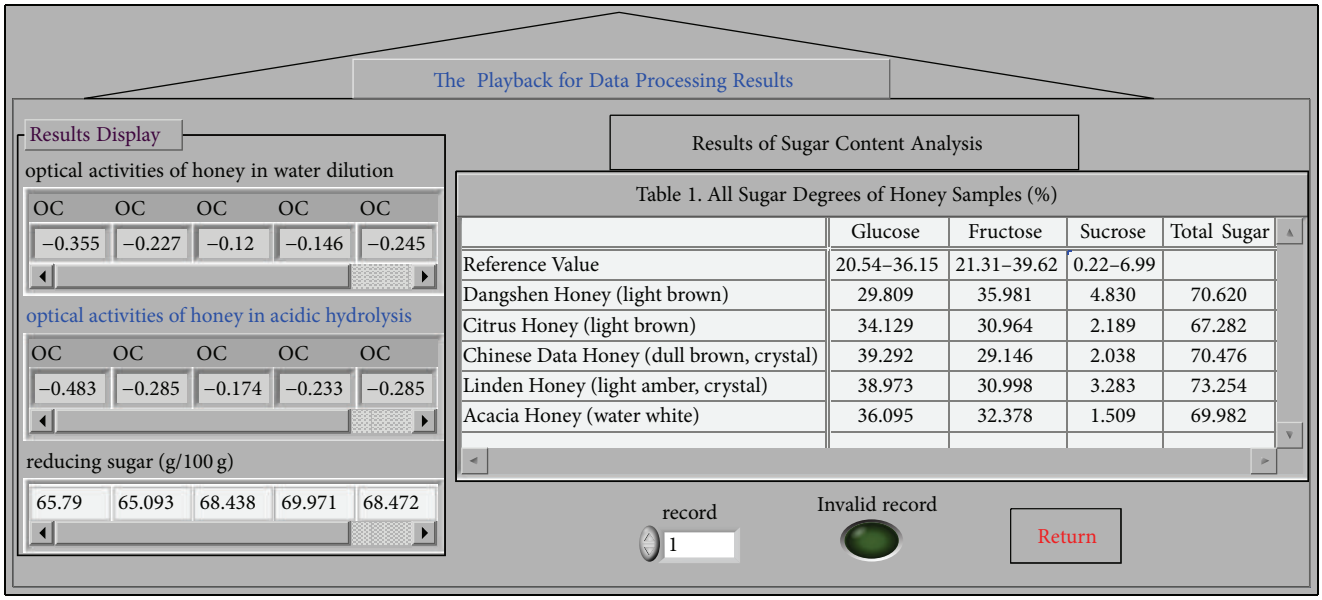

Figure 6: The playback panel for data results.

The analysis on the sugar degree of honey is preceded as follows. Firstly, the total contents (A) of the reducing sugar in all honey samples were measured using potassium ferricyanide method. Then, 25.00 g (in accurate weight) of citrus honey, linden honey, Chinese date honey, Dangshen Honey, and acacia honey (origin: Sichuan) was put into 100 mL volumetric flasks, respectively, and diluted using distilled water to constant volume. The honey solutions obtained were then discolored using activated carbon for 3 hours. After being filtered, we take two shares of 10 mL sample solution from each discolored solution and put these sample solutions in different 100 mL volumetric flasks. One of the solution samples was diluted with 10 mL 3 mol/L H_2SO_4, adding distilled water to the constant scale, and another was directly added into distilled water to the scale and placed overnight. In the following day, we measured the optical activities of the solutions diluted by acid and water at a constant temperature of 20°C. Afterwards, we input the reducing sugar parameter measured at the beginning and the optical activities of the honey solutions diluted and clicked "Yielding the Results." The sugar degree data processing results of each honey sample were obtained, as shown in Figures 5 and 6. A reference value using China's standard sugar degree determination using the HPLC method is shown in Figure 6, glucose displays

the highest content above reference value (like 39.292% and 38.973%), and it is easily crystallized; the contents of glucose and fructose are all higher than 65%, while sucrose contents are all less than 5%. The results obtained were consistent with the results obtained using China's standard sugar degree determination method. Therefore, it is proved that this VI system is applicable to the batch detection of a variety of honey samples.

5. Conclusions

The application results suggested that the LabVIEW-based optical activity measurement VI system conveniently solved the hardware problems of conventional chemical optical instrument such as the interface with computer and realized the automatic data acquisition and data storage functions. Moreover, using the rich function library of LabVIEW, users can compile different LabVIEW programs for this system to profoundly analyze and process the optical activity data measured and obtain corresponding measurement results in real time. It was applicable to the batch detection in the sugar degree analysis of honey. In addition, this system showed friendly man-machine interface and convenient operation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are pleased to acknowledge the financial support of this startup project for high-level talents of Guizhou Institute of Technology (Grant no. XJGC20150101). They also acknowledge the financial support of this research by the Natural Science Research Project of Guizhou Provincial Education Office (Grant nos. [2013]180 and [2013]181) and the support from Guizhou Province Science and Technology Fund (Grant nos. [2013]2006 and [2013]2007).

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 491406, 8 pages
http://dx.doi.org/10.1155/2015/491406

Research Article

Protective Effects of Intralipid and Caffeic Acid Phenethyl Ester on Nephrotoxicity Caused by Dichlorvos in Rats

Muhammet Murat Celik,[1] Ayse Alp,[2] Recep Dokuyucu,[3] Ebru Zemheri,[4] Seyma Ozkanli,[4] Filiz Ertekin,[5] Mehmet Yaldiz,[6] Abdurrahman Akdag,[7] Ozlem Ipci,[6] and Serhat Toprak[6]

[1]*Department of Internal Medicine, Medical Faculty, Mustafa Kemal University, 31000 Hatay, Turkey*
[2]*Department of Biochemistry, The Government Hospital of Obstetrics and Gynecology, 31000 Hatay, Turkey*
[3]*Department of Medical Physiology, Medical Faculty, Mustafa Kemal University, 31000 Hatay, Turkey*
[4]*Department of Pathology, Medeniyet University Goztepe Training and Research Hospital, 81054 Istanbul, Turkey*
[5]*Department of Internal Medicine, Ministry of Health Batman Regional Government Hospital, 72000 Batman, Turkey*
[6]*Department of Medical Pathology, Medical Faculty, Mustafa Kemal University, 31000 Hatay, Turkey*
[7]*Department of Chemistry, Science and Arts Faculty, Mustafa Kemal University, 31000 Hatay, Turkey*

Correspondence should be addressed to Muhammet Murat Celik; muratcelikdr@yahoo.com

Received 28 September 2014; Revised 2 February 2015; Accepted 17 February 2015

Academic Editor: Shixin Deng

The protective effects of Caffeic Acid Phenethyl Ester (CAPE) and intralipid (IL) on nephrotoxicity caused by acute Dichlorvos (D) toxicity were investigated in this study. Forty-eight Wistar Albino rats were divided into 7 groups as follows: Control, D, CAPE, intralipid, D + CAPE, D + IL, and D + CAPE + IL. When compared to D group, the oxidative stress index (OSI) values were significantly lower in Control, CAPE, and D + IL + CAPE groups. When compared to D + IL + CAPE group, the TOS and OSI values were significantly higher in D group ($P < 0.05$). When mitotic cell counts were assessed in the renal tissues, it was found that mitotic cell count was significantly higher in the D group while it was lower in the D + CAPE, D + IL, and D + IL + CAPE groups when compared to the control group ($P < 0.05$). Also, immune reactivity showed increased apoptosis in D group and low profile of apoptosis in the D + CAPE group when compared to the Control group. The apoptosis level was significantly lower in D + IL + CAPE compared to D group ($P < 0.05$) in the kidneys. As a result, we concluded that Dichlorvos can be used either alone or in combination with CAPE and IL as supportive therapy or as facilitator for the therapeutic effect of the routine treatment in the patients presenting with pesticide poisoning.

1. Introduction

Organophosphorus pesticides (OPs) have been widely and effectively used for applications in agricultural settings, public health, commerce, and individual households worldwide in order to increase efficiency of agricultural production and maintain hygienic conditions [1, 2]. Dichlorvos (2, 2-dichlorovinyl phosphate) (D) is an OP that is widely used worldwide. Since its commercial introduction in 1961, D has been increasingly used in many countries and produced important benefits by controlling internal and external parasites in livestock and domestic animals as well as insects in houses and fields [3]. However, the extensive applications of D inevitably cause environmental, soil, and crop pollution. Consequently, human exposure to low levels of D became chronic via contaminated food and water. Recently, the effects of D on human health have raised increasing attention in community [4]. The clinical signs and symptoms associated with acute D poisoning are generally attributable to acetylcholine (ACh) accumulation following the inhibition of acetylcholinesterase (AChE). Overstimulation of the ACh causes the clinical signs and symptoms including muscarinic, nicotinic, and central nervous system toxic effects [5]. In addition, acute cholinergic effects may cause irreversible and progressive neurological deficits in both humans and animals [6].

Several antidotes have been evaluated for the routine treatment of OP poisoning and the currently recommended

drugs are atropine and pralidoxime chloride [7]. Atropine has been used as antidote against OPs over past decades, as it effectively antagonizes the muscarinic receptors, but not nicotinic receptor, against toxic effects of Ach [5]. Some studies have demonstrated that D has toxic effects such as hepatotoxicity, renal toxicity, and neurotoxicity. However, new methods and drug investigations are needed for support or protective clinical treatment against nephrotoxicity caused by OP toxication.

Recently, oral IL emulsion was introduced as a novel method in the treatment of intoxication from several lipophilic agents. Since it was shown to be effective in bupivacaine toxicity, IL may be a promising approach for other lipophilic drug intoxications, including herbicides and pesticides. Moreover, it has been suggested that IL binds lipophilic agents and confines liposoluble toxic elements. It is also reported that it is administered by a bolus dose of 1.5 mL/kg and an infusion dose of 0.25 mL/kg/min in liposoluble drugs (clomipramine, propranolol, bupropion, haloperidol, and organophosphates). However, IL is not currently used in the treatment protocols because of insufficient evidence in intoxications [8].

Caffeic acid phenethyl ester (CAPE) is a compound that is structurally similar to the flavonoid found in bee propolis. It is an active component of propolis extract, which is one of the reactive oxygen species (ROS) that occurs as a result of oxidative stress in toxic failure and ischemia-reperfusion injury and has tissue protective effect [9]. CAPE reaches to required blood concentration when administered intraperitoneally [10] and CAPE, at 10 mmol/kg concentration, inhibits the xanthine oxidase system and the formation of ROS [9, 11].

This study concluded that CAPE and IL are capable to exert protective effects against nephrotoxicity caused by acute D toxicity in rats. Therefore, this study shed light on the literature in terms of the prophylactic use of these two agents in the disorders induced by oxidative damage and distant organs damage.

2. Materials and Methods

2.1. Animals, Care, and Nutrition. The study was approved (ethic number: 2012-065) by Necmettin Erbakan University, Experimental Medical Research Center's Experimental Animals Ethics Committee, Konya, Turkey. The forty-eight mature Wistar Albino rats weighing 200–250 g were randomly divided into seven groups. The animals were kept under laboratory conditions of 12-hour light-dark cycle at a room temperature (21°C ± 2°C).

2.2. Animals and Treatment. The forty-eight rats were randomly divided into seven groups ($n = 6$) as Control (C), Dichlorvos ($n = 7$), intralipid ($n = 7$), CAPE ($n = 7$), Dichlorvos + intralipid ($n = 7$), Dichlorvos + CAPE ($n = 7$), and Dichlorvos + intralipid + CAPE ($n = 7$) groups. Doses and durations of medication were adjusted according to the literature so the rats were given Dichlorvos (4 mg/kg/day via oral route) [3, 12], intralipid (18.6 mL/kg via oral route) [8, 13], and CAPE (10 μmol/kg via intraperitoneal route) [10, 11]. IL and CAPE were administered immediately after D administration as single dose. Rats were sacrificed under ketamine/xylazine (90/10 mg/kg) anesthesia. Kidney tissues of the rats that were removed were stored at −70°C for biochemical analysis whereas a part of kidney specimen was kept in formaldehyde solution for histologic examination.

2.3. Biochemical Analysis. The TAS of supernatant fractions was evaluated by using a novel automated and colorimetric measurement method developed by Erel. Hydroxyl radicals, the most potent biological radicals, are produced in this method. In the assay, the ferrous ion solution present in reagent 1 is mixed with hydrogen peroxide, which is present in reagent 2. The radicals produced subsequently, such as brown-colored dianisidinyl radical cations produced by the hydroxyl radicals, are also potent radicals. Using this method, the antioxidative effect of the sample is measured against the potent-free radical reactions initiated by the produced hydroxyl radicals. The assay has excellent precision values (lower than 3%). The TAS results are expressed as nmol Trolox equivalent/mg protein. The TOS of supernatant fractions was also evaluated by using a novel automated and colorimetric measurement method developed by Erel. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is amplified by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion produces a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of nanomoles of H_2O_2 equivalent/milligram of protein. The units of TOS and TAS were micromoles of H_2O_2 equivalent/gram protein and millimole of H_2O_2 equivalents/gram protein, respectively. The Oxidative stress index (OSI) value was calculated as follows: OSI = TOS/TAS [14, 15]. Ellagic acid attenuates oxidative stress on brain and sciatic nerve and improves histopathology of brain in streptozotocin-induced diabetic rats [16].

2.4. Histopathologic Analysis

2.4.1. Hematoxylin and Eosin Method. For histopathologic examinations, routine histologic paraffin block preparation method was used after fixation of kidney samples that are kept in 10% formalin. 5 μm thick sections were obtained from paraffin blocks by using a microtome (Leica Rotary; Leica Microsystems GmbH, Wetzlar, Germany). Collected sections were stained with hematoxylin-eosin (H&E) and examined under light microscope (×100) and photos were captured. Histopathology of the tissue samples was rated semiquantitatively according to normal tissue composition. In the pathological examinations of the sample parameters of brush border loss, extravasation, tubular cast structures, nucleus loss in the tubule epithelial cells, tubular dilatation and interstitial accumulation of lymphocytes, and tubular necrosis were rated from 0 to 5 points; thus, normal kidneys

and other applied kidneys were compared. This scoring (0: normal tissue, 1: blown tubular epithelium cell areas, vacuolar degradation, and necrosis, less than 25% of cases, 2: 25–50% of similar cases, 3: 50–75% of similar cases, 4: more than 75% of similar cases, 5: complete cortical necrosis) was carried out according to similar scoring of the other studies in kidney [17].

2.4.2. Immunohistochemistry Method. Immunohistochemical examination was performed on a Leica Bond-Max automated IHC/ISH platform (Leica Microsystems Inc., Buffalo Grove, Illinois). Four-micrometer paraffin sections were dewaxed in a Bond Dewax solution and rehydrated in alcohol and Bond Wash solution (Leica Microsystems). Antigen retrieval was performed using a high pH (ER2) retrieval solution for 15 minutes followed by endogenous peroxidase blocking for 5 minutes on the machine. Anti-mouse monoclonal antibody Bcl-2 (C-2: sc-7382, Santa Cruz Biotechnology, Inc., in dilution 1 : 200), anti-mouse monoclonal antibody Bax (B-9: sc-7480, Santa Cruz Biotechnology, Inc., in dilution 1 : 100), and anti-mouse caspase-3 (CPP32) monoclonal antibody (clone JHM62, Leica Biosystems Ltd., Newcastle) were applied at 1 : 50 dilution for 60 minutes at room temperature. Detection was performed using the Bond Polymer Refine Red Detection system (Leica Microsystems) with a 15-minute postprimary step followed by 25-minute incubation with alkaline phosphatase-linked polymers. Sections were then counterstained with hematoxylin on the machine, dehydrated in alcohols, and mounted with mounting medium (Sakura Finetek USA, Inc., Torrance, California). Prepared tissues were observed by histopathologists blinded to the experimental study groups. The numbers of apoptotic cells were counted in ten randomly selected microscope fields under a ×400 magnification in a blind fashion. The average number of stained neurons for each set of ten fields was calculated and expressed as the number of the positive cells/high-power field.

2.5. Statistical Evaluation. SPSS 11.5 software was used for statistical analysis. Data were expressed as mean ± SD. Kruskal-Wallis test was applied to determine the abnormal distribution followed by post hoc Tukey's and Mann-Whitney U test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Biochemical Analysis

3.1.1. TAS Levels. The TAS level was significantly lower in the D group than those in the other groups ($P < 0.05$). the TAS values were significantly higher in Control, CAPE, and D + IL + CAPE groups when compared to the D group ($P < 0.05$). Also the TAS values were significantly higher in the CAPE and IL groups when compared to the D + IL + CAPE group ($P < 0.05$) (Table 1).

3.1.2. TOS Levels. The TOS values were significantly lower in the Control, IL, CAPE, D + CAPE, and D + IL + CAPE groups when compared to D group ($P < 0.05$), while no significant difference was observed among other groups ($P > 0.05$) (Table 1). Also, the TOS values were significantly higher in the D group compared to the D + IL + CAPE group ($P < 0.05$) (Table 1).

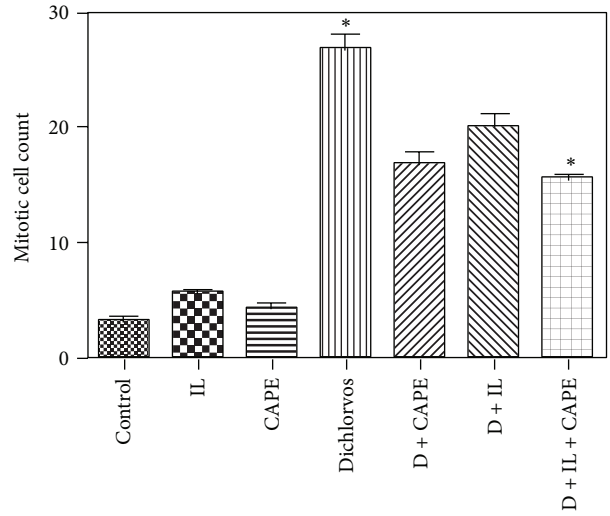

Figure 1: Histopathologic results for kidney tissues. Mitotic cell count. ANOVA test (intralipid (IL), Dichlorvos (D)).

3.1.3. OSI Levels. The OSI values were significantly lower in the Control, CAPE, and D + IL + CAPE group when compared to the D group ($P < 0.05$) while no significant difference was observed among other groups (Table 1). Also, the OSI values were significantly higher in the D group when compared to the D + IL + CAPE group ($P < 0.05$) (Table 1).

3.2. Histopathologic Results. When the mitotic counts were assessed in the renal tissues, it was found that mitotic count was higher in the D group (Figures 1 and 2(b)) and significantly lower in the D + CAPE (Figure 2(c)), D + IL, and D + IL + CAPE (Figure 2(d)) groups compared to the Control group (Figure 2(a)) (Figure 2, H&E) (Table 2).

Using caspase-3, Bcl-2, and Bax, immune reactivity showed increased apoptosis in the kidneys from the D group (Figures 3 and 4(b)) and low profile of apoptosis in the D + CAPE group (Figure 4(c)). The apoptosis level was significantly lower in the D + IL + CAPE group (Figure 4(d)) than the D group (Figure 4). The apoptosis was evaluated in the renal cells and it was found that there were 10.2 ± 1.49 apoptotic cells in the Control group, 11.7 ± 1.49 in CAPE, 14.8 ± 2.54 in IL, 151.3 ± 6.49 in D, 78.8 ± 3.67 in D + CAPE, 127.4 ± 4.89 in D + IL, and 72.1 ± 3.02 in D + CAPE + IL. This indicated that the apoptosis rate was lower in the D + CAPE and D + CAPE + IL groups compared to the D group (Table 3, Figure 3).

4. Discussion

When organophosphate is absorbed through the skin or by means of digestion, mucosal membranes, conjunctiva, or

Table 1: Comparison of postmedication total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) levels in renal tissues.

	TAS (mmol Trolox Eq./g protein)	TOS (mmol H_2O_2 Equiv./g protein)	OSI (H_2O_2/Trolox)
Control	1.50 ± 0.16	132.9 ± 16.71	88.35 ± 7.79
CAPE	1.49 ± 0.20	120.1 ± 30.36	93.2 ± 13.62
IL	1.30 ± 0.30	133.8 ± 9.55	107.5 ± 28.36
Dichlorvos	1.17 ± 0.08[a]	169.6 ± 8.61[b]	126.9 ± 13.24[c]
Dichlorvos + CAPE	1.31 ± 0.06	137.1 ± 24.87	104.6 ± 21.08
Dichlorvos + IL	1.33 ± 0.07	141.7 ± 25.41	106.3 ± 18.42
Dichlorvos + IL + CAPE	1.44 ± 0.12[d]	135.3 ± 21.19[e]	94.55 ± 18.41[f]

Data are presented as mean ± SD. Kruskal-Wallis and post hoc Tukey's and Mann-Whitney *U* tests were used. The mean difference is significant at the level of 0.05. ($P < 0.05$).

[a]Compared with the Dichlorvos group, the TAS values in the Control, CAPE, and D + IL + CAPE groups were significantly higher ($P = 0.001$).

[b]Compared with the Dichlorvos group, the TOS values in Control, IL, CAPE, D + CAPE, and D + IL + CAPE groups were significantly lower ($P = 0.01$).

[c]Compared with the Dichlorvos group, the OSI values in Control, CAPE, and D + IL + CAPE groups were significantly lower ($P = 0.001$).

[d]Compared with the Dichlorvos + IL + CAPE group, the TAS values in the Dichlorvos group were significantly lower ($P = 0.001$).

[e,f]Compared with the Dichlorvos + IL + CAPE group, the TOS and OSI values in the Dichlorvos group were significantly higher ($P = 0.01$).

D (Dichlorvos), IL (intralipid), and CAPE (caffeic acid phenethyl ester).

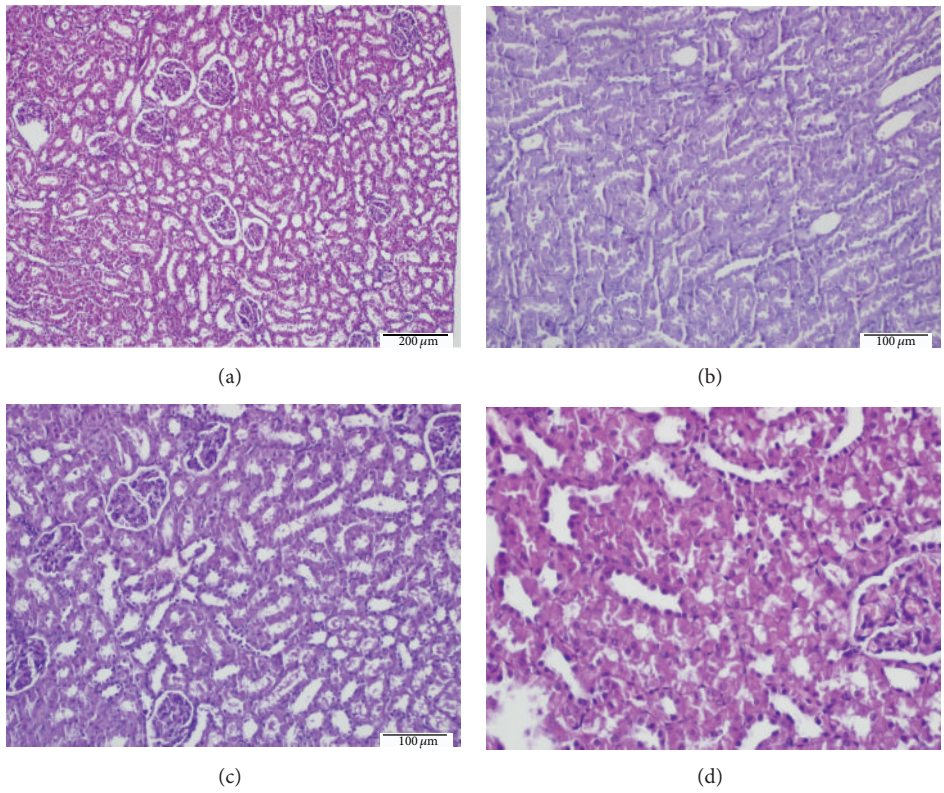

Figure 2: Evaluation of mitosis (hematoxylin and eosin method). (a) Control rats with normal renal histology (H&E, ×100). (b) Common mitotic increase in the rats with Dichlorvos (H&E, ×100) (D). (c) Decreasing mitotic density and regenerative changes in the rats given CAPE following Dichlorvos (H&E, ×200) (D + CAPE). (d) Relative decrease in the mitotic density and regenerative changes in the rats given CAPE and IL following Dichlorvos (H&E, ×200) (D + CAPE + IL).

respiration, organophosphate (OP) intoxication leads to quite a serious clinical picture, even leading to sudden onset of respiratory failure which requires admission to intensive care unit [18, 19]. OP has several toxic effects on other systems such as neurotoxicity, myocardial toxicity, embryotoxicity, hepatotoxicity, immunotoxicity, genetic toxicity, and nephrotoxicity [20, 21].

The oral acute LD50 dose of D, which is an OP compound, is 80 mg/kg in rats [3]. Previous studies showed that the application of D with a dose of 7.2 mg/kg leads to pathologic

Table 2: Histopathologic results for kidney tissues, mitotic cell count.

	Mean ± SD	Min–Max
Control	3.1 ± 1.34	1–5
CAPE	4.4 ± 0.97	3–6
IL	5.5 ± 0.97	4–7
D	26.5 ± 3.99	20–32
D + CAPE	16.86 ± 2.41	13–20
D + IL	20.14 ± 2.61	16–24
D + IL + CAPE	15.43 ± 1.27	14–17
Control versus D, D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
Dichlorvos versus D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
CAPE versus D, D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
IL versus D, D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
D + IL versus D + IL + CAPE	$P < 0.001$	

Kruskal-Wallis and post hoc Tukey's and Mann-Whitney U tests were used.

Table 3: Histopathologic results for kidney tissues, apoptotic cell count.

	Mean ± SD	Min–Max
Control	10.2 ± 1.49	8–12
CAPE	11.7 ± 1.49	10–14
Intralipid (IL)	14.8 ± 2.54	11–18
Dichlorvos (D)	151.3 ± 6.49	140–160
D + CAPE	78.8 ± 3.67	75–85
D + IL	127.4 ± 4.89	119–134
D + IL + CAPE	72.1 ± 3.02	68–76
Control versus D, D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
Dichlorvos versus D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
CAPE versus D, D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
IL versus D, D + CAPE, D + IL, and D + IL + CAPE	$P < 0.0001$	
D + IL versus D + IL + CAPE	$P < 0.0001$	
D + CAPE versus D + IL + CAPE	$P < 0.01$	

Kruskal-Wallis and post hoc Tukey's and Mann-Whitney U tests were used.

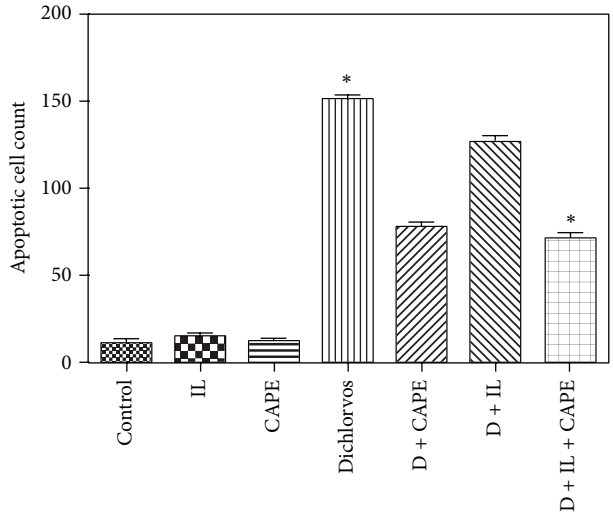

Figure 3: Histopathologic results for kidney tissues. Apoptotic cell count. ANOVA test (intralipid (IL), Dichlorvos (D)).

and biochemical changes in the renal cells [22]. Also, some other studies showed that an oral dose of 4 mg/kg in rats induces a decrease in sperm motility [3] and endometrial injury [12]. Therefore, the rats were administered with D (4 mg/kg/day via oral route) in the present study. Ben Amara et al. reported that OPs lead to kidney injury and increased malondialdehyde (MDA) levels, cystatin C levels, and plasma creatinine and uric acid levels and that these injuries can be ameliorated via vitamin E and selenium [23]. On the other hand, Silfeler et al. revealed that paraquat intoxication leads to pancreatic injury and that CAPE (10 μmol/kg via intraperitoneal route) is effective in reversing this injury [11]. Therefore, the rats were given CAPE (10 μmol/kg via intraperitoneal route) in the present study.

Similarly, Tuzcu et al. claim that IL diminishes the pancreatic injury caused by malathion, an OP, and that IL exerts these effects through decreasing malathion absorption by acting as a chelate with malathion in the stomach [8]. Similar to the above studies, in the D group of the present study, histopathologic analysis revealed significant nucleus

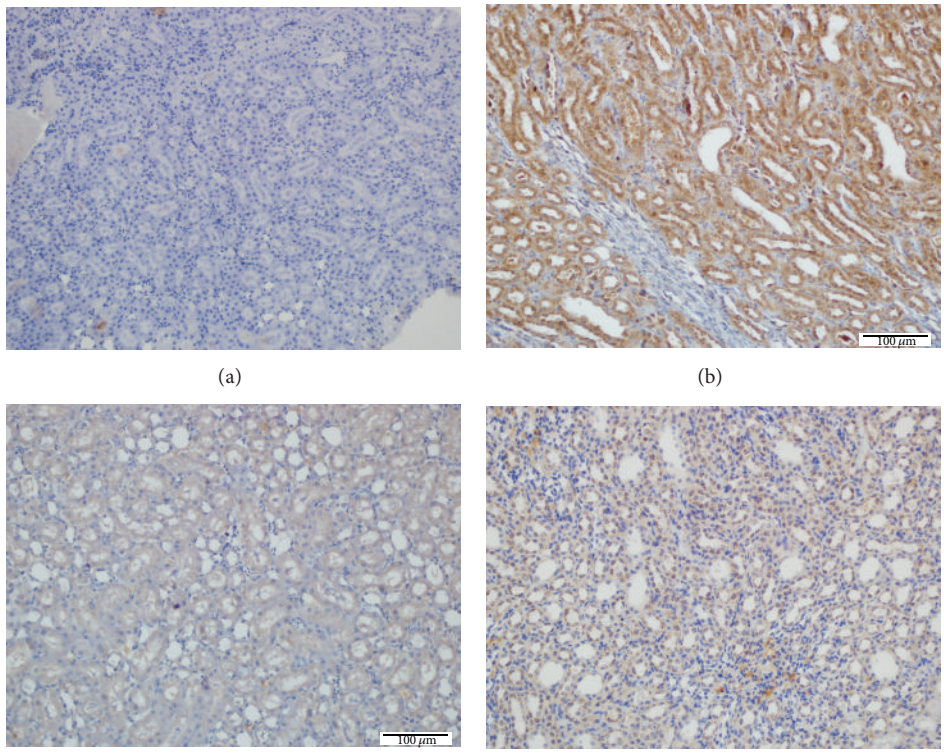

Figure 4: Evaluation of apoptosis by Bcl-2, Bax, and caspase-3 (immunohistochemical method). (a) Unusual development of immune reactivity in Control rats (IHC, ×200). (b) Immune reactivity shows increased apoptosis in the kidneys given Dichlorvos (IHK, ×200). (c) Low profile of apoptosis in the D + CAPE group (IHC, ×200). (d) The apoptosis level in the D + IL group is only lower than in the D group (IHC, ×400).

loss in the tubule epithelial cells, tubular dilatation and interstitial accumulation of lymphocytes, and tubular necrosis. Quasi-normal renal tissue with blown tubular epithelium cell areas, vacuolar degradation, and necrosis less than 25% was observed in the D + CAPE and IL groups.

Toxic cell death is known to appear by only two main mechanisms: necrosis and apoptosis, which can be divided by their characteristic morphological and biochemical situations. These aspects of apoptosis are observed in various tissues from animal models, and while there is widespread necrosis occurring within the area of injury, apoptosis also plays an important role in toxic cell death in various tissues [24].

Hou et al. have demonstrated that rat exposure to D caused renal injury, including renal tubular, glomerular filtration, and oxidative stress. Also, these toxic effects were also regulated by high-dose quercetin. In that study, histopathological examination revealed that D induced extensive cell vacuolar denaturation; however, milder histopathological alterations were observed in the kidney tissues of rats by combined D + quercetin (50 mg/kg bw) [22]. Fiore et al. showed that D-induced apoptosis is mediated by pesticide's capacity to induce monopolar spindle-associated mitotic cell arrest, which, in turn, promotes apoptosis directly from mitosis [25]. In a study conducted by Alp et al., CAPE was demonstrated to be an effective agent in protection against injuries in liver, lungs, and kidney caused by diazinon which is an OP [26].

The results of above referred studies were in agreement with our results. In present study, the D group revealed cytoplasmic hypereosinophilic changes, loss of intracellular boundaries, nuclear pyknosis, diffusely increased mitotic activity in histopathologic examinations, and diffusely increased apoptosis in immunohistochemical analysis. However, in the rats given D + CAPE, D + IL, and D + IL + CAPE the mitotic density was decreased and regenerative changes were observed. Similarly, in the immunohistochemical analysis, apoptosis was increased in the D group while it was lower in D + CAPE, D + IL, and D + IL + CAPE groups compared to the D group. These results show that CAPE and IL may exclusively ameliorate the renal injury caused by D in the present study.

When biochemical results were evaluated in some studies, it was reported that OPs cause increase of oxidative stress, ROS, and depletion of antioxidant enzymes [27]. Celik and Suzek showed that the sublethal concentrations of D have toxic effects on MDA content and antioxidant defense system such as reduced glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in various tissues of rats exposed to 0.0225- and 0.0450-millimole (mmol) D in drinking water [28]. Also, Cao et al. showed that the activities of GST, SOD, and catalase

(CAT) were decreased dramatically in the D-treated group when compared with the flavonoid extracts group and the D + flavonoid extracts group [29].

Our results were in agreement with abovementioned studies. The present study showed that the administration of D causes increase in the TOS levels and decrease in the TAS levels compared to the Control, IL, CAPE, D + CAPE, and D + IL + CAPE groups. Also, the TAS levels in D + CAPE, D + IL, and D + IL + CAPE groups were found higher than in the D group. In the present study, histopathological results were consistent with biochemical results.

Based on our results, it can be concluded that CAPE and IL are similarly capable of preventing the renal injuries caused by D by means of their antioxidant effects. Therefore, we suggest that it can be used either alone or in combination with CAPE and IL or it can be used as supportive therapy or as facilitator for the therapeutic effect of the routine treatment in the patients presenting with pesticide poisoning. However, further studies are needed about protective effects of CAPE and IL on OP intoxications.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

Acknowledgment

This study with 1206M0126(404) ID number was supported by Mustafa Kemal University Scientific Research Project Coordination.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 175696, 14 pages
http://dx.doi.org/10.1155/2015/175696

Research Article

^{1}H HR-MAS NMR Spectroscopy and the Metabolite Determination of Typical Foods in Mediterranean Diet

Carmelo Corsaro,[1,2] Domenico Mallamace,[3] Sebastiano Vasi,[2] Vincenzo Ferrantelli,[4] Giacomo Dugo,[3,5] and Nicola Cicero[3,5]

[1] *Istituto per i Processi Chimico-Fisici del CNR di Messina, Viale F. Stagno D'Alcontres 37, 98158 Messina, Italy*
[2] *Dipartimento di Fisica e Scienze della Terra, Università di Messina, Viale F. Stagno D'Alcontres 31, 98166 Messina, Italy*
[3] *Dipartimento di Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti e della Salute, Università di Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy*
[4] *Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Via G. Marinuzzi 3, 90129 Palermo, Italy*
[5] *Science4life SRL Academic Spin-off, Università di Messina, Viale F. Stagno D'Alcontres 31, 98166 Messina, Italy*

Correspondence should be addressed to Carmelo Corsaro; ccorsaro@unime.it

Received 4 February 2015; Revised 29 April 2015; Accepted 1 May 2015

Academic Editor: Shao-Nong Chen

NMR spectroscopy has become an experimental technique widely used in food science. The experimental procedures that allow precise and quantitative analysis on different foods are relatively simple. For a better sensitivity and resolution, NMR spectroscopy is usually applied to liquid sample by means of extraction procedures that can be addressed to the observation of particular compounds. For the study of semisolid systems such as intact tissues, High-Resolution Magic Angle Spinning (HR-MAS) has received great attention within the biomedical area and beyond. Metabolic profiling and metabolism changes can be investigated both in animal organs and in foods. In this work we present a proton HR-MAS NMR study on the typical vegetable foods of Mediterranean diet such as the Protected Geographical Indication (PGI) cherry tomato of Pachino, the PGI Interdonato lemon of Messina, several Protected Designation of Origin (PDO) extra virgin olive oils from Sicily, and the Traditional Italian Food Product (PAT) red garlic of Nubia. We were able to identify and quantify the main metabolites within the studied systems that can be used for their characterization and authentication.

1. Introduction

In the last two decades, there was a remarkable increase in studies concerning food science performed by means of Nuclear Magnetic Resonance (NMR) spectroscopy [1, 2]. The reason for NMR success in food analysis lies essentially in the possibility to study complex matrices, obtaining a large number of information on metabolites within a single experiment, with minimal or no sample preparation [2]. In fact, even if other widely used analytical techniques such as Gas Chromatography (GC) have a higher sensitivity, they need quite sophisticated extraction procedures [3, 4]. Furthermore, advanced NMR hardware and user-friendly software have been developed as well as bidimensional techniques that allow easy metabolite identification [5]. The area below each proton NMR signal is directly proportional to the numbers of nuclei so its knowledge allows the determination of the quantitative chemical composition. Finally, its use in synergy with multivariate statistical analyses permitted a number of relevant studies on food metabolomics and chemometrics especially after the increasing needs for the control on food quality and safety [6, 7].

In order to obtain good quality NMR spectra, the system under study must be liquid. In fact, nonliquid systems are characterized by strong anisotropic interactions that cannot be averaged out and produce unresolved broad peaks. In particular, the NMR spectral sensitivity and resolution are limited by those mechanisms that provoke line-broadening effects also with high magnetic fields. Dipolar coupling and susceptibility heterogeneity are examples of these kinds of

mechanisms that occur within biological samples. Therefore, one technique that can average multiple line-broadening mechanisms was developed [8] to limit and resolve these problems, particularly strong for solid-state NMR. This technique is known as magic angle spinning (MAS) NMR spectroscopy (see Section 2) and, taking advantage of "geometric constrains," can be used to acquire high-resolution (HR) spectra of heterogeneous samples such as tissues and cells. This allows the determination of the metabolic profile of the studied system under the considered conditions. Therefore, HR-MAS NMR has become more popular in food science and in the biological and biomedical fields [9, 10].

As a matter of fact, many studies performed by means of HR-MAS on different organs and tissues have been reported, demonstrating—for example, the ability of this technique to discriminate between malignant and benign disease [11–13]. Moreover, the possibility to follow the metabolic changes has led to apply the HR-MAS technique in different fields ranging from the characterization and authentication of different foods [14] to the study of the cellulose degradation happening over centuries in ancient documents [15].

The experimental results we present in this work concern the application of this interesting and powerful technique to the study of the metabolic profile of some typical foods of the Mediterranean diet. In the early 1960s, in Greece and southern Italy, adult life expectancy was among the highest in the world and rates of coronary heart disease, certain cancers, and other diet-related chronic diseases were among the lowest. This was attributed to the particular diet adopted in those regions and today known as Mediterranean diet [17].

The Mediterranean diet is principally characterized by the consumption of olive oil and wine together with numerous plant foods (vegetables, breads, other forms of cereals, potatoes, beans, nuts, and seeds), fresh fruit (e.g., citrus), fish, and cheese. Poultry is consumed from low to moderate amounts; zero to four eggs are consumed weekly and red meat is consumed in low amounts. Furthermore, garlic, onions, and herbs were used as condiments. This diet is able to provide all of the known essential micronutrients (i.e., vitamins and minerals), fiber, and other plant food substances believed to promote health [17]. It is noteworthy that the Mediterranean diet was inscribed in 2013 on the Representative List of the Intangible Cultural Heritage of Humanity (UNESCO).

Among the different foods of the Mediterranean diet, we focused our attention on four important food products characterized by a Protected Geographical Status [18]: the PGI (Protected Geographical Indication) cherry tomato of Pachino, the PGI Interdonato lemon of Messina, several PDO (Protected Designation of Origin) extra virgin olive oils (eVOOs) from Sicily, and the PAT (Traditional Italian Food Product) red garlic of Nubia.

The European Union (EU) has restrictive laws about the food safety policy aimed at protecting consumer health and interests while guaranteeing the smooth operation of the single market. In particular, the EU ensures that control standards are established and adhered to regarding food and food product hygiene, animal health and welfare, and plant health and preventing the risk of contamination from external substances. It also establishes the bases for an appropriate labelling, in line with the approach "From the Farm to the Fork," thereby guaranteeing a high level of safety for foodstuffs and food products marketed within the EU, at all stages of the production and distribution chains.

Indeed, in this paper we present HR-MAS NMR results on the mentioned four typical food products of the Mediterranean diet. The increasing demand of quality control by consumers pushes the development analytical techniques able to characterize the metabolic profile of a particular food. We were able to identify and quantify the main metabolites within the studied systems that can be considered their fingerprint. In fact, the used technique can reveal and quantify a number of metabolites even on few amounts of samples and without any chemical treatment. In spite of its quite low sensitivity, the rapidity and easiness of the HR-MAS technique, together with the reduction of chemical consumption and waste production, make the methodology very attractive for industry.

2. Materials and Methods

2.1. Instrumental. ^{1}H one and two-dimensional NMR experiments were conducted at atmospheric pressure by using a Bruker Avance spectrometer operating at 700 MHz, ^{1}H resonance frequency, in the experimental configuration known as magic angle spinning (MAS). This technique was developed to reduce the two main line-broadening mechanisms that are important in acquiring spectra of a tissue or cell sample, namely, dipolar coupling and heterogeneous isotropic susceptibility [8]. Spinning the sample at the magic angle $\theta \sim 54.74^\circ$ by few thousands of Hertz averages, these interactions to zero and high-resolution spectra can be achieved for semisolids samples.

Our experiments were performed at the temperature of 300 K calibrated against the standard CH_3OH reference (4% CH_3OH in CD_3OD) with an accuracy of 0.2 K. Temperature calibration is very important for this kind of experiments because of the heat produced by the high rotational speed. In fact, the real sample temperature is higher with respect to that read by the thermocouple. For each experiment we use a 4 mm-diameter zirconia sample holder (rotor) with a spherical insert for a total volume of 50 μL and a Kel-F rotor cap. We use deuterated solvents (D_2O and $CDCl_3$) in order to have a lock signal for a chemical shift reference and for a fine optimization of the static magnetic field homogeneity. Furthermore, the use of deuterated solvent is necessary in order to avoid any excessive proton signal from the solvent itself. In aqueous preparation we use a 1 mM solution of D_2O with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard for the quantification of the assigned metabolites. High purity reagents were bought from Sigma-Aldrich Co. (Saint Luis, MO, USA). The acquired spectra were processed (Fourier transform, phase correction, and baseline adjustment) by means of the standard routines of the software package Xwinnmr version 3.5 (Bruker Biospin, Deutschland). Peaks assignment was performed by means of literature data and of a well-established software package: NMR Suite Professional version 7.1 (Chenomx, Alberta, Canada). This latter

software is based on a highly sophisticated targeted profiling technology which allows an easy deconvolution of complex NMR spectra and the corresponding quantification of the identified compounds. For the complete and unambiguous assignment of some compounds we performed standard two-dimensional NMR techniques such as COSY and HSQC. For those metabolites not included in the software database (such as gallic acid) (see Section 3.2) and also for a confirmation of the Chenomx output, we used the standard Bruker program "nmrquant" for the quantification of metabolites. The NMR technique is less sensitive with respect to other well-established analytical techniques such as GC and ICP-MS (Inductively Coupled Plasma Mass Spectrometry) [19] and can quantify metabolites whose concentration is usually above one part per million. The quantification is obtained by using a reference compound of known concentration within the studied solution (1 mM DSS in D_2O in our case). The area below each proton signal is proportional to the amount of the corresponding substance, so that by the knowledge of the chemical structure and molecular weight of the assigned metabolites, it is possible to obtain the molar concentration by a simple proportion between the peak areas. By means of the Chenomx software, all the spectral contributions belonging to the considered molecule should fit the experimental spectrum whereas by using nmrquant, the most intense and resolved peaks should be used for metabolites quantification. Finally, we can state that the advantages of the used method rely on the rapidity of the analysis without the needs for any sample treatment that allows, at least in principle, the reuse of the sample. Furthermore, the method is precise and allows observing a great number of compounds simultaneously. On the contrary, the method is not very sensitive for the detection of compounds whose concentration is below one part per million. Precise extraction procedures are needed in order to observe particular compounds (or secondary metabolites) at very low concentration.

In the following subsections, we describe the sample preparation and experimental procedures for each single food products we have analyzed.

2.2. PGI Cherry Tomato of Pachino. The PGI cherry tomato of Pachino is produced within an area located in the south east of Sicily (Italy) that includes the entire municipality of Pachino and Portopalo di Capo Passero and part of the territories of Noto and Ispica. We analyzed 14 cherry tomato samples of Pachino and 14 of dubious provenience (non-Pachino) including 2 coming directly from Beijing (China) [20]. For a statistically significant outcome we analyzed at least 5 samples for each kind of tomato. The PGI cherry tomatoes of Pachino were provided by Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" (http://www.izssicilia.it/) which is the official institution recognized by the EU as to able to certificate this food product.

All samples were studied in the red stage which is when more than 90% of the surface, in the aggregate, is red. In doing so we reduce the eventual metabolic differences due to different ripening stage [21, 22]. We diluted 6 mg of freeze-dried tomato in 100 μL of a 1 mM solution of DSS in D_2O. Then, we vortexed for a couple of minutes and put fifty microliters into the rotor that in this case was spun at 6000 Hz. The duration of the hard pulse was of 8 μs with a relative attenuation of 3 dB, the spectral width was 10 kHz, the acquisition time was 2.9 s, the points in the time domain were 64 k, the number of transient was 128, and the relaxation time was 2 s for a total time of about 10 min per experiment. For the reduction of the residual signal of water we use the standard Bruker presaturation pulse sequence *zgpr* with a presaturation pulse attenuation of 60 dB. When processing the spectra we considered 32 k points in the frequency domain.

2.3. PGI Interdonato Lemon of Messina. The PGI Interdonato lemon of Messina is traditionally also known as "limone fino" (fine lemon) and "limone speciale" (special lemon). Interdonato lemons have an oval shape with a yellow peel. Interdonato lemons own a strong fragrance and an acidic taste. They are rich in sugars, vitamin C, and flavonoids, which are very important in human metabolism. We have analyzed 10 different Interdonato lemon samples cultivated in Sicily and 10 cultivated in Turkey at the same ripening stage. For each sample we have repeated the measurements on 6 different replicates in order to have a statistically significant outcome. The PGI Interdonato lemons were provided by 4 different companies belonging to the "Consorzio di tutela del limone Interdonato di Sicilia IGP". We extracted the lemon juice by a simple mechanical procedure and diluted 20 μL of juice in 30 μL of 1 mM DSS dissolved in D_2O directly into the rotor with a spherical insert and a Kel-F rotor cap. The sample was kept at ambient temperature (300 K) by a cold N_2 flow and a heating element. We used the following experimental parameters: rotor spinning rate 6000 Hz, duration of the hard pulse 8 μs, spectral width 10 kHz, acquisition time 2.9 s, 64 k points in the time domain, 128 transients, and 2 s of relaxation time. We use the standard Bruker presaturation pulse sequence "zgpr" to achieve a reduction of the residual water signal. The total time necessary for each experiment was of about 10 min and we used 32 k points in the frequency domain for processing the spectra.

2.4. PDO Extra Virgin Olive Oil of Sicily. 16 samples of different eVOOs were selected from different geographical areas of Sicily and in particular 8 from the province of Trapani (TP), 5 from that of Messina (ME), and 3 from that of Agrigento (AG). Among these we consider the following PDO cultivars: Valle del Belice (TP), Val di Mazara (TP), Valli Trapanesi (TP), and Valdemone (ME). All samples, after being carefully kept away from light and possible temperature changes that would alter the nature of the oils, were analyzed by taking 30 μL of $CDCl_3$ and 20 μL of sample, placed in the rotor. The spectral width used was of 20 ppm (~14 kHz), the repetition time was 5 s, and the number of transients was 128. The duration of the hard pulse was of 5 μs with an attenuation of 3 dB. The rotation speed of the rotor was set to 7000 Hz and the total time was of about 15 min per experiment.

The main signals of the typical proton spectrum of eVOOs come from fatty acids [23–25]. We used two different methods based on peaks integration for the determination of

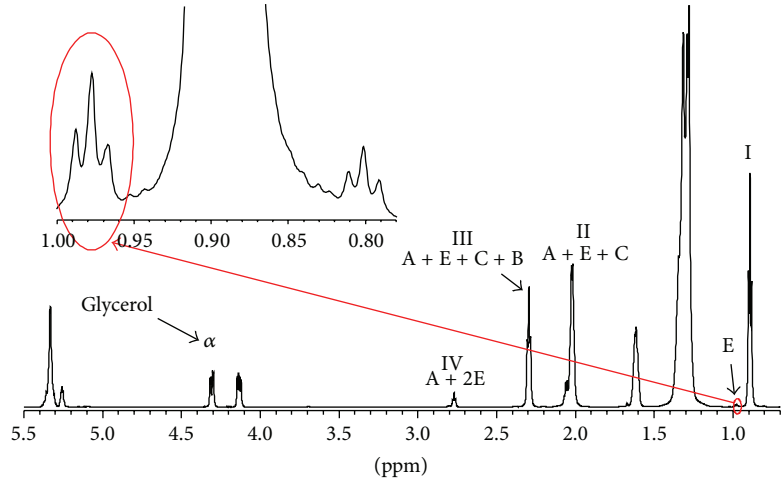

Figure 1: Typical proton NMR spectrum of eVOOs with highlighted the characteristic peaks of fatty acids and glycerol moieties used for the determination of fat percentage. In addition, the expansion shows the ^{13}C satellites of the methyl peak at 0.85 ppm (I) superimposed to that of linolenic at 0.98 ppm (E).

the fatty acids composition. In particular, Barison et al. [23] consider that all fatty acyl chains are esterified to a common moiety, glycerol, in order to form triacylglycerols. This means that it is possible to obtain the fatty acid composition by the inherent connection between the areas of the characteristic signals of each fatty acyl chain and one of the glycerol backbone (α) in the ^1H NMR spectra (Figure 1, e.g., signals at 4.27 ppm) [23]. In particular, fatty acids can be esterified up to three times to the same glycerol moiety and this fact must be taken into account in order to calculate the correct amount. For example, it is possible to have up to three linolenic acid groups esterified to the same glycerol moiety so a ratio of 22.2 α glycerol to 100 linolenic acid hydrogens should be used when integrating the signal E (0.98 ppm) in Figure 1. Special attention should be given to the integral determination (limits, slope, etc.). For example, in our case (700 MHz) the ^{13}C satellites of most intense peaks have to be subtracted by the integrated region in order to obtain correct values (e.g., as shown in the expansion of the linolenic (E) signal at 0.98 ppm reported in Figure 1) [24]. Accordingly, the percentage of linoleic (A) acid can be determined by integrating the signal at about 2.74 ppm (signal IV = A+2E in Figure 1), which refers to the methylene hydrogens between two double bonds or olefins [23].

Therefore, by setting the integral of the chosen glycerol signal to 33.3, the relative area found for the signal at 2.74 ppm directly gives the percentage of linoleic plus linolenic acids. Since linoleic (A) and linolenic (E) acids have, respectively, two and four methylene hydrogens between olefins, and the percentage of linoleic acid is obtained by subtracting twice the content of linolenic acid that was previously determined [23]. The signal (II in Figure 1) at about 2.02 ppm refers to the methylene α olefin hydrogens of all unsaturated fatty acids, and being the ratio of 2 α glycerol hydrogens to 12 possible α olefin hydrogens, if the signal of α glycerol hydrogens is set to 16.7, the area of the signal at 2.02 ppm provides the percentage of all unsaturated fatty acids: linolenic (E), linoleic (A), and oleic (C). The percentage of oleic acid can be then obtained by subtracting from the value found, the contributions of the unsaturated acids previously obtained [23]. Finally, by setting again the integral of the chosen glycerol signal to 33.3, that of the signal at 2.28 ppm (from six α carbonyl hydrogens of all fatty acids esterified to the glycerol moiety) is approximately 100. Therefore, the percentage of saturated fatty acids can then be determined by subtracting from the area of the signal at 2.28 ppm, the contributions of the unsaturated oleic, linoleic, and linolenic acids found earlier.

The second method that we used was introduced by Vigli et al. [25] by considering that the content of linoleic acid can be determined by referring the intensity of its characteristic methyl signal at 0.95 ppm (signal E in Figure 1) to the intensity of the methyl signal at 0.85 ppm (signal labeled I in Figure 1) belonging to all acids except linolenic [25]. Consider

$$[\text{linolenic}] = \frac{E}{(E + I)}. \tag{1}$$

The relative amount of linoleic acid can be determined by subtracting from the signal of diallylic protons at 2.73 ppm (signal IV in Figure 1) the relative amount of linolenic calculated earlier as well as the oleic acid content that can be determined by referring the allylic protons centered at 2.02 ppm (signal II in Figure 1) to all fatty chains as measured from the intensity of the C-2 protons around 2.3 ppm (signal III in Figure 1) [25]. One has

$$\begin{aligned}
[\text{linoleic}] &= \frac{(3IV - 4E)}{3III} \\
[\text{oleic}] &= \frac{II}{2III} - [\text{linoleic}] - [\text{linolenic}] \\
[\text{saturated}] &= \frac{I}{(E + I)} - [\text{linoleic}] - [\text{oleic}] \\
&\quad - [\text{linolenic}] .
\end{aligned} \tag{2}$$

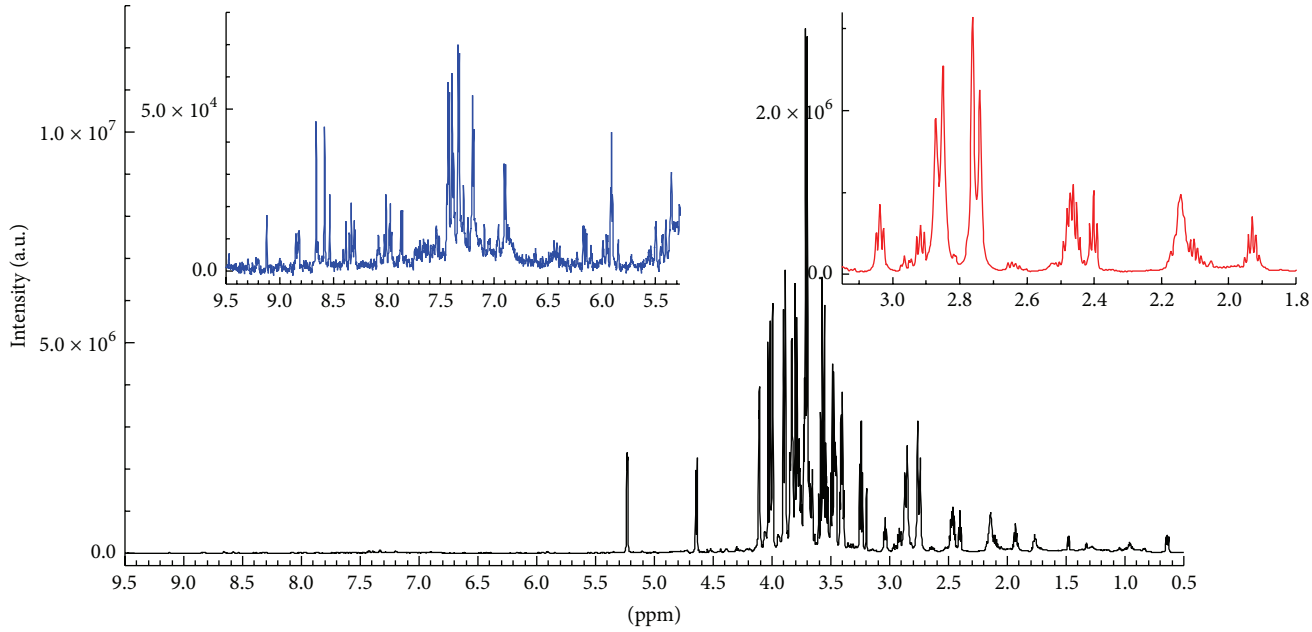

FIGURE 2: The complete typical proton spectrum of a PGI cherry tomato of Pachino sample is reported in the main plot. The expansions report the enlargement of the phenolic region (left side, blue line) and part of the aminoacidic region (right side, red line).

2.5. Red Garlic of Nubia. The red garlic of Nubia is cultivated within the municipality of Paceco (Trapani) and in particular in the Integral Natural Reserve of Saline in Trapani. It is a food product registered to the Italian Ministry of Agricultural, Food, and Forestry Policies as a Traditional Italian Food Product (PAT). We have prepared the garlic samples by accurately cutting a thin strip of about 20 mg, rolling it directly into the rotor together with 30 μL of 1 mM DSS in D_2O. In our one-dimensional experiments we used 32 k points in the time domain and a spectral width of 14 ppm (~10 kHz). The repetition time was set to 3 s and the number of transients was 256. The duration of the hard pulse was of 6.4 μs with an attenuation of 3 dB. Also in this case, to reduce the residual water signal, we use the standard Bruker presaturation pulse sequence *zgpr*. The rotation speed of the rotor was set to 7000 Hz and the total duration was of about 20 min per experiment. We have performed the experiments on 6 samples and 6 different replicates in order to have a statistically significant outcome.

3. Results and Discussion

3.1. PGI Cherry Tomato of Pachino. Tomato (*Solanum lycopersicum*) is probably the most consumed fresh vegetable all over the world. Tomato is low in calories and shows antioxidant, antitumoral, and antidepressive properties [26] due to the relatively high concentration of lycopene, ascorbic acid, vitamin E, flavonoids, and so forth. The first tomato accredited by the PGI certificate (Council Regulation (EEC) number 2081/92) and one of the most counterfeit food product is the Sicilian cherry tomato of Pachino. Its special taste comes from the right combination of sugars, organic acids, free amino acids, and salts [20, 27].

We were able to identify and quantify the molar concentration of the main metabolites that can be observable by means of our NMR technique. In such a way we aim to obtain a metabolic fingerprint of this protected foodstuff that allows for its characterization and authentication. Figure 2 reports in the main plot the complete typical proton spectrum of a PGI cherry tomato of Pachino sample. The expansions are the enlargement of the phenolic region (left side, blue line) and part of the aminoacidic region (right side, red line). One can note the high resolution of the obtained spectra especially in the phenolic region that is usually characterized by high noise level.

We used for peaks assignment literature data [21, 22, 28] and the above mentioned software package NMR Suite Professional version 7.1 (Chenomx, AB, Canada) that allows also the determination of metabolites concentration [20]. We followed the same procedures also for the non-Pachino cherry tomato samples in order to execute a multivariate statistical analysis in terms of the Principal Components Analysis (PCA). This kind of multivariate statistical analysis, being based on an unsupervised pattern recognition technique, allows the identification of differences and similarities between NMR metabolic fingerprints.

In particular, NMR spectra were processed by means of a custom-written ProMetab 3.3 software [29] in MATLAB version R2009b (The Math Works, Natick, MA, USA); spectra were binned from 0.7 to 10.0 ppm with 0.005 ppm bin size; the residual water signal (4.65–4.95 ppm) was excluded; spectra were normalized to the total area and were generalized by log transformation (with a transformation parameter, $\lambda = 10^{-6}$) to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks [29]. Finally we use the software package Unscrambler X version 10.0.1

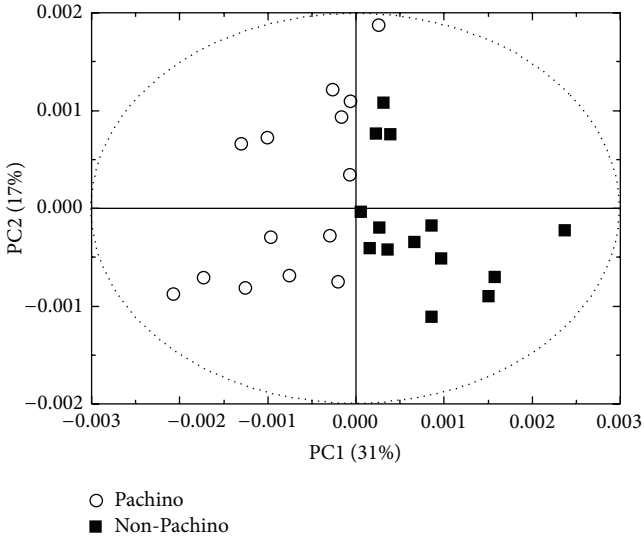

Figure 3: Score plot of the PCA analysis performed on cherry tomato samples. The Principal Component 1 (PC1) discriminates between Pachino and non-Pachino cherry tomatoes sample.

Table 1: Comparison between the statistically significant average metabolite molar concentrations of Pachino cherry tomatoes and of non-Pachino ones. Besides, we signed in bold the metabolites with the higher concentration.

Metabolite	Pachino cherry tomatoes (mM)	Non-Pachino cherry tomatoes (mM)
GABA	**0.89**	0.34
Alanine	0.26	**0.54**
Aspartic acid	**0.81**	0.50
Fructose	**19.4**	14.6
Glucose	**13.4**	10.8
Glutamic acid	**1.26**	0.78
Glutamine	**1.59**	1.04
Guanosine	0.033	**0.098**
Methanol	0.61	**1.35**
Trigonelline	**0.051**	0.023
Tryptophan	**0.068**	0.032
Tyrosine	**0.058**	0.022

(Camo Software AS, Oslo, NO) for the PCA analysis with cross validation; data were mean-centered and the singular value decomposition (SVD) algorithm was used.

Our aim is to establish if there are any metabolites that can account for sample differentiation. We report in Figure 3 the results of the PCA analysis in the form of a score plot, where samples that are metabolically similar cluster together. As it can be noticed, the Principal Component 1 (PC1) is able to separate Pachino from non-Pachino cherry tomatoes except for one sample.

The final identification of the metabolites that can account for sample differentiation can indeed be obtained by analyzing the loadings corresponding to the PC1. The loadings of a Principal Component represent the weight by which each standardized original variable should be multiplied to get the component score. In particular, positive loadings values represent metabolites that are predominant in non-Pachino samples and vice versa [20]. Therefore, we were able to identify those metabolites whose concentration can determine the sample clustering. Student's *t-test* analysis, performed by means of the software package Microsoft Excel (Microsoft Co., WA, USA), allows to determine only the statistically significant (p value less than 0.05) changes of metabolites that we report in Table 1. Sugars, GABA, glutamic acid, trigonelline, tryptophan, and tyrosine concentration is higher in Pachino cherry tomatoes whereas that of alanine, guanosine, and methanol is higher in non-Pachino ones.

We want to stress that our result should be independent of factors such as the annual weather variations that can provoke some metabolic changes. In fact, it was shown that despite the marked variability showed only by antioxidants content, greenhouse-growing conditions in Sicily induce the accumulation of relatively high levels of ascorbic acid, phenolic compounds, and carotenoids in cherry tomatoes for most of the year [30]. Moreover, very recent studies on the response of tomato to constraining the intensity of solar radiation showed that the tomato plant's metabolism has a strong adaptation to cope with the limitation in light availability such as increasing the specific leaf area and reducing respiration. This was only of little concern to the fruit quality, because no effect of constraining the intensity of solar radiation on the concentration of total dry matter, sugars, and lycopene in the fruits was observed [31].

3.2. PGI Interdonato Lemon of Messina. Lemon (*Citrus limon* (L.) Burm.), similarly to tomato, is one of the most consumed fresh fruit. Lemon is low in calories and displays antioxidant and antineoplastic properties [32] that depend on the relatively high concentration of potassium, magnesium, calcium, vitamin C, phenolic compounds, and so forth. In particular, lemon is the third most important health-promoting fruit rich in phenolic compounds as well as vitamins, minerals, dietary fiber, essential oils, and carotenoids. Furthermore, it is widely used also by the food industry as raw materials or flavoring additives for a wide variety of products. Indeed, lemons have a strong commercial value for fresh products market and food industry [32].

The cultivar of our interest, known as "Interdonato," represents a hybrid between a cedar and a lemon. It is cultivated in the province of Messina (Italy) within an area delimited by the Ionian Sea and the Peloritans Mountains and it is one of the few citrus accredited by the European PGI certificate (Commission Regulation (EC) number 1081/2009).

In particular, we have studied the one-dimensional proton spectrum of lemon juice for different samples of both PGI Interdonato lemon of Messina and Interdonato lemon from Turkey [33]. We have assigned and quantified the main metabolites that are present in these two hybrids by means of literature data [34] and the above mentioned Chenomx software package. In fact, other studies performed on the different tissues of lemon with the HR-MAS technique are

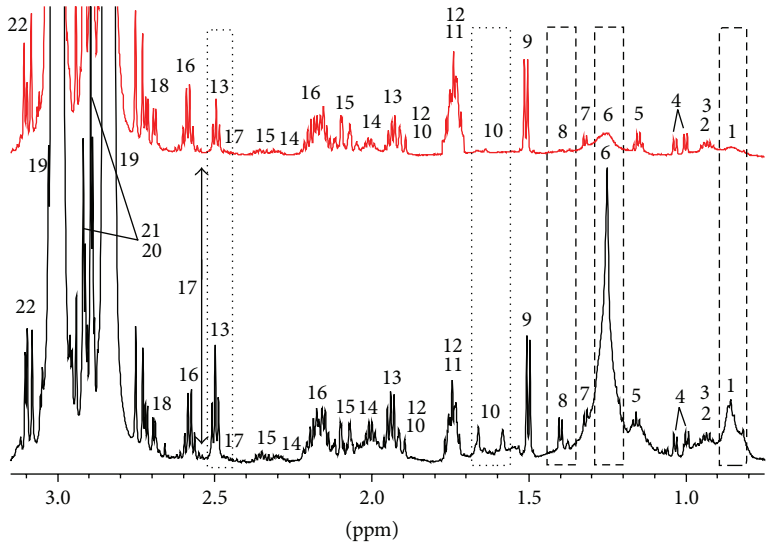

Figure 4: Comparison between the one-dimensional proton spectra of the lemon juice from PGI Interdonato lemon of Messina (red line) and Interdonato Turkish lemon (black line) in the region of amino acids. 1: saturated fatty acids, 2: isoleucine, 3: leucine, 4: valine, 5: ethanol, 6: unsaturated fatty acids, 7: threonine, 8: lactic acid, 9: alanine, 10: arginine, 11: DSS, 12: lysine, 13: GABA, 14: proline, 15: glutamic acid, 16: glutamine, 17: isocitric acid, 18: aspartic compound, 19: citric acid, 20: malic acid, 21: asparagine, and 22: stachydrine.

present in literature [34] that agree with our results. However, in this mentioned study no metabolites quantification was performed.

Figure 4 reports the comparison between the one-dimensional proton spectrum of the PGI Interdonato lemon (red line) and that of the Turkish one (black line) in the chemical shift region of amino acids. In the figure, all the identified metabolites are numbered and the most evident spectral differences are highlighted by means of rectangular shapes. In detail, in Turkish lemon there is a greater amount of both saturated (1) and unsaturated (6) fatty acids, lactic acid (8), arginine (10), and γ-aminobutyric acid or GABA (13). In contrast, asparagine (21) and malic acid signals (20), even if their peaks are cut in the figure, are more intense in PGI Interdonato lemon of Messina.

In lemon juice the major contribution of unsaturated fatty acids comes from oleic, linoleic, and linolenic acids whereas that of saturated fatty acids comes from palmitic and stearic acids [35]. Organic acids such as citric, isocitric, and lactic acids mainly contribute to determine the lemon acidity that plays the major role in the criteria assessing the commercial acceptability of the fruit. Citric acid (peaks in Figure 4 at 2.85 and 3.00 ppm cut because of their extreme intensity) content in lemon juice is about 5% to 6% [35].

For what concerns the sugars, namely, sucrose, fructose, and glucose, that represent the major component of carbohydrates in citrus fruits and hold the key to sweetness of the juice, the spectral comparison is reported in Figure 5. Fructose and β-glucose have a higher concentration in PGI Interdonato lemon of Messina whereas sucrose content is essentially identical. Another metabolite that is very important for nutritional consideration and that displays the same concentration in the two hybrids is vitamin C. The question mark at about 4.15 ppm in Figure 5 represents a metabolite that displays different chemical shift and that we were not able to assign with certainty but should belong to some malonic compounds.

Some minor but important metabolites that we were able to assign are myoinositol (peaks at about 3.28, 3.53, 3.62, and 4.06 ppm), scyllo-inositol (peak at about 3.34 ppm), and stachydrine (peak at about 3.11 ppm). Inositols are present in many vegetable species as minor components and have a positive physiologically activity in human [36]. Many important studies have demonstrated the importance of inositols in the treatment of several diseases such as the polycystic ovary syndrome [37]. Furthermore, myoinositol content and myoinositol/fructose ratio have been found to provide information on the quality and genuineness of orange juice [38]. Stachydrine is an osmoprotectant or osmoprotective compound, which helps organisms to survive extreme osmotic stress [39]. Our evaluation of stachydrine content (about 0.6 mM) (see Table 2) in both Interdonato lemon juices agrees with that of a recent work on the effect of stachydrine on endothelial cell senescence under high glucose stimulation [39]. Finally, the methanol peak at about 3.36 ppm is well evident in the spectrum of Interdonato Turkish lemon but it is not so intense in that of the PGI Interdonato lemon of Messina. Even if we are dealing with low concentrations we want to stress that an excess of methanol is not well tolerated by the human body since it interferes with liver metabolism where it is oxidized.

Also in the phenolic region (Figure 6) we were able to identify and quantify a good number of metabolites. Here we observe signals coming from nucleosides compounds (33), trigonelline (31), tryptophan (37), tyrosine (34), phenylalanine (38), gallic acid (38), and so forth. Note that phenolic compounds possess antitumoral and health properties [40, 41]. In particular, we want to stress the

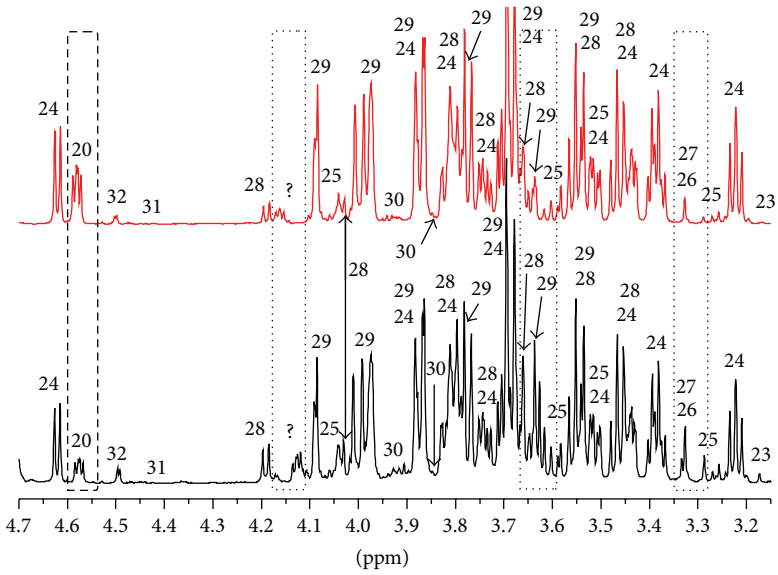

FIGURE 5: Comparison between the one-dimensional proton spectra of the lemon juice from PGI Interdonato lemon of Messina (red line) and Interdonato Turkish lemon (black line) in the region of the sugars. 20: malic acid, 23: choline, 24: glucose, 25: myoinositol, 26: scyllo-inositol, 27: methanol, 28: sucrose, 29: fructose, 30: serine, 31: trigonelline, and 32: ascorbic acid.

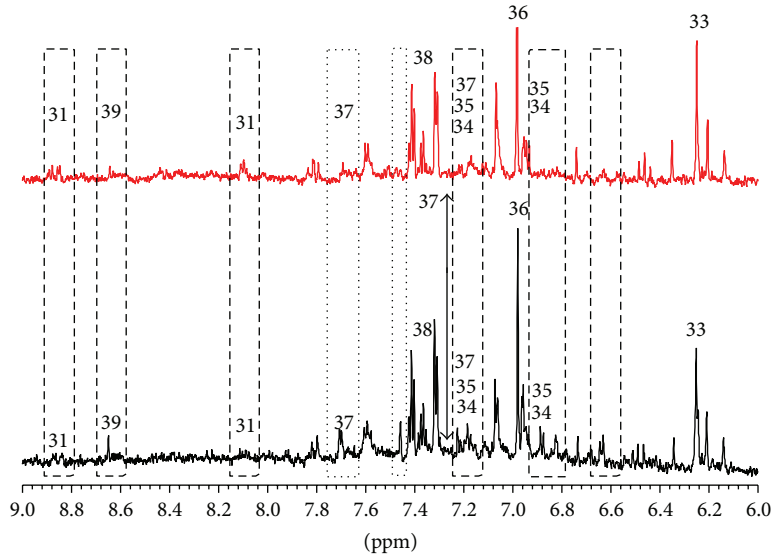

FIGURE 6: Comparison between the one-dimensional proton spectra of the lemon juice from PGI Interdonato lemon of Messina (red line) and Interdonato Turkish lemon (black line) in the region of phenolic compounds. 31: trigonelline, 33: nucleosides, 34: tyrosine, 35: tyramine, 36: gallic acid, 37: tryptophan, 38: phenylalanine, and 39: adenosine monophosphate (AMP).

relatively high concentration, for both hybrids, of gallic acid which is a hydroxybenzoic acid present in food of plant origin and exhibit antioxidative properties [42]. Gallic acid is one example of metabolite identified by means of HSQC experiments.

In Table 2, we report the molar concentration, together with the standard deviation, of those metabolites whose p value is below 0.005 and so that can be taken into account for sample differentiation. In particular, the metabolic differences can be considered due to the different geographical origin of the two hybrids of Interdonato lemon.

3.3. PDO Extra Virgin Olive Oil of Sicily. Olive oil is the principal source of fat in the Mediterranean diet. Olive oil contains a large proportion of monounsaturated fat, is relatively low in saturated fat, and is another source of the antioxidant vitamin E [43]. These characteristics make olive oil preferable to animal fats just from the standpoint of health [44, 45]. In fact, diets high in monounsaturated fat seem to reduce the risk of atherogenesis and coronary heart diseases, because they increase the concentration of high-density lipoproteins (HDLs) without increasing that of the low-density lipoproteins (LDLs) [46].

Table 2: Average metabolites molar concentration of PGI Interdonato lemon of Messina and Interdonato lemon of Turkey juices.

Metabolite	PGI Interdonato lemon of Messina (mM)	Interdonato lemon of Turkey (mM)
Alanine	0.95 ± 0.01	1.49 ± 0.02
AMP	0.016 ± 0.001	0.039 ± 0.002
Arginine	1.1 ± 0.1	1.61 ± 0.02
Asparagine	16.3 ± 1.0	10.8 ± 0.8
Choline	0.032 ± 0.004	0.102 ± 0.014
Fructose	70.4 ± 1.3	38.1 ± 0.6
GABA	1.05 ± 0.05	2.4 ± 0.1
Glucose	65.4 ± 2.0	35.7 ± 1.1
Isoleucine	0.097 ± 0.007	0.287 ± 0.009
Lactic acid	0.11 ± 0.01	0.61 ± 0.02
Leucine	0.177 ± 0.012	0.467 ± 0.028
Malic acid	16.6 ± 1.3	6.9 ± 0.2
Methanol	0.19 ± 0.01	1.08 ± 0.11
Myoinositol	4.8 ± 0.2	3.2 ± 0.3
Phenylalanine	0.17 ± 0.01	0.28 ± 0.01
Proline	0.58 ± 0.02	1.79 ± 0.04
Scyllo-inositol	0.67 ± 0.01	0.96 ± 0.02
Stachydrine	0.59 ± 0.02	0.69 ± 0.025
Threonine	0.27 ± 0.02	0.74 ± 0.02
Tryptophan	0.057 ± 0.006	0.13 ± 0.01
Tyramine	0.019 ± 0.001	0.044 ± 0.003
Tyrosine	0.013 ± 0.001	0.044 ± 0.004
Valine	0.29 ± 0.02	0.55 ± 0.02

Several cultivars of Sicilian eVOOs have been certified with the Protected Designation of Origin (PDO) certificate by the European Commission such as Valdemone, Valle del Belice, Valli Trapanesi, and Val di Mazara. We aim to study their peculiar characteristics in terms of fatty acids concentration and of minor compounds such as terpenes and aldehydes [47].

We have studied the fat composition of several eVOOs produced in Sicily by means of proton HR-MAS NMR, by means of two different but almost equivalent NMR methodologies (based on peak integration) described in Section 2.4. We obtained essentially the same results, for both methods (NMR1 and NMR2) that are reported in Table 3 and compared with the corresponding values obtained by means of Gas Chromatography on some of them [48]. The results are very promising and confirm that ^{1}H NMR spectroscopy can be considered a very useful tool for assessing virgin olive oil quality and genuineness [49].

Furthermore, we investigate the possibility to discriminate between samples coming from different Sicilian provinces. To this purpose, we executed the PCA analysis on the processed spectra and the corresponding score plot is reported in Figure 7. Even though there is no clear distinction between different Sicilian regions, being the results superimposed to each other, the two samples grown in the province of Trapani, highlighted with an orange ellipse, belong to two Spanish cultivars: Arbosana and Arbequina.

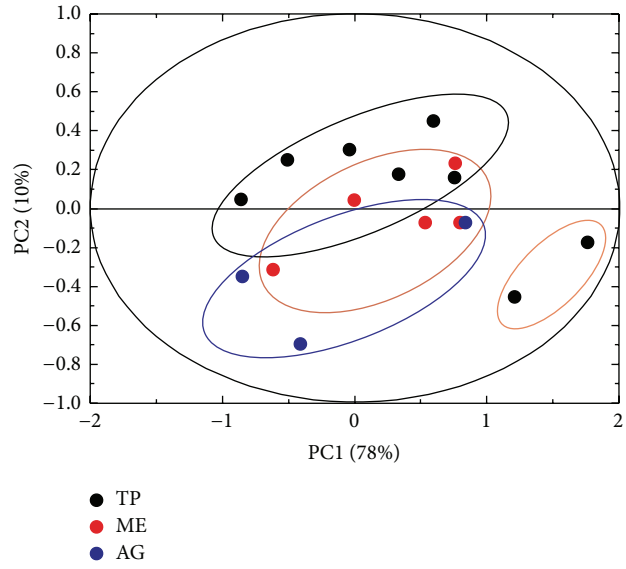

Figure 7: Score plot of the PCA analysis performed on Sicilian eVOOs samples. The results from different Sicilian regions are superimposed to each other; however the orange ellipse on the bottom right side refers to two Spanish cultivars grown in the province of Trapani: Arbosana and Arbequina.

These two samples show a minor concentration of oleic acid and a major concentration of saturated fatty acids with respect to the original Sicilian cultivars. It is noteworthy that oleic acid is considered to be antithrombotic compared with saturated fatty acids [45]. Furthermore, Arbosana and Arbequina eVOOs possess a minor amount of squalene as pointed out by Figure 8 where the comparison between the spectra of Arbequina and Valle del Belice cultivars is reported. Indeed in Figure 8 the expansion on the left side (green arrow) shows that even though the peak at about 2.83 ppm (representing the total amount of fatty acids) (see Methods Section 2.4) has the same intensity for the two cultivars, that at about 2.02 ppm is more intense for the Valle del Belice sample rather than for Arbequina. Moreover, the expansion on the right side (blue arrow) shows that the peak at about 2.83 ppm, corresponding to squalene, is more intense for Valle del Belice rather than for Arbequina. Squalene is a hydrocarbon and a triterpene involved in the synthesis of all plant and animal sterols. It is known that squalene assumption for olive oil consumption reduces the risk of cancer [50].

3.4. Red Garlic of Nubia. Garlic (*Allium sativum L.*) and garlic supplements are consumed in many cultures for their healthy effects. Since the ancient times, garlic was consumed as a remedy for different alimentary disorders and infections [51]. In fact, in literature, there are many studies that investigate garlic preparations and their properties [52].

Table 3: Fatty acids percentage in Sicilian eVOOs determined by the two considered methods compared with that obtained by GC on some of them. See text for labels explanation.

Cultivar	Linolenic acid (E)			Linoleic acid (A)			Oleic acid (C)			Saturated acids		
	NMR1	NMR2	GC	NMR1	NMR2	GC	NMR1	NMR2	GC	NMR1	NMR2	GC
Arbequina (TP)	0.61	0.59	0.64	10.07	10.04	10.39	70.20	68.80	68.42	18.50	20.20	19.45
Arbosana (TP)	0.55	0.55	0.51	6.50	6.47	5.95	74.34	72.95	72.42	19.50	19.50	19.30
Dop Valle del Belice (TP)	0.91	0.93	\	8.65	8.59	\	76.45	75.65	\	14.66	13.88	\
FSI17 (TP)	1.15	1.14	1.08	8.28	8.24	7.92	75.77	75.13	72.36	15.32	14.35	16.02
Valli Trapanesi (TP)	0.57	0.56	\	8.69	8.68	\	78.09	77.69	\	12.83	12.51	\
Nocellara del Belice (TP)	0.73	0.72	\	9.53	9.34	\	77.73	75.94	\	14.01	13.27	\
Nocellara del Belice 2 (TP)	0.51	0.50	\	8.26	8.16	\	78.65	77.45	\	13.79	13.38	\
Nocellara del Belice 3 (TP)	0.56	0.56	0.55	8.94	9.44	9.80	77.20	77.10	76.70	13.30	12.90	12.95
Santagatese (ME)	0.76	0.73	\	9.77	9.45	\	78.61	75.84	\	14.17	13.24	\
Ogliarola Messinese (ME)	0.67	0.65	\	7.04	6.89	\	82.09	80.06	\	12.41	11.75	\
Acquedolci Santagatese (ME)	1.08	1.01	\	15.51	14.83	\	71.54	68.16	\	16.46	14.97	\
Dop Valdemone (ME)	0.59	0.58	\	9.17	9.10	\	79.23	78.43	\	11.71	11.31	\
Dop Valdemone 2 (ME)	0.94	0.93	\	8.49	8.34	\	79.65	77.99	\	12.69	11.80	\
Cerasuola (AG)	0.59	0.61	0.58	10.25	10.15	10.63	77.00	76.90	76.16	12.16	12.34	12.62
Biancolilla 1 (AG)	0.86	0.83	0.78	9.22	8.99	9.64	75.72	73.62	71.56	16.71	15.73	16.47
Biancolilla 2 (AG)	0.82	0.80	\	10.01	9.80	\	76.00	74.10	\	15.40	14.50	\

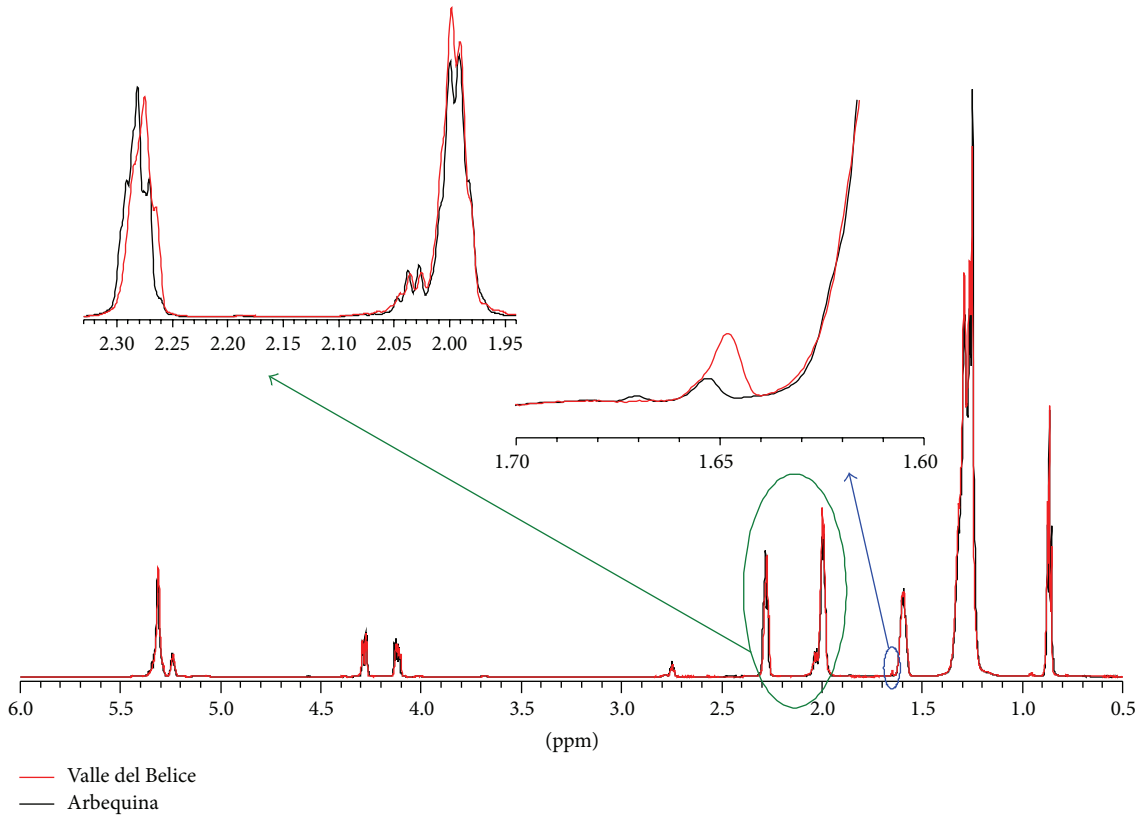

Figure 8: The comparison between the HR-MAS NMR spectra of Arbequina and Valle del Belice cultivars. The expansion on the left side (green arrow) shows that the peak at about 2.83 ppm has the same intensity for the two cultivars, whereas those at about 2.02 ppm and 2.83 ppm are more intense for the Valle del Belice sample rather than for Arbequina.

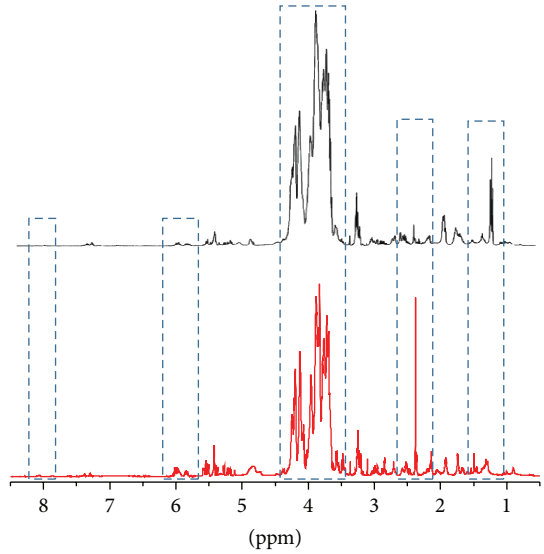

Figure 9: The comparison between the HR-MAS NMR spectra, of two different experiments on two red garlic samples: the red garlic of Nubia (red line, measured by us with a 700 MHz spectrometer) and the red garlic of Sulmona (black line, measured by Ritota et al. [16] with a 400 MHz spectrometer). The highlighted regions represent the most important similarities and differences.

The most important garlic compounds that have beneficial effects on human health are the organosulphur ones [52]. The peculiar garlic fragrance arises from allicin and other oil-soluble sulfur components. However, once garlic is cut or crushed, compounds in the intact garlic are converted into hundreds of organosulfur compounds in a short period of time. For example, alliin is converted to allicin by alliinase. Allicin is an effective antimicrobial agent that can be found in limited amounts only in freshly crushed garlic [53]. Another important allylic compound is S-Allyl Cysteine (SAC) that has a strong antifungal action and that seems to be highly present just in the red garlic of Nubia (see the region at about 6 ppm of NMR spectra in Figure 9).

We have determined the molar concentration of the main metabolites of the red garlic of Nubia by studying the one-dimensional proton spectrum obtained by means of HR-MAS NMR. The peak assignment was particularly difficult in the carbohydrates and allylic regions of the NMR spectra for the superposition of signals belonging to many similar chemical species. Our results confirm and extend those obtained by means of the same technique by Ritota et al. [16] on white and red Italian garlic. In Figure 9 we report the comparison between the HR-MAS NMR proton spectra of two different Italian red garlics: the red garlic of Nubia (measured by us with a 700 MHz spectrometer) and the red garlic of Sulmona (measured by Ritota et al. [16] with a 400 MHz spectrometer).

In the figure, we have highlighted five different chemical shift regions that are relevant for the comparison. The region centered at about 4 ppm belongs to carbohydrates and is similar for both spectra. The regions centered at about 6 and 8 ppm have instead a different intensity (higher for the Nubia sample) and correspond to allylic compounds and riboflavin, respectively. The major differences between the spectra of the two red garlics are showed in the first two regions. In particular, it is noteworthy that only in the spectrum of the red garlic of Sulmona (black line in Figure 9) there is a sharp triplet at about 1.23 ppm that the authors [16], together with a signal at about 3.95 ppm, assign to an unknown compound. We believe that these signals belong to diethylthiophosphate, that is, an organophosphorus compound widely used as pesticide because of easy degradation in the environment. On the other side, the peak at about 2.4 ppm, belonging to pyruvate, is much more intense in red garlic of Nubia with respect to the other red garlic considered. In the following table (Table 4), we report the molar concentration of the identified metabolites that were quantified by means of the above described procedures. We list only the metabolites whose concentration has a standard deviation less than 15%.

4. Conclusions

In this work, we have presented our studies, by means of the powerful NMR technique known as HR-MAS, on the characterization of some food products typical of the Mediterranean diet. In particular, we have analyzed the metabolic profile of the PGI cherry tomato of Pachino and we were able to identify few metabolites that can be considered for sample authentication. For example, in this protected foodstuff, we have found a higher concentration of GABA, sugars (fructose and glucose), glutamic compounds (glutamate and glutamine), and phenolic compounds (trigonelline, tyrosine, and tryptophan) with respect to non-Pachino cherry tomatoes. Furthermore, we have characterized the metabolic profile of juice from the PGI Interdonato lemon of Messina and compared it with that of the juice from the same hybrid cultivated in Turkey. We find for both hybrids high levels of sugars (sucrose, fructose, and glucose), citric acid, vitamin C, gallic acid, and inositols (mioinositol and scyllo-inositol).

The major source of fat in the Mediterranean diet comes from the consumption of eVOOs. For this reason, we have studied the fatty composition of several Sicilian cultivars (including few PDO samples) by means of two different methods both based on peaks integration. The results are consistent with those obtained by means of Gas Chromatography and confirm the power of NMR technique for quick quantitative chemical analysis. Moreover, we were able to discriminate between cultivars grown in the same province (Trapani) but coming from different nations (Italy and Spain), just for the different amount of oleic and unsaturated fatty acids and also for squalene content.

Finally, we have characterized the metabolic profile of the PAT red garlic of Nubia, quantifying the principal metabolites. In addition, we have compared its proton HR-MAS NMR spectrum with that of another Italian red garlic and have revealed that both garlics possess the same amount of carbohydrates. However, the red garlic of Nubia has a bigger amount of riboflavin, pyruvate, and allylic compounds. On the other side, only the red garlic of Sulmona shows a peak that could belong to diethylthiophosphate, a widely used pesticide.

Table 4: Average metabolites molar concentration of red garlic of Nubia.

Metabolite	mM
2-Phenylpropionate	0.9
4-Aminobutyrate	1.36
Alanine	0.65
Arginine	12.6
Ascorbate	1.2
Asparagine	10.3
Aspartate	0.88
Caprate	1.6
Choline	1.1
Citrate	3.03
Cystine	0.33
Ethanol	0.97
Formate	0.12
Fructose	15.4
Fumarate	0.049
Glucose	0.98
Glutamate	5.1
Glutamine	6.4
Glycine	4.7
Histidine	0.14
Isoleucine	0.37
Leucine	0.45
Lysine	1.02
Malate	1.45
Malonate	0.16
Maltose	0.4
Methanol	2.16
Methionine	0.33
Nicotinate	0.004
O-Phosphocholine	0.82
Pantothenate	0.19
Phenylalanine	0.11
Proline	1.5
Pyruvate	10.4
Riboflavin	0.87
Serine	30.1
Sucrose	21.0
Threonine	1.3
Thymine	0.25
Trigonelline	0.3
Tryptophan	0.06
UDP-glucose	0.073
Valine	0.63

In conclusion, the overall results allow appreciating the enormous potential of the used technique that is able to reveal and quantify a number of metabolites (characteristic of the particular food product condition), even on few amounts of samples and without any chemical treatment. The NMR technique is a rapid (few minutes of signal acquisition), nondestructive (no need of sample treatment), and reliable methodology to be used in an official method eventually in conjunction with other traditional analytical techniques such as GC. We want to stress that the consequence of insisting on NMR spectroscopy for food products characterization leads to the reduction of chemical consumption and waste production, which is important from both the economic and environmental points of view. All these characteristics also make the methodology very attractive for industry.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge the "NMR Center for Soft Matter CNR-UNIME" at the Department of Physics and Earth Sciences, University of Messina. Furthermore, they acknowledge the "Consorzio di tutela del Limone Interdonato di Sicilia IGP" and the academic spin-off of University of Messina Science4life for the scientific support.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 230170, 8 pages
http://dx.doi.org/10.1155/2015/230170

Research Article

Chemical Analysis of Suspected Unrecorded Alcoholic Beverages from the States of São Paulo and Minas Gerais, Brazil

Giuseppina Negri,[1] Julino Assunção Rodrigues Soares Neto,[2] and Elisaldo Luiz de Araujo Carlini[1]

[1]*Brazilian Information Center on Psychotropic Drugs (CEBRID), Department of Preventive Medicine, Federal University of São Paulo (UNIFESP), Botucatu Street 740, 04023-900 São Paulo, SP, Brazil*
[2]*Brazilian Information Center on Psychotropic Drugs (CEBRID), Department of Psychobiology, Federal University of São Paulo (UNIFESP), Botucatu Street 586, 04023-900 São Paulo, SP, Brazil*

Correspondence should be addressed to Giuseppina Negri; gnegri@terra.com.br

Received 1 December 2014; Revised 21 January 2015; Accepted 10 February 2015

Academic Editor: Shao-Nong Chen

Our study analyzed 152 samples of alcoholic beverages collected from the states of São Paulo and Minas Gerais, Brazil, using gas chromatography with flame ionization detection (GC-FID) and mass spectrometry (GC-MS), Fourier transform infrared spectroscopy (FT-IR), and inductively coupled plasma atomic emission spectrometry (ICP-AES). The methanol content varied from 20 to 180 ppm in 28 samples, and the limit of the accepted level of 200 ppm was exceeded in only one sample. High content of cyanide derivatives and ethyl carbamate, above the accepted level of 150 ppb, was observed in 109 samples. Carbonyl compounds were also observed in 111 samples, showing hydroxy 2-propanone, 4-methyl-4-hepten-3-one, furfural, and 2-hydroxyethylcarbamate as main constituents. Copper was found at concentrations above 5 ppm in 26 samples; the maximum value observed was 28 ppm. This work evaluated the human health risk associated with the poor quality of suspected unrecorded alcohols beverages.

1. Introduction

Public health problems caused by excessive consumption of alcoholic beverages are a serious concern in many countries [1], and Brazil is no exception [2–5]. This situation may worsen, due to the sale of unrecorded alcoholic beverages. According to World Health Organization [1] "unrecorded" is an overview category that includes any alcohol not taxed as alcoholic beverage or registered in the jurisdiction where it is consumed.

Cachaça is a beverage exclusive to Brazil, having an alcohol content of 38–48% v/v at 20°C; which is obtained by distilling fermented sugarcane juice and has particular sensory characteristics [6]. During the alcoholic fermentation of sugarcane with wild, unselected yeasts, ethanol and carbon dioxide are formed as major products [7, 8].

However, an excessive amount of by-products considered as contaminants may be present in alcoholic beverages from several countries, when the production process, such as distillation is not carried out in accordance with quality control [7, 9]. For example, high concentrations of ethyl carbamate (EC) [9–11] have been found in alcoholic beverages, especially in spirits derived from cyanogenic plants, such as cachaça derived from sugarcane [11] and tiquira from Cassava (*Manihot esculenta*) [4]. Cyanide derivatives can be formed through the thermal cleavage and enzymatic reaction of cyanogenic glycosides found in these plants [4, 9–11]. Other by-products found in cachaça are higher alcohols with 3–5 carbon atoms [12–14], ethyl esters [15], acetates [16, 17], organic acids [8], and carbonyl compounds, such as 5-hydroxymethylfurfural and furfural [6].

In Brazil, good manufacturing practices and quality control for alcoholic beverages are regulated by the Ministry of Agriculture, Livestock and Supply (MAPA). According to research carried out by Minas Gerais government, about 95% of alembics are informal; that is, 100 million among the

200 million liters of beverages produced in Minas Gerais are unrecorded [18]. According to a study carried out by Getúlio Vargas Foundation (FGV), it is estimated that the beverage consumed in Brazil was 13,6 billions of liters and the unrecorded beverages correspond to 20.3% [19].

Brazil ranks 84th in the Human Development Index (HDI: 0.718) [20]. Its total population in 2010 was 190,755.799 inhabitants, with a population density (PD) of 22.4 inhabitants/km^2 and a life expectancy of 73.48 years [20]. The city of São Paulo has a PD of 7,387.69 inhabitants/km^2 and a municipal human development index (HDI) of 0.841 [21], and Diadema has a PD of 12,519.10 inhabitants/km^2 and an HDI of 0.79 [21]. In Minas Gerais State, the selected cities have the following. Salinas has a PD/HDI: 20.75/0.699, Belo Horizonte has a PD/HDI: 7,167.02/0.839, Patrocínio has a PD/HDI: 28.69/0.799, Passa Quatro has PD/HDI: 56.21/0.832, and Itanhandu has PD/HDI: 98.87/0.795 [21].

The downside of the excessive alcohol consumption by Brazilians was observed in different individual and social aspects of the society, such as unemployment, increased violence, increase in venereal diseases, and AIDS. According to Nappo and Galduróz [22] the presence of alcohol was detected in the blood of 15.2% of the deceased persons examined at the Institute of Forensic Medicine from São Paulo city between 1987 and 1992. Unrecorded alcoholic beverages can be considered an aggravating factor to this problem. Thus, as a measure to reduce the harmful use of alcohol, one of the goals emphasized by the World Health Organization [1] is the need to reduce the impact of alcohol from informal origin on public health through the adoption of appropriate measures. Therefore, it is pertinent to carry out the chemical analyses of unrecorded beverages to protect the health of Brazilian people. This paper describes the results of chemical analyses of alcoholic beverages deemed unrecorded from the states of São Paulo (SP) and Minas Gerais (MG).

2. Experimental Procedures

2.1. Collection. Sixty-five samples of alcoholic beverages were collected from two cities in the state of São Paulo (SP), and 87 from five cities in Minas Gerais, from 2010 to 2012, for a total of 152 samples. In São Paulo state 25 samples were collected in São Paulo city and 40 in Diadema city. In Minas Gerais State 9 samples were collected in Salinas, 18 in Belo Horizonte, 22 in Patrocínio, 19 in Passa Quatro, and 19 samples in Itanhandu. They were collected from homes, from bars, and at parties. In Brazil there are many popular parties, such as "rodeio," "baladas," and "carnaval," where the unrecorded beverages have very high consumption. The criteria that drove the collection of the samples were the absence of labels or the absence of MAPA registration on the label, no tax seal, low price, or inadequate packaging.

This research was approved by Committee of Ethics in Research of UNIFESP (CEP N° 0195/12).

2.2. Chemical Analyses. The chemical analyses were carried out on 114 samples of cachaça, 18 of whiskey, 9 of liqueur, 7 of vodka, 2 of tequilas, 1 of wine, and 1 of beer. The methodologies used in these analyses were based on the AMPHORA (Alcohol Measures for Public Health Research Alliance) methodologies, which were developed for analyses of unrecorded alcohols [23–26].

2.2.1. Analysis of Methanol and Higher Alcohols Using Gas Chromatography with a Flame-Ionization Detector (GC/FID). The quantification of methanol and higher alcohols was performed by GC/FID [27] using a Shimadzu gas chromatography, GC-17A model, equipped with a DB 624 capillary column (30 m × 0.55 mm × 0.25 μm). The temperatures of the detector and injector were set at 250°C, the injection mode was set to a flow split of 1 : 25, and the injection volume was set to 1.00 μL of sample. The column oven temperature was programmed with an initial temperature of 40°C, which was maintained during 5 min. The temperature was increased to 250°C at a rate of 10°C/min and was kept for 10 min. Compounds were identified by comparing the retention times to those of analytical standards. The standard curves for methanol and higher alcohols were plotted using 10, 20, 40, 80, 100, 150, 200, and 250 μg of standard alcohols dissolved in 100 mL anhydrous alcohols as external standards. Chromatographic analysis of each standard solution was performed three times, and the calibration curve was plotted as the peak area of the chromatogram on the y-axis against that of the standard compound (μg) on the x-axis [26, 27]. The data points were fitted to a best-fit line using the linear regression method.

2.2.2. Analysis of Ethanol, Cyanide Derivatives, and Carbonyl Compounds Using Fourier Transform Infrared Spectroscopy (FT-IR). FT-IR spectra were recorded at room temperature (ca. 25°C) using a Bomem spectrometer by scanning over the frequency range 4000–400 cm^{-1} at a resolution of 5 cm^{-1}. Ethanol shows three bands: an intense band at 1046 cm^{-1} and two other bands of medium intensity, centered at 1086 and 879 cm^{-1}, respectively [23, 28]. The ethanol quantification was carried out using the analytical band at 1046 cm^{-1} (Figure 1) in the FT-IR. For the determination of ethanol content (percent by volume—%vol.), linear regression analysis of the relative peak absorption versus concentration for standard ethanol/water solutions with ethanol contents of 10%, 20%, 30%, 40%, 50%, and 60% was used in order to obtain calibration curves [23, 28]. Beverage alcoholic samples that showed turbid were filtered to prevent disturbances in the optical path length of the cuvette. Under the experimental conditions, the analytical signal increased linearly with the ethanol concentrations. The precision of the procedure was estimated by average concentration of three FT-IR determinations of each sample. The results among the three determinations for each sample did not exceed 1.1%. The results obtained using FT-IR were correlated with those obtained using GC-FID and showed similar values.

Cyanide derivatives and carbonyl compounds in alcoholic beverages show absorptions in the FT-IR spectra due to various functional groups [23, 28]. Thus, this analysis was also important for assessing the presence of carbonyl compounds and cyanide derivatives, which are precursors of EC.

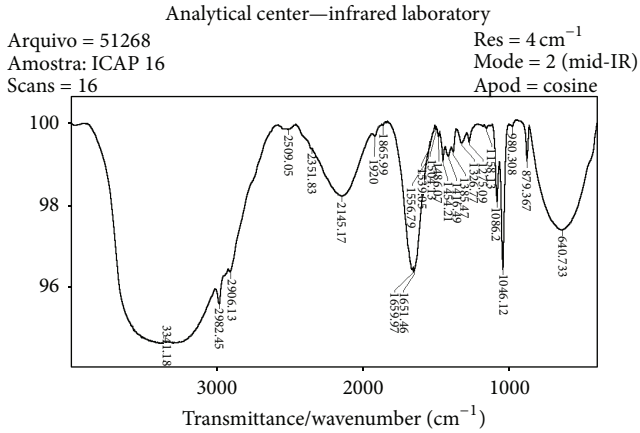

Figure 1: IR spectrum of a sample of Brazilian cachaça showing the main bands: at 3341–2906 cm^{-1} attributed to O–H stretching of alcohols, water, carboxylic acids, and amides; at 2145 cm^{-1} attributed to the asymmetric vibration of NCO^{-} in cyanate-copper complexes; at 1659–1651 cm^{-1} attributed to CO stretching vibrations conjugated with double bonds (C=C) adjacent to a carbonyl group; at 1086 and 1046 cm^{-1} attributed to stretching vibrations of hydrogen-bonded C–OH alcoholic groups.

2.2.3. Copper Analysis Using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The copper content was analyzed using Spectro Arcos SOP-FHS12 equipment. The samples were diluted with a 1% solution of nitric acid and ethanol (4%, v/v), which were added to the calibration solution [25–27]. The operating conditions were frequency 38 MHz; double diffraction net 352 line mm^{-1}; generator 1280 W; plasma was formed in a stream of argon gas of 13 L min^{-1}; and cone spray nebulizer pressure 58 psi.

2.2.4. Analysis of Carbonyl Compounds in Two Samples of Cachaça Using Gas Chromatography Mass Spectrometry (GC/MS). Two unrecorded beverage samples that showed high concentrations of carbonyl compounds were analyzed by GC-MS with the aim of identifying the carbonyl compounds that produced the band at 1651–1659 cm^{-1} (attributed to CO stretching vibrations conjugated with double bonds adjacent to a carbonyl group) in the FT-IR spectra (Figure 1). This analysis was carried out using a Shimadzu GCMS-QP505A gas chromatography coupled to a quadrupole mass selective spectrometer. The chromatographic conditions were an injection mode with a flow split of 1 : 25 with an injection volume of 1.00 μL of sample. The separation was performed using a DB 624 capillary column (30 m × 0.55 mm × 0.25 μm). The column oven temperature was programmed with an initial temperature of 40°C, which was maintained during 5 min. The temperature increased to 300°C at a rate of 10°C/min and was kept for 10 min. MS analyses were made in the electron impact (EI) mode (70 eV). Helium was used as the carrier gas with a flux of 1.5 mL min^{-1} and a split ratio of 100 : 1; linear velocity of 63 cm/sec, total flow 77.3 mL/min., and solvent cut time of 3.0 min. The MS conditions were filament current, 0.3 mA; detector voltage, −0.7 kV, ion source temperature, 300∘C; interface temperature, 300∘C; scan speed 2 scans s^{-1}. The mass range was from m/z 29–300 μ and the chromatogram was acquired in total ion current (TIC). The carbonyl compounds were identified through comparison of their mass spectra with those reported in the GC-MS computer database (Wiley 275, Wiley 229, and NIST 21) and literature data. Beside this, standard compounds, such as formic acid, acetic acid, and furfural were coinjected in order to confirm the identification of compounds. The determination of its relative amounts was based on the regions under the corresponding chromatogram peak.

Table 1: Number of samples among the 152 samples of alcoholic beverages collected from the states of São Paulo (number of samples 65) and Minas Gerais (number of samples 87), Brazil, which are unlabeled and did not display a MAPA registration number or tax seal.

Items considered	Samples from São Paulo (N = 65)	Samples from Minas Gerais (N = 87)
Missing label	44	49
MAPA registration number	12	1
Tax seal	0	24
Inadequate packaging	2	1
Low price	7	12

3. Results

Among the 65 samples collected from SP (Table 1), only 21 had labels. Furthermore, only 12 displayed a MAPA registration number. Similar results were obtained for the 87 samples collected from MG, all of which showed signs of being unrecorded, with 49 unlabeled. Only one sample displayed a MAPA registration number. There were no significant differences between the results obtained from the two states. Therefore, all data were pooled, and only those for which concentrations exceeded the legally admissible limits were emphasized.

3.1. Methanol and Higher Alcohols Analyzed by GC/FID. Methanol was quantified in only 54 of the 65 samples collected in SP, due to impurities present in the remaining samples, methanol, around 80 ppm, was detected in three cachaça and one liqueur samples. The wine sample showed a methanol content of 240 ppm, which is above the legal limit of 200 ppm (Table 2). In MG, methanol was detected in 24 samples, of which 19 were cachaça with levels ranging from 42 to 169 ppm, one vodka (180 ppm), one tequila (120 ppm), and three whiskey samples, one with 26 ppm and two with 79 ppm (Table 2), all below the legal limit of 200 ppm.

In the samples collected from SP, the higher alcohols found are 2-butanol, *n*-butanol, *n*-propanol, and isoamyl alcohol, with levels ranging from 2 to 40 ppm, in 47 of the 54 samples analyzed. In MG, 2-butanol, *n*-butanol, *n*-propanol, and isoamyl alcohol were found in 84 of the 87 samples with

Table 2: Results obtained in chemical analyses carried out in 65 samples of alcoholic beverages from state of São Paulo* and 87 from state of Minas Gerais, Brazil. Methanol content (limit 200 ppm), ethanol content in percentage by volume (38% to 48%), and copper (limit 5 ppm).

States	Number of samples	Methanol (ppm)			Ethanol (%)			Copper (ppm)	
		<20	20 to 180	>240	>38	20% to 38%	5% to 20%	<5	5 to 28
São Paulo	65*	49	4	1	32	11	22	54	11
Minas Gerais	87	63	24	0	36	13	38	72	15

*For analyses of methanol content in samples from São Paulo State, among the 65 samples, 11 were not analyzed, due to impurities.

Table 3: Carbonyl compounds identified through GC-MS analysis in two Brazilian cachaça samples.

Retention time	EI-MS data (m/z)* (percentage)	Proposed structure	Peak percentage**
2.2	46 (100), 45 (80)	Formic acid	2%/3%
2.6	60 (80), 45 (100), 43 (90)	Acetic acid	4%/2%
2.8	74 (1), 43 (100), 31 (40)	Hydroxy 2-propanone	4%/3%
11.2	96 (20), 95 (100), 39 (60)	furfural	4%/6%
12.6	126 (1), 97 (90), 69 (60), 41 (100), 39 (80)	4-Methyl-4-hepten-3-one	32%/29%
15.43	105 (1), 73 (60), 60 (100), 43 (70)	2-Hydroxyethylcarbamate	19%/40%
16.5	126 (1), 97 (5), 82 (50), 81 (100), 53 (50), 39 (40)	5-Hydroxymethyl furfural (5-HMF)	28%/6%

*Data obtained (molecular ions and fragments) by mass spectrum analysis.
**Percentages determined by peak areas in the chromatograms.

levels also ranging from 2 to 40 ppm, which is below the legal limit of 360 ppm.

3.2. Ethanol, Cyanide Derivatives, and Carbonyl Compounds Analyzed by FT-IR. Ethanol content reported as the "percent by volume" (% vol) was determined using two different methods GC-FID and FT-IR [23–28]. However, as GC/FID and FT-IR showed similar yields, the results of ethanol content were based on the FT-IR spectroscopy data. Ethanol shows three bands: an intense band at 1046 cm^{-1} and two other bands of medium intensity, centered at 1086 and 879 cm^{-1}, respectively. These bands are due to vibrational transitions of the C–O–H system: C–O stretching vibration (p-OH) and O–H bending vibration out of plane [25, 29]. The ethanol quantification was carried out using the analytical band at 1046 cm^{-1} (Figure 1) in the FT-IR. Only 32 out of 65 samples collected from São Paulo had ethanol contents above 38% (Table 2), which are in accordance with the legal limit. In 11 samples, the ethanol content was lower than 38% (20% to 38%), while in the other 22 the ethanol content varied from 5% to 20% (Table 2). In MG, 36 of the 87 samples analyzed contained an ethanol content above 38%, in 13 samples less than 38% (20% to 38%) was detected, while for the other 38 samples, the ethanol content varied from 5% to 20%.

The presence of ethyl carbamate (an ester of carbamic acid) was observed through its cyanide derivatives precursors, the hydrocyanic acid (HCN) and cyanic acid (HCNO and its tautomeric form HOCN). The FT-IR spectra of various samples of unrecorded beverages showed a large band in 2145 cm^{-1} that was attributed to the asymmetric vibration of the cyanate $(NCO)^-$ in the cyanate-copper complexes [10, 11, 29, 30], as can be seen in Figure 1. According to Baffa Junior et al. [10], the formation of EC from HCN is based on the complexation of HCN to copper, followed by its oxidation to HCNO, which reacts with ethanol to form EC. Copper acts as an important catalyst in the conversion of HCN into EC in cachaça [29, 30].

In SP, 24 of the 65 tested samples showed the presence of cyanide derivatives, while in MG, cyanide derivatives were present in 85 out of 87 samples. It is interesting to mention that among the samples from SP, the presence of cyanide derivatives was observed only in cachaças mixed with plants used in folk medicine of Brazil, whereas in the samples from MG, this contaminant was found in almost all samples, including whiskey and vodka. In SP, 26 out of the 65 samples tested showed a high content of carbonyl compounds, while in MG, a high content of carbonyl compounds was present in 85 out of 87 samples.

3.3. Copper Content Using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). In the samples of cachaça from SP, the copper content ranged from 1.0 to 28.0 ppm, with 11 samples exhibiting copper contents ranging from 5.0 to 28.0 ppm, upper limit 5.0 ppm. Out of these, only one with a copper content of 7.72 ppm displayed the MAPA registration number. In samples from MG, the copper content ranged from 1.0 to 26.0 ppm, with 15 samples exhibiting copper contents ranging from 5.0 to 28.0 ppm (Table 2). This is different from the results for the whiskey, tequila, and vodka samples, with average within the normal level, lower than 5 ppm.

3.4. Analysis of Carbonyl Compounds in Two Samples of Cachaça Using GC-MS. Carbonyl compounds present in cachaça were identified in the analyses of two samples of cachaça, which showed a high content in FT-IR analyses, using GC-MS, and the results are shown in Table 3. The carbonyl compounds were identified through comparison of their mass spectra with those reported in the GC-MS computer database (Wiley 275, Wiley 229, and NIST 21),

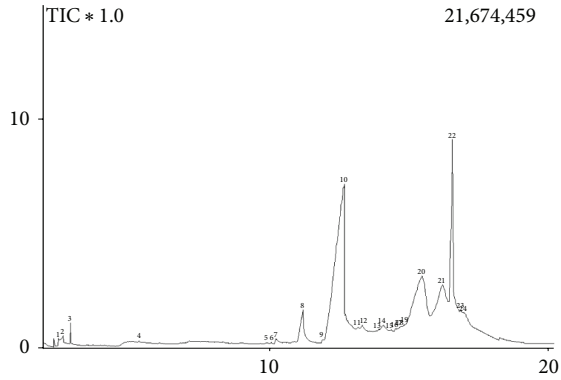

Figure 2: Total ion chromatogram obtained in the analyses of cachaça sample from Minas Gerais through GC-MS. The numbers corresponding to the following compounds. 1: formic acid. 2: acetic acid. 3: hydroxy 2-propanone. 8: furfural, 10: 4-methyl-4-hepten-3-one. 20: 2-hydroxyethylcarbamate, 22: 5-hydroxymethyl furfural (5-HMF).

standard compounds, such as formic acid, acetic acid, and furfural and literature data [31]. The obtained chromatogram is shown in Figure 2. The Mass Spectrometry analyses were carried out in the electron impact (EI) mode (70 eV). The main constituent found in these two cachaças samples was proposed as being 4-methyl-4-hepten-3-one, which showed a molecular ion at *m/z* 126 ($C_8H_{14}O$) (Table 3). The presence of this unsaturated ketone was corroborated by the band at 1656 cm^{-1} observed in FT-IR, as can be seen in Figure 1. The presence of the 5-hydroxymethylfurfural (5-HMF) was detected mainly through the base peak at *m/z* 81, which was attributed to the ion $(C_5H_5O)^+$ formed by furan ring with a methyl group, through comparison with literature data [32].

The presence of 2-hydroxyethylcarbamate [11, 33] was observed through the molecular ion at *m/z* 105 and base peak at *m/z* 60, which could be attributed to the ion (CH_2NO_2) indicating the presence of carbamate moiety. Carbamates derivatives had been studied through Tandem mass spectrometry measurement [33].

4. Discussion

The unrecorded beverages have attracted the attention of WHO, due to the presence of compounds such as methanol and ethyl carbamate and its precursors, furfural, 5-hydroxymethylfurfural, acrolein, and other toxic compounds. There have been discussions on whether or not these substances can produce serious illness effects in humans [24, 25, 34, 35]. Many studies were carried out to evaluate the chemical composition of unrecorded alcohol from Nigeria, Lithuania, Hungary, Poland, Guatemala, Vietnam, and Brazil, with the aim to investigate the possible health impact of unrecorded alcohol [24, 25, 34, 35]. In Brazil, Nagato et al. [36] carried out the analyses of methanol, ethanol, and higher alcohol content in 608 samples of alcoholic beverages confiscated by the police, from 1993 to 1999. Among them, 391 were counterfeit, being the ethanol content below the accepted levels in all of them. In two of the samples, high methanol content (14 g/100 mL and 10 g/100 mL) was detected, which is much above the accepted level of 0.02 g/100 mL.

In our study, the contamination with methanol, higher alcohols, and copper was analyzed in 152 samples of unrecorded alcoholic beverages from SP and MG. Beside this the contamination with cyanide derivatives and carbonyl compounds, as well as the content of ethanol, was also evaluated. A similar extensive study was carried out in Russia involving 81 samples of unrecorded alcohol [37].

As shown in Table 1, the missing labels, the absence of tax seal, MAPA registration number, inadequate packaging, and low price indicated that the samples collected show clear evidence of being unrecorded, which was corroborated by the results of chemical analysis (Tables 2 and 3). Many of the beverages exhibited ethanol contents lower than 38%, which was also observed by other authors [6–8, 38] in the analyses of Brazilian cachaça. Low alcohol levels could be related to the conditions during storage of the beverages, such as the temperature, humidity, and porosity of the barrel [6–8, 38].

Methanol is the most toxic alcohol, with documented cases of poisonings, including optic nerve damage and fatal intoxications [8, 38, 39]. In our sample, the methanol levels were below the 200-ppm limit for all unrecorded beverages, except one wine sample, which is consistent with several analyses of cachaça samples collected from southern Minas Gerais and other regions of Brazil [6–8, 38, 39]. The incidence of methanol poisoning was infrequent but had received high media exposure [24–27]. Higher alcohols have also been found in low levels as reported by other authors [12]. Higher concentrations of methanol, isobutanol, 1-propanol, and isoamyl alcohol were found in illicitly distilled spirits from Hungary [35]. High amounts of these alcohols could cause hepatic damage, contributing to the high level of alcohol-induced liver cirrhosis [40].

Ethyl carbamate (EC) is a carcinogen (group 2A), and its legal limit is 150 ppb [11]. EC is formed from the reaction of ethanol and compounds containing carbamoyl groups. The main EC precursors are commonly generated from arginine metabolism by *Saccharomyces cerevisiae* or lactic acid bacteria, which is accompanied by the fermentation process [10, 11, 41, 42]. High EC content, reaching levels as high as hundreds of micrograms per liter, was detected in Brazilian sugarcane spirits [42]. Such high concentrations were found in samples from the state of Minas Gerais and Pernambuco in many authors [10, 11, 29, 30, 41, 42].

In destillation process of cachaça, the head fraction comprises compounds such as methanol, acetaldehyde, and ethyl carbamate with more solubility in ethanol than in water. The heart fraction comprises mainly ethanol and higher alcohols, while the tail fraction comprises compounds such as acetic acid and 5-hydroxymethylfurfural (HMF), which are less volatile than ethanol [43]. In Brazil, most producers used direct-fire alembic, in which the heat produced by burning sugarcane bagasse reaches temperatures near 100°C. In this temperature, the fermented sugarcane formed an azeotropic mixture consisting of ethanol and water, whose boiling point is below 100°C [43]. Although this temperature is below the EC boiling point (186°C), it does not prevent

that cyanide derivatives, hydroxymethylfurfural, furfural, and ethyl carbamate will be carried out to the distillate [11, 43]. In addition, this system used to heat the stills does not provide a constant rate of heat transfer [11].

The contamination with ethyl carbamate was also found in many spirits from Hungary, Poland, and other European countries [24–27, 34, 35]. This problem occurs when the homemade beverages are produced from fruit materials or medicinal plants that contained cyanogenic glycosides, without the application of measures to prevent this contamination [24–27]. Cachaça distilled in alembics contains a higher content of EC, due to the dissolution of the basic copper carbonate from the inner wall of the still by the acid vapors formed, during the distillation process, which cause the corrosion and release of copper into the beverages, favoring EC formation [6, 11, 42]. Generally, sugarcane spirits produced in pot still distillation had lower EC values than those from continuous distillation columns [11].

Cachaça may contain considerable concentrations of As, Pb, and Cu, which are harmful for human health if ingested in high quantities. Cachaça is distilled in copper stills and copper contamination can take place. High accumulation of copper in the organism could cause Wilson's disease [44] and consequently toxic effects, because copper, is not excreted by the liver. This disease, if untreated, can lead to brain and liver damage. Copper catalyzes the formation of cyanate, which react with ethanol to form ethyl carbamate [10, 11]. The Brazilian law establishes a limit for copper content of 5 ppm, prohibits the use of copper stills for the production of alcoholic beverages, and requires the use of stainless steel stills. Our study found 26 cachaças (11 from SP and 15 from MG) with high copper levels (Table 2), consistent with the results obtained by other authors [6, 11, 29, 30].

Various aldehydes, formaldehyde, acetaldehyde, propionaldehyde (acrolein), furfural, and 5-hydroxymethylfurfural (5-HMF), together with formic acid and acetic acid, are obtained as by-products during the production of cachaça [6, 24–27, 38]. 5-HMF is cytotoxic in high concentrations and irritating to the eyes, upper respiratory tract, skin, and mucous membranes [45]. As far as we know among the compounds listed in Table 3, hydroxy 2-propanone, 4-methyl-4-hepten-3-one, and 2-hydroxyethylcarbamate were not previously reported in cachaça.

5. Conclusion

This study revealed the following facts. (1) In the Brazilian market, alcoholic beverages are present without MAPA registration number, unlabeled, and with suspiciously low price. These unrecorded alcoholic beverages are certainly acquired and consumed by the population. (2) Many of these unrecorded beverages have low alcohol content and consequently high water content. (3) Chemical assays showed the presence of various highly toxic contaminants, mainly cyanide derivatives. Therefore, the consumption of these unrecorded alcoholic beverages may be an aggravating factor that adversely affects the health of Brazilians people. (4) Adequate sanitary and legal measures should be taken to correct these problems. (5) More severe quality control of beverages should be carried out according to WHO. (6) Further, the population should be informed about the risk of consumption of unrecorded beverages.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

The authors wish to express their sincere thanks to UNIFESP, CEBRID, and ICAP (International Center for Alcohol Policies) for financial support. They also declare that they did not receive personal payment for their work and are the only persons responsible for the content wrote in this paper.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 102125, 10 pages
http://dx.doi.org/10.1155/2015/102125

Research Article

Determination of Polyphenols, Capsaicinoids, and Vitamin C in New Hybrids of Chili Peppers

Zsuzsa Nagy,[1] Hussein Daood,[2] Zsuzsanna Ambrózy,[1] and Lajos Helyes[1]

[1] *Institute of Horticulture, Faculty of Agriculture and Environmental Sciences, Szent István University, Páter Károly Street 1, Gödöllő 2100, Hungary*
[2] *Regional Knowledge Centre, Szent István University, Páter Károly Street 1, Gödöllő 2100, Hungary*

Correspondence should be addressed to Zsuzsa Nagy; nagy.zsuzsa@mkk.szie.hu

Received 8 April 2015; Revised 9 July 2015; Accepted 27 July 2015

Academic Editor: Shao-Nong Chen

Six hybrids were subjected to chromatographic analyses by HPLC for the determination of phytochemicals such as capsaicinoid, polyphenol, and vitamin C. The dynamics of ripening of 4 of the hybrids were also characterised. Seven capsaicinoids could be separated and determined; the major compounds were nordihydrocapsaicin, capsaicin, and dihydrocapsaicin, while homocapsaicin and homodihydrocapsaicin derivatives were detected as minor constituents. Capsaicin content ranged between 95.5 ± 4.15 and 1610.2 ± 91.46 μg/g FW, and the highest value was found in Bandai (*C. frutescens*) at the green ripening stage. The major capsaicinoids had a decreasing tendency in Bandai and Chili 3735 hybrids, while no change was observed in Beibeihong and Lolo during ripening. Nine polyphenol compounds were detected including 8 flavonoids and a nonflavonoid compound in the pods of all hybrids. The major components were naringenin-diglucoside, catechin, and vanillic acid-derivative and luteolin-glucoside. Naringenin-diglucoside ranged from 93.5 ± 4.26 to 368.8 ± 30.77 μg/g FW. Except vanillic acid-derivative, dominant polyphenols increased or remained unchanged during ripening. As for vitamin C, its content tended to increase with the advance in ripening in all hybrids included in this study. The highest value of 3689.4 ± 39.50 μg/g FW was recorded in Fire Flame hybrid.

1. Introduction

The components evolving pungency in chili peppers have been established as a mixture of acid amides of vanillylamine and C8 to C13 fatty acids, also known as capsaicinoids [1]. Capsaicinoids are secondary metabolites and are synthesised by glands at the join of the placenta and the pod wall of pungent peppers [2]. The effect of capsaicinoids on human health has been widely investigated. For instance, it is beneficial in low concentration against gastric injuries [3], stimulates cation channels (Na^+, K^+, and Ca^{2+}) in sensory receptor membrane [4], evokes pain, and activates autonomic reflexes [5]. Environmental factors and the circumstance of cultivation influence capsaicinoid content of the pods [6, 7], while probably a higher impact on pungency by the genotype is present [8, 9]. Besides, the amount and proportion of capsaicinoids are changing during the ripening process of the pods [10–13]. Flavonoids represent a significant subgroup of polyphenols [14] and naturally occur in high concentration in wild mint [15] and grape [16] while generally pungent peppers have moderate level of polyphenol content. The health protective attributions of them are mainly associated with preventing cancer through inhibiting certain enzymes and suppressing angiogenesis [17]. The polyphenol content in pungent peppers is found to be influenced by genotype and the ripening process [18–20]. Ascorbic acid, the main component of vitamin C, is very abundant in fresh *Capsicum* species and has been found to be beneficial in maintaining collagen synthesis and healthy immune-system and also has antitumor properties [21–23]. The content of ascorbic acid is highly varying among cultivars and ripening stages [24, 25]; in addition, the utilised agricultural techniques play significant role in the final amount of ascorbic acid in the pods [26].

Numerous cultivars of pungent pepper are nowadays available; however, many of them have not been analysed for their quality and nutritional components. The objective of the present work is to investigate capsaicinoid, polyphenol, and vitamin C content in six hybrids of chili pepper (Bandai,

Beibeihong, Lolo, Chili 3735, Fire Flame, and Star Flame) using recently developed liquid chromatographic method in the determinations. In addition, characterisation of ripening stages of four hybrids was aimed.

2. Material and Methods

2.1. Plant Material. The plants were cultivated with convention horticultural practices in the experimental field of Szent István University, Gödöllő, Hungary. Bandai F_1 (Bandai) and Beibeihong 695 F_1 (Beibeihong) which belong to *Capsicum frutescens* and Lolo 736 F_1 (Lolo) and Chili 3735 F_1 (C3735) which belong to *Capsicum annuum* were all purchased from East-West Seeds Company, from Thailand, while Star Flame and Fire Flame (both *Capsicum annuum*) were purchased from Seminis, Hungary. The pods of Bandai, Beibeihong, Lolo, C3735, and Fire Flame are red when fully ripe, while Star Flame has vivid yellow pods. Peppers with intermediate pungency level were selected for the investigation because those have multiple utilization methods. Those peppers involved in the recent study have limited data available for breeders and growers; thus, it makes them important for research work. Star Flame and Fire Flame are commercially available in certain European countries but not yet in Hungary.

2.2. Capsaicinoid Determination. The determination of capsaicinoid content was made following the method of Daood et al. [27]. Three grams of well-blended pepper sample were crushed in a crucible mortar with quartz sand. To the macerate 50 mL of methanol (analytical grade) was added and the mixture was then transferred to a 100 mL Erlenmeyer flask. The mixture was subjected to 4 min long ultrasonication (Raypa, Turkey) and then filtered through filter paper (Munktell, Germany). The filtrate was more purified by passing through a 0.45 mm PTFE syringe filter before injection on the HPLC column.

After suitable dilution, the extract was injected to Nucleodur C18, Cross-Linked (ISIS, from Macherey-Nagel, Düren, Germany). The separation was performed with isocratic elution of 50 : 50 water-acetonitrile and a flow rate of 0.8 mL/min. Fluorometric detection of capsaicinoid was carried out at EX: 280 nm and EM: 320 nm.

Peaks referring to different capsaicinoids were identified by comparing retention times and mass data (Daood et al. [27]) of standard material (purified from pungent red pepper, with 99% purity, by Plantakem Ltd., Sándorfalva, Hungary) with those appearing on chromatogram of samples. Capsaicinoid compounds are referred as follows: nordihydrocapsaicin (NDC), capsaicin (CAP), dihydrocapsaicin (DC), homocapsaicin 1-2 (HCAP1-2), and homodihydrocapsaicin 1-2 (HDC1-2). Scoville heat unit (SHU) was calculated by the following algorithm:

$$\begin{aligned}(\mathrm{CAP}\times 16,1)+(\mathrm{DC}\times 16,1)+(\mathrm{NDC}\times 9,3)\\ +[(\mathrm{HCAP1}+\mathrm{HCAP2})\times 8,6]\\ =\text{Scoville heat unit.}\end{aligned}\quad(1)$$

All variables are expressed in μg/g dry weight basis [28].

2.3. Polyphenol Determination. Five grams of well-blended pepper sample were replaced into an Erlenmeyer flask and then 10 mL distilled water was added to the sample and subjected to ultrasonication force using ultrasonic bath for 30 sec. Then, 15 mL of 2% acetic acid in methanol was added to the mixture which was shaken by a mechanical shaker for 15 min. The mixture then was kept overnight at 4°C. Next day after filtrating the mixtures, a further cleanup ofthe filtrates was made by passing through the mixture a 0.45 μm PTFE HPLC syringe filter. That followed by injection on the HPLC column for the analysis of phenols. Nucleosil C18, 100, Protect-1 (Macherey-Nagel, Düren, Germany), 3 μm, 150 × 4.6 column was used. The gradient elution was done using 1% formic acid (A) in water, acetonitrile (B), and flow rate of 0.6 mL/min. Gradient elution started with 98% A and 2% B and changed in 10 min to 87% A and 13% B and in 5 min to 75% A and 25% B and then in 15 min to 60% A and 40% B; finally it turned in 7 min to 98% A and 2% B. The peaks that appeared on the chromatogram were identified by comparing their retention times and spectral characteristics with available standards such as catechin, quercetin-3-glucoside, kaempferol, luteolin-glucoside, and naringenin-glucoside (Sigma-Aldrich Ltd., Hungary). Quantitation of phenol components having maxima absorption at 280 nm were quantified as catechin equivalent and flavonoids were quantified as kaempferol-equivalent at 350 nm [29, 30]. The standard material was singly injected as external standard as well as being cochromatographed (spiking) with the samples.

2.4. Ascorbic Acid Determination. Five grams of well-homogenised sample were disrupted in a crucible mortar with quartz sand. To the macerate 50 mL of metaphosphoric acid (analytical grade) was gradually added and the mixture was then transferred to a 100 mL Erlenmeyer flask closed with stopper and then filtered. The filtrate was purified in addition by passing through a 0.45 mm PTFE syringe filter before injection on HPLC column. The analytical determination of ascorbic acid was performed on C18 Nautilus, 100-5, 150 × 4.6 mm (Macherey-Nagel, Düren, Germany) column with gradient elution of 0.01 M KH2PO4 (A) and acetonitrile (B). The gradient elution started with 1% B in A and changed to 30% B in A in 15 min; then; it turned to 1% A in B in 5 min. The flow rate was 0.7 mL/min. The highest absorption maxima of ascorbic acid under these conditions were detected at 265 nm. For quantitative determination of ascorbic acid standard materials (Sigma-Aldrich, Budapest, Hungary) were used. Stock solutions and then working solutions were prepared for each compound to make the calibration between concentration and peak area.

2.5. HPLC Apparatus. A Hitachi Chromaster HPLC instrument, which consists of a Model 5110 Pump, a Model 5210 Auto Sampler, a Model 5430 Diode Array detector, and a Model 5440 Fluorescence detector, was used for the determination of all compounds.

2.6. Validation of Applied Methods. Since the methods used in the different chromatographic determinations are derived

Table 1: Some validation parameters for the HPLC determinations of the major polyphenols, ascorbic acid, and capsaicinoids.

	LOD μg/mL	LOQ μg/mL	Linearity range μg/mL	Linearity curve	R^2
Catechin	2.625	8.75	0–50	$y = 0.331x - 2.5895$	0.899
Naringenin-diglucoside	0.0318	0.106	0–50	$y = 0.3906x - 3.0556$	0.899
Quercetin-3-glucoside	1.083	3.61	0–50	$y = 0.2188x - 0.781$	0.983
Luteolin-glucoside	1.018	3.39	0–50	$y = 0.1912x - 0.381$	0.979
Kaempferol-derivative	0.0208	0.069	0–50	$y = 0.4402x - 3.444$	0.899
Ascorbic acid	2.500	0.750	30–120	$y = 0.2736x - 2.4305$	0.997
Nordihydrocapsaicin*	0.003*	0.008*	0–0.07–1.1*	$y = 2000 + 07x + 3000 + 06$*	0.997*
Capsaicin*	0.004*	0.01*	0.1–5*	$y = 2000 + 07x + 3000 + 06$*	0.998*
Dihydrocapsaicin*	0.002*	0.007*	0.3–6*	$y = 2000 + 07x + 3000 + 06$*	0.998*

*From previously published research work on HPLC determination of capsaicinoids by Daood et al. [27].

from the literature (validated protocols) we dealt with only measuring the limit of detection (LOD) and quantification (LOQ) and linearity curves of different compounds under the conditions of our laboratories. The LOD and LOQ were calculated from standard solutions and samples as the concentrations of analytes at peak/noise of 3 times and 10 times, respectively. Linearity curves were made plotting concentration in μg/mL against peak areas.

2.7. Dry Matter Determination. Three grams of fresh pepper samples were dried at 65°C until constant weight. The dry matter content was measured as a proportion of fresh and dried fruit weight.

2.8. Statistical Analyses. For each independent variable a one-way linear model (LM) was fitted, where "ripening stage" was set as explanatory (factor) variable. Prior to model fitting assumptions were checked by plot diagnosis. In the analysis of the major compounds (SHU, CAP, naringenin-diglucoside, ascorbic acid, and dry matter) among the six hybrids another LM was made, where "hybrid" was set as explanatory (factor) variable. Post hoc comparison was made by Tukey HSD test. All statistical analyses were performed in IBM SPSS 22 software (IBM Co., USA) and Microsoft Excel (Microsoft Co., USA). α was set at 0.05 in the entire study.

3. Results and Discussion

To adapt the applied chromatographic protocols under the conditions of our laboratories, certain parameters such as LOD, LOQ, and linearity curve were studied. The values depicted in Table 1 show that the used methods are accurate enough to carry on precise and sensitive determination of polyphenols, capsaicinoids, and ascorbic acid. This statement is based on the low levels of LOQ, LOD found for all tested compounds. The concentration of such compounds in our samples is much higher than the levels of LOQ and LOD. Moreover, values obtained for regression coefficient indicated that the methods can be applied at wide range of concentrations for different compounds in chili samples.

3.1. Pungency. The major components evolving pungency in our hybrids are NDC, CAP, and DC. Besides, we could identify the homologues of CAP and DC which are HCAP1, HCAP2 and HDC1, HDC2, respectively (Figure 1). All of them are branched-chain alkyl vanillylamides. Kozukue et al. [31] detected the 7 compounds, in addition to nonivamide which is a straight-chain nonoyl vanillylamide analog of CAP [1]. In Beibeihong advance in ripening did not affect the major capsaicinoids (CAP, NDC, and DC shown in Table 2), while it influenced HCAP1 and HDC1 (both $p \leq 0.032$) including a slight decrease from green to colour-break stage and then a low increase at the final stage. In Bandai, unlike Beibeihong the ripening affected the major and minor capsaicinoids as well (all $p \leq 0.027$). The changing of CAP included a notable decrease between the initial stage and the colour-break stage. On DC, NDC, and HDC2 a gradual decrease was measured. A straight increasing of HDC1 was observed, while on HPC1 the same tendency like that in HPC1 of Beibeihong was observed.

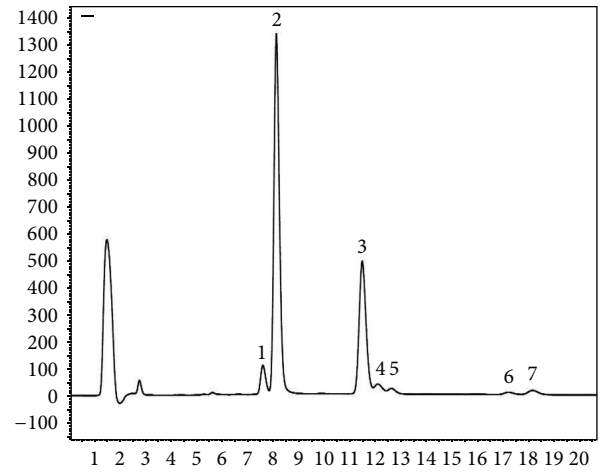

Figure 1: HPLC profile of capsaicinoid components separated from red stage of Bandai hybrid using cross-linked C18 column with acetonitrile-water elution and fluorescence detection. 1: NDC, 2: CAP, 3: DC, 4: HCAP1, 5: HCAP2, 6: HDC1, and 7: HDC2. For more information see text.

Focusing on the major compounds of capsaicinoids, Bandai hybrid could be characterised with pungency loss, while in Beibeihong those compounds did not change during

Table 2: Change in content of capsaicinoid compounds in chili hybrids as a function of ripening. The values represent means in μg/g fresh base weight ± standard deviation ($n = 3$).

Hybrid	Ripening stage	NDC (μg/g)	CAP (μg/g)	DC (μg/g)	HCAP1 (μg/g)	HCAP2 (μg/g)	HDC1 (μg/g)	HDC2 (μg/g)
Beibeihong	Green	51.8 ± 3.90a	294.5 ± 19.72a	326.5 ± 51.20a	3.5 ± 2.01ab	20.0 ± 2.37a	10.6 ± 3.01ab	26.3 ± 2.60a
	Colour-breaker	60.6 ± 15.01a	254.6 ± 31.90a	263.4 ± 25.92a	1.8 ± 0.70a	25.3 ± 5.13a	9.0 ± 0.82a	29.5 ± 6.42a
	Orange	61.7 ± 6.65a	261.9 ± 26.12a	269.3 ± 14.06a	3.6 ± 0.97ab	28.5 ± 4.64a	10.7 ± 0.50ab	26.7 ± 2.09a
	Red	63.2 ± 15.12a	311.8 ± 63.25a	272.7 ± 74.99a	5.7 ± 0.51b	23.7 ± 3.62a	13.9 ± 0.5b	23.9 ± 3.79a
	F-value	0.61	1.44	1.12	5.41	2.26	4.89	0.94
	p value	0.626	0.302	0.394	0.025	0.158	0.032	0.464
Bandai	Green	102.9 ± 14.17ab	1610.2 ± 91.46b	780 ± 36.03b	13.1 ± 5.61ab	8.9 ± 0.99a	12.8 ± 1.22a	30.2 ± 2.29ab
	Colour-breaker	102.2 ± 1.21ab	1182.2 ± 82.56a	725.2 ± 32.03ab	6.3 ± 1.83a	18.5 ± 3.69b	12.7 ± 0.57a	31 ± 3.41ab
	Orange	115.6 ± 5.26b	1104.9 ± 77.27a	635.2 ± 32.36a	11.5 ± 0.51ab	27.2 ± 3.33c	15 ± 0.46ab	37.9 ± 3.41b
	Red	81.5 ± 6.91a	1176.1 ± 112.1a	600.4 ± 87.11a	20.1 ± 6.24b	14.3 ± 2.39ab	16.3 ± 2.06b	27.3 ± 3.34a
	F-value	8.59	18.93	7.40	5.27	22.70	5.95	6.10
	p value	0.007	0.001	0.011	0.027	<0.001	0.020	0.018
Lolo	Green	18.7 ± 2.42a	222.5 ± 69.33a	139.2 ± 50.97a	0.5 ± 0.23b	0.4 ± 0.08a	1.8 ± 0.15a	9.2 ± 1.19a
	Colour-breaker	26.2 ± 3.94a	95.5 ± 4.15a	96.7 ± 9.29a	0.4 ± 0.04b	2.4 ± 0.77b	1.9 ± 0.08a	12.6 ± 1.31a
	Red	22.2 ± 5.14a	197 ± 92.13a	119.8 ± 53.03a	0 ± 0a	3.2 ± 0.15b	1.9 ± 0.27a	12.5 ± 3.27a
	F-value	2.69	3.05	0.74	12.12	29.94	0.86	2.54
	p value	0.146	0.122	0.516	0.008	0.001	0.467	0.159
C3735	Green	31.3 ± 1.46b	259.6 ± 39.15b	183.4 ± 23.27b	UDL	7 ± 0.79a	1.6 ± 0.21b	8.2 ± 0.58ab
	Colour-breaker	35.9 ± 1.64b	168.9 ± 33.86ab	148.2 ± 24.21b	UDL	12.6 ± 4.16a	1.9 ± 0.08b	9.8 ± 1.01b
	Red	18.2 ± 6.21a	126.3 ± 35.95a	88.9 ± 6.38a	UDL	12.6 ± 3.7a	1.3 ± 0.07a	7.2 ± 1.31a
	F-value	17.39	10.50	17.58	—	3.00	15.03	5.00
	p value	0.003	0.011	0.003	—	0.125	0.005	0.053
Fire Flame	Red	15.5 ± 3.28	234.3 ± 45.23	109.7 ± 19.9	1.2 ± 0.13	1 ± 0.23	0.9 ± 0.13	5.9 ± 1.24
Star Flame	Yellow	21.9 ± 5.36	440.8 ± 17.22	135.9 ± 20.28	2.5 ± 0.08	0.2 ± 0.08	1.4 ± 0.25	6.4 ± 1.10

The same letter indicates no significant difference in capsaicinoid content between ripening stages in the given hybrid according to Tukey HSD post hoc test; UDL: under detection limit.

ripening. In the study by Gnayfeed et al. [12] CAP reached the highest value in F-03 cultivar (*C. annuum*) at the initial green stage, similarly found in Bandai, but its content in F-03 did not change significantly with ripening. The obtained results suggest even in the same species (*C. frutescens*) that the hybrids have a different characteristic in ripening regarding capsaicinoid contents. It is in accordance with findings of Merken and Beecher [30] who also measured the maximal capsaicinoid content in 3 different *C. frutescens* peppers in 3 variant times after flower budding.

In Lolo the ripening slightly affected but not significantly CAP, while it increased HCAP2 ($p = 0.001$). After the colour-break stage the amount of HCAP1 decreased ($p = 0.008$) to undetectable level. In C3735 ripening decreased NDC, CAP, DC, HDC1 (all $p \leq 0.011$), and nonmarginally HDC2, while HCAP1 was absent or under detection limit at all ripening stages. Therefore, most of the compounds showed a decreasing tendency during ripening of C3735, so a remarkable pungency loss was observed. On the contrary, those compounds remained unchanged in Lolo.

Iwai found the peak 40 days after flowering and then a gradual decrease of capsaicinoid content in a *C. annuum* pepper. Because of the different scale used by Iwai et al. [32], it is difficult to compare to our data, but probably the 40 days after flowering is roughly equal to the green stage we used. Gnayfeed et al. [12] observed in *C. annuum* cultivars that capsaicinoids reached maximum level at the colour-break stage and then started declining in Hungarian spice pepper (*C. annuum*), which is a characteristic of pungency change that we did not observe. The change in capsaicin content during ripening of pepper may relate to activity of some enzymes that interfere in the ripening dynamics. The amount of capsaicinoids has been investigated in relation with several enzymes [10, 33, 34]. Contreras-Padilla and Yahia [10] showed that peroxidase activity started increasing, when the amount of capsaicinoid started to decrease in Habanero and de Arbol, while in Piquin it began to increase before the decrease of capsaicinoid. They concluded that peroxidase enzyme is involved in capsaicinoid degradation and that attribution is a genotypic characteristic. Iwai et al. [32] found higher phenylalanine ammonia-lyase activity in green stage than in red stage. In addition, Bernal et al. [33] observed that the operation of capsaicinoid synthetase enzyme is more influenced by the availability of precursors and the conditions of forming than its substrate specificity. The capsaicinoid composition and content are the result of the above referred enzymes.

A study concerning the maturation of Habanero (*C. chinense*) proved that green pod contains four times less capsaicin than ripe red ones [13], while we found less

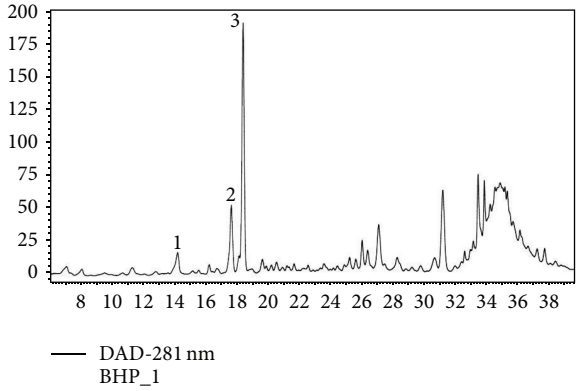

Figure 2: HPLC profile of polyphenols detected separated on Protect-1 C18 column and detected at 280 nm. 1: vanillic acid-derivative, 2: catechin, and 3: naringenin-diglucoside.

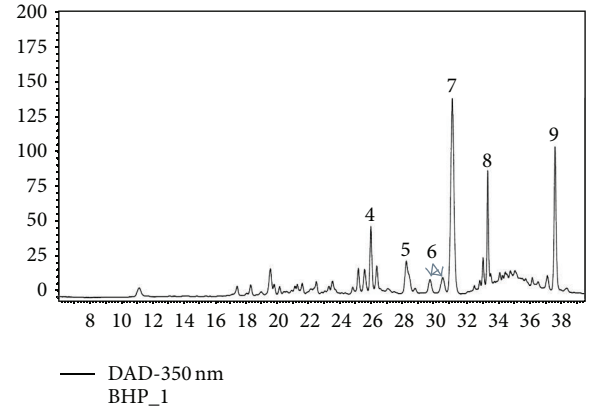

Figure 3: HPLC profile of polyphenols detected separated on Protect-1 C18 column and detected at 350 nm. 4: luteolin-rutinoside, 5: quercetin-glucoside, 6: quercetin-glycosides (the sum of these compounds is used in Table 3), 7: luteolin-glucoside, 8: myricetin, and 9: kaempferol-derivative.

difference and even more capsaicin in green stage (e.g., Bandai); however, none of our investigated hybrids belong to *C. chinense*. They also reported that DC content is seven times less in green pods as compared to red ones, while we found only a slight decrease of DC between the green and red stages.

3.2. Polyphenols. Since there is no available standard for myricetin and vanillic acid in our laboratory, they were tentatively identified based on comparison of their spectral characteristics and retention behaviour on the HPLC column with those found in the literature.

Due to the high content of vanillic acid-derivative, catechin, and naringenin-diglucoside, those compounds were found to be the dominant polyphenols, which have maxima absorption at 280 nm (Figure 2). The minor compounds were luteolin-rutinoside, quercetin-glucoside, quercetin-glycosides, myricetin, and kaempferol-derivative; all were detected with maxima absorption at 350 nm and also luteolin-glucoside occurs in higher concentration and is detected at 350 nm (Figure 3).

In Beibeihong, ripening increased catechin, luteolin-rutinoside, quercetin compounds, myricetin, and kaempferol-derivative (all $p \leq 0.02$ shown in Table 3), while it decreased vanillic acid content ($p < 0.001$). In Bandai ripening increased all compounds (all $p \leq 0.002$) except vanillic acid and luteolin-rutinoside which statistically remained unchanged during ripening stages. In quercetin-glucoside, myricetin, and kaempferol-derivative the highest values were measured in the middle of the ripening. Most of the studies regarding polyphenol constitution of pungent pepper focus on the green (initial) and red (final) ripe stages but omit the intermediate or colour-break stage. Howard et al. [20] found that quercetin decreased, while luteolin did not change with ripening of Tabasco (*C. frutescens*). On the contrary, we found an increase of quercetin-related compounds in both *C. frutescens* hybrids and also an increase of luteolin-rutinoside in Beibeihong and of luteolin-glucoside in Bandai.

In Lolo the ripening significantly decreased vanillic acid ($p < 0.001$) but increased catechin, luteolin-rutinoside, luteolin-glucoside, and myricetin (all $p < 0.001$). In C3735 vanillic acid decreased ($p = 0.007$) while catechin, naringenin-diglucoside, and myricetin increased (all $p \leq 0.019$). Howard et al. stated that quercetin had either increasing or decreasing tendency depending on cultivar; also no change was observed during maturity stages of certain cultivars on *C. annuum* peppers. We could only confirm the last statement that none of the quercetin-related compounds changed when the pods changed from green to red in *C. annuum* peppers studied.

According to Materska and Perucka [19] the most abundant flavonoid compounds in the green stage were quertecin-3-O-L-rhamnoside and luteolin-related compounds, and with ripening those phytochemicals decreased. In the present work particularly in red stage contained higher amounts of luteolin-related in Lolo, while in quercetin-glycoside content no change was detected in both *C. annuum* hybrids.

The disappearances of flavonoids are parallel to capsaicinoids accumulation [35] because the synthesis of flavonoids may converge with the capsaicinoid pathways [36]. The only nonflavonoid phenolic acid detected in our peppers is vanillic acid, and it is the only polyphenol compound which decreased or stayed unchanged during ripening, while the flavonoids mostly increased with advance of ripening. At the same time the major capsaicinoids generally decreased or did not change even with ripening. Kawada and Iwai [37] found a direct relation between DC and vanillic acid; they fed rats with DC and then detected vanillic acid in a notable amount in the urine of the rats. This experiment may also support our findings that vanillic acid is certainly related to capsaicinoids and has similar dynamics during ripening in pungent pepper.

According to Tsao [14], flavonols (kaempferol, quercetin, and myricetin) consist of highly conjugated bindings and a 3-hydroxy group, whose attributions are considered very important in evolving high antioxidant activity. In our hybrids the highest levels of the latter flavonoids were obtained at the orange or red stage that makes the pepper of higher nutritive value.

Table 3: Change in content of polyphenol compounds in different chili hybrids as a function of ripening. The values represent means in μg/g fresh weight base ± standard deviation ($n = 3$).

Hybrid	Ripening stage	Vanillic acid-derivative (μg/g)	Catechin (μg/g)	Naringenin-diglucoside (μg/g)	Luteolin-rutinoside (μg/g)	Quercetin-glucoside (μg/g)	Quercetin-glycosides (μg/g)	Luteolin-glucoside (μg/g)	Myricetin (μg/g)	Kaempferol-derivative (μg/g)
Beibeihong	Green	109.5 ± 9.84b	50.4 ± 1.86a	349.5 ± 13.09a	11.3 ± 0.53a	12.5 ± 2.07a	3.7 ± 0.14a	62.9 ± 2.78a	10.3 ± 0.74a	22.6 ± 1.13a
	Colour-breaker	145.7 ± 9.71c	135.3 ± 3.97b	477.4 ± 52.69b	8.5 ± 0.73a	13.4 ± 1.19a	4.7 ± 0.12ab	79 ± 2.78a	23.5 ± 1.13b	67.3 ± 5.26c
	Orange	114.9 ± 9.66b	153.5 ± 5.56c	431.3 ± 39.72ab	9.7 ± 0.67a	9.1 ± 0.07a	4.9 ± 0.62b	84.6 ± 13.18a	24.9 ± 0.46b	69.7 ± 3.86c
	Red	79.2 ± 11.08a	132.7 ± 3.85b	368.8 ± 30.77a	17.0 ± 3.37b	21.1 ± 2.69b	5.5 ± 0.68b	90.1 ± 16.97a	31.9 ± 6.77b	51.1 ± 5.22b
	F-value	21.88	390.61	7.54	13.47	23.64	7.62	3.45	20.45	79.18
	p value	<0.001	<0.001	0.01	0.02	<0.001	0.01	0.071	<0.001	<0.001
Bandai	Green	96.3 ± 0.25a	96.9 ± 7.67a	130.3 ± 4.82a	13.2 ± 1.06a	4.6 ± 0.66a	5.6 ± 0.24a	84.6 ± 2.37a	25.7 ± 1.58a	62.8 ± 3.33a
	Colour-breaker	89.7 ± 7.98a	134.6 ± 17.02b	202 ± 17.77b	15.1 ± 3.96a	14.2 ± 0.61ab	7.8 ± 0.91ab	91.4 ± 13.75a	117.1 ± 8.21b	289.5 ± 45.59bc
	Orange	92 ± 14.56a	166.4 ± 17.16b	254.2 ± 38.95b	14.8 ± 3.68a	17.4 ± 2.99c	10.2 ± 1.38bc	107.3 ± 23.49a	110.4 ± 19.41b	307.8 ± 53.01c
	Red	101.6 ± 5.67a	175.2 ± 12.32c	276.5 ± 16.65c	21.6 ± 10.69a	12.8 ± 0.97b	11.9 ± 1.15c	157.8 ± 15.26b	52.9 ± 12.94a	200.7 ± 20.83b
	F-value	1.05	19.03	23.73	1.15	33.24	22.39	13.41	38.70	28.15
	p value	0.42	<0.001	<0.001	0.385	<0.001	<0.001	0.002	<0.001	<0.001
Lolo	Green	72.2 ± 0.85c	45 ± 2.71a	116.8 ± 7.28a	2.1 ± 0.49a	5.7 ± 0.83b	5 ± 0.78b	25.7 ± 5.05a	2 ± 0.51a	UDL
	Colour-breaker	50.3 ± 2.85a	51.4 ± 3.09a	160.8 ± 14.93b	3.2 ± 0.36a	2.8 ± 0.08a	2.9 ± 1.03a	53.4 ± 4.52b	4.8 ± 0.62b	UDL
	Red	64.2 ± 4.15b	171.5 ± 6.14b	117.8 ± 7.18a	7.3 ± 1.22b	6.1 ± 1.28b	4.5 ± 0.28ab	75.9 ± 2.48c	8.9 ± 0.57c	9.1 ± 3.77
	F-value	42.59	836.79	17.35	37.55	12.72	6.49	108.95	113.35	—
	p value	<0.001	<0.001	0.003	<0.001	0.007	0.032	<0.001	<0.001	—
C3753	Green	73.1 ± 5.46b	22.6 ± 1.28a	123.6 ± 6.23a	2 ± 0.7a	1.7 ± 0.39a	16.8 ± 2.38a	17 ± 2.57a	3.8 ± 0.96a	UDL
	Colour-breaker	51 ± 8.55a	64.2 ± 10.49b	148.3 ± 40.01ab	1.5 ± 0.37a	1.3 ± 0.24a	17.3 ± 3.44a	13.5 ± 2.66a	10 ± 1.09b	21 ± 1.71a
	Red	56 ± 6.67a	124.5 ± 6.18c	217.2 ± 8.36b	UDL	1.1 ± 0.23a	19.8 ± 2.55a	17.5 ± 2.78a	11.3 ± 0.94b	16.4 ± 2.85a
	F-value	13.06	157.47	8.32	0.44	2.84	0.95	1.99	48.91	6.007
	p value	0.007	<0.001	0.019	0.661	0.135	0.44	0.12	<0.001	0.070
Fire Flame	Red	27.8 ± 2.07	26.6 ± 1.40	141.6 ± 4.17	2.8 ± 0.27	2.7 ± 0.16	4.2 ± 0.22	44.4 ± 2.76	18.2 ± 0.43	UDL
Star Flame	Yellow	24.2 ± 1.37	17.6 ± 0.24	93.5 ± 4.33	9.5 ± 0.47	2.0 ± 0.14	4.1 ± 0.62	60.6 ± 1.34	18.4 ± 1.29	UDL

The same letter indicates no significant difference in polyphenol content between ripening stages in the given hybrid according to Tukey HSD post hoc test; UDL: under detection limit.

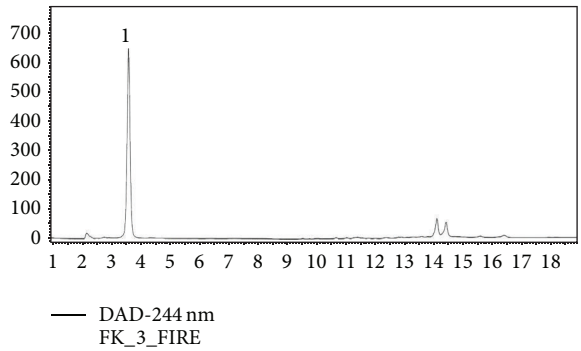

Figure 4: HPLC profile of vitamin C determination. The separation was performed on C18 Nautilus column with PDA detection at 244 nm. 1: L-ascorbic acid.

3.3. Ascorbic Acid. By the applied HPLC method only L-ascorbic acid was found in the extract of all hybrids (Figure 4). It was found that ascorbic acid increased during ripening in all hybrids ($p \leq 0.001$ shown in Table 4). In Beibeihong and Bandai after green stage a more notable increase was observed than after the colour-break stage where the ascorbic acid gradually increased, while in Bandai at the red stage the average of ascorbic acid was less than in orange stage. In Lolo the green and colour-break stage did not differ significantly, while the red stage contained the most. In C3735 a straight increase was observed. The increasing tendency in the investigated hybrids is in accordance with that found in previous works [12, 20, 24, 25] which concluded that the more ripened the pods were, the more ascorbic acid could be measured from them. With ripening the pepper pods store more reducing sugars [36], which are the precursors of L-ascorbic acid [38], and that explains the increasing vitamin C content with ripening in all hybrids included in our study. On the contrary, Shaha et al. [18] showed a different dynamics of the ascorbic acid accumulation, because they found the highest level in yellow (intermediate) stage and the declining level in the red mature stage. That agrees with our finding in Bandai, where the highest average values (1005.2 ± 100.73 μg/g) were observed in the orange or colour-break stage (937.9 ± 78.04 μg/g), although these are not significantly higher than that determined in red stage (787.4±131.21 μg/g). Probably it is also due to the high standard deviation present in the red stage.

The recommended daily allowance (RDA) is 60 μg FW; according to Dias [39] 100 g fresh chili provides about 143.7 μg vitamin C. Focusing on the hybrids of the recent study at the green stage all of them failed to reach this value, while at colour-break stage Beibeihong and C3735 reached it and finally at the red stage all of them achieved the RDA.

3.4. Comparison of Major Compounds among the 6 Hybrids. The comparison among the hybrids has been done on the main parameters: CAP, ascorbic acid, naringenin-diglucoside, Scoville heat unit, and dry matter (shown in Table 5), at the final stage of the hybrids, which is generally considered as the most valuable in nutrition and having the most processing possibility. A higher dry matter signifies a better fruit quality and also a higher nutritional concentration when fresh weight basis is used to express nutritional parameters. We measured 25–30% dry matter content in *C. frutescens*, which produces more seeds and smaller pods, while in the peppers belonging to *C. annuum* this value lessens to 14.1–15.8%.

The CAP content was found to be statistically the same in all red coloured *C. annuum* hybrids, while the yellow hybrid Star Flame (234.3 ± 45.23 μg/g) contained more, and Bandai (1176.1 ± 112.1 μg/g) the most ($p < 0.001$). Our findings roughly agree with the result of Sanatombi and Sharma [40] who showed that the cultivars belonging to *C. annuum* contain less capsaicin than others of *Capsicum frutescens*. Beibeihong was an exception, because it statistically contained the same amount as *C. annuum* hybrids. Focusing on the Scoville heat units, the highest CAP value in Bandai corresponds to the highest SHU (98090.8 ± 9920.74) observed among the hybrids investigated. Bernal et al. [33] measured 87300–276500 SHU in ripe *C. frutescens* peppers, but in Bandai hybrid the value found was close to the lower level determined by the authors. Among *C. annuum* hybrids, Star Flame was found to be a prominent pepper regarding

Table 4: Change in content of ascorbic acid in different chili hybrids as a function of ripening. The values represent means in μg/g fresh weight base ± standard deviation ($n = 3$).

Hybrid	Ripening stage	Ascorbic acid (μg/g)
Beibeihong	Green	355 ± 64.85a
	Colour-breaker	1503.4 ± 358.31b
	Orange	2085.7 ± 252.2bc
	Red	2483.8 ± 570.74c
	F-value	19.74
	p value	<0.001
Bandai	Green	329.5 ± 58.88a
	Colour-breaker	937.9 ± 78.04b
	Orange	1005.2 ± 100.73b
	Red	787.4 ± 131.21b
	F-value	30.09
	p value	<0.001
Lolo	Green	111.3 ± 14.01a
	Colour-breaker	451.5 ± 115.56a
	Red	1940.9 ± 533.57b
	F-value	28.57
	p value	0.001
C3735	Green	315.1 ± 59.91a
	Colour-breaker	1522.5 ± 127.47b
	Red	2468.2 ± 58.93c
	F-value	449.65
	p value	<0.001
Fire Flame	Red	3689.4 ± 39.50
Star Flame	Yellow	3154.8 ± 160.61

The same letter indicates no significant difference in ascorbic acid content between ripening stages in the given hybrid according to Tukey HSD post hoc test.

Table 5: Capsaicin, ascorbic acid, and naringenin-diglucoside content (μg/g fresh weight base), pungency unit of Scoville, and dry matter of different chili hybrids. The values represent means ± standard deviation ($n = 3$).

Hybrid	Capsaicin (μg/g)	Scoville heat unit	Ascorbic acid (μg/g)	Naringenin-diglucoside (μg/g)	Dry matter
Beibeihong	311.8 ± 63.25ab	37999.8 ± 5761.66a	2483.8 ± 570.74bc	368.8 ± 30.77f	25.8 ± 0.82d
Bandai	1176.1 ± 112.1c	98090.8 ± 9920.74c	787.4 ± 131.21a	276.5 ± 16.65e	30.2 ± 0.41c
Lolo	197 ± 92.13a	33188.2 ± 5229.83a	1940.9 ± 533.57b	117.8 ± 7.18ab	14.0 ± 0.61a
C3735	126.3 ± 35.95a	23730.9 ± 3174.95a	2468.2 ± 58.93bc	217.2 ± 8.36d	15.8 ± 0.93b
Fire Flame	234.3 ± 45.23a	40417.3 ± 7830.33a	3689.4 ± 160.61d	141.6 ± 4.19c	14.1 ± 0.34ab
Star Flame	440.8 ± 17.22b	66201.2 ± 7132.51b	3154.8 ± 160.61cd	93.5 ± 4.26a	14.4 ± 0.58ab
F-value	94.64	48.43	27.62	146.17	357.48
p value	<0.001	<0.001	<0.001	<0.001	<0.001

The same letter indicates no significant difference in the major components between the fully ripe stages of the 6 hybrids according to Tukey HSD post hoc test.

SHU (66201.2 ± 7132.51) comparing to the measurements of Topuz and Ozdemir [41] 9720 ± 2061.8 and Giuffrida et al. [42] 21034 ± 3579. Beibeihong and Bandai have not been investigated by pungency profiles before. Comparing to Tabasco (also belonging to *C. frutescens*) the SHU measured by Giuffrida et al. [42] (21348 ± 867) is below our values of the latter hybrids, although CAP content determined by Giuffrida et al. [42] (917 ± 34 μg/g) is between the values measured in Bandai (1176.1 ± 112.1 μg/g) and Beibeihong (311.8 ± 63.25 μg/g). Interestingly, Bandai hybrid had the highest CAP content at the same time; it also had the lowest ascorbic acid amount. Topuz and Ozdemir [41] described in pungent peppers that the content of ascorbic acid and capsaicinoid is positively related, which we could not underline in case of Bandai. The highest ascorbic acid was measured in ripe Fire Flame (3689.4 ± 160.61 μg/g) and this value is well above the one measured in Hungarian spice pepper where approximately 1800 μg/g converted to fresh weight basis [12], and it is more than the one detected in New Mexican-type chili peppers 2766 μg/g [25].

Naringenin-diglucoside content ranged from 93.5 ± 4.26 to 368.8 ± 30.77 μg/g and had higher values in *C. frutescens* hybrids compared to *C. annuum* hybrids, probably because of the higher dry matter content of such peppers. Naringenin (belonging to flavanones), being an initial compound in the chain of flavonoid synthesis [14], explains the high content present in our samples. Other studies found also naringenin-glucosides as a dominant flavonoid in peel of pungent pepper [43] and in sweet pepper alike [44].

4. Conclusion

The investigated new hybrids can be regarded to be good sources of phytochemicals for future applications. We recommend using the red coloured hybrid Fire Flame to produce chili products with high content of vitamin C. On the other hand, when heat principles (capsaicinoid) for food and pharmaceutical industries are required, the use of Star Flame and Bandai can be suggested, as they contain a level of capsaicin around 440.8 ± 17.22 μg/g and 1610.2 ± 91.46 μg/g, respectively. In order to get the maximum level of the bioactive phytochemicals such as vitamin C, capsaicinoid, and polyphenol it is important to characterize the ripening dynamics of each of these new hybrids. For example, the highest level of capsaicin could be found in the green stage of ripening of Bandai and C3735 hybrids, while in the other hybrids pungency was similar in all ripening stages.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This study was funded in part by Research Centre of Excellence: 8526-5/2014/TUDPOL Szent István University and KTIA_AIK_12-1-2012-0012 projects.

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Hindawi Publishing Corporation
Journal of Analytical Methods in Chemistry
Volume 2015, Article ID 247685, 21 pages
http://dx.doi.org/10.1155/2015/247685

Review Article

Sesquiterpene Lactones from *Artemisia* Genus: Biological Activities and Methods of Analysis

Bianca Ivanescu,[1] Anca Miron,[2] and Andreia Corciova[3]

[1]*Department of Pharmaceutical Botany, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa", 16 Universitatii Street, 700150 Iasi, Romania*
[2]*Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa", 16 Universitatii Street, 700150 Iasi, Romania*
[3]*Department of Drug Analysis, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa", 16 Universitatii Street, 700150 Iasi, Romania*

Correspondence should be addressed to Bianca Ivanescu; biancaivanescu@yahoo.com

Received 10 April 2015; Revised 23 August 2015; Accepted 25 August 2015

Academic Editor: Shixin Deng

Sesquiterpene lactones are a large group of natural compounds, found primarily in plants of *Asteraceae* family, with over 5000 structures reported to date. Within this family, genus *Artemisia* is very well represented, having approximately 500 species characterized by the presence of eudesmanolides and guaianolides, especially highly oxygenated ones, and rarely of germacranolides. Sesquiterpene lactones exhibit a wide range of biological activities, such as antitumor, anti-inflammatory, analgesic, antiulcer, antibacterial, antifungal, antiviral, antiparasitic, and insect deterrent. Many of the biological activities are attributed to the α-methylene-γ-lactone group in their molecule which reacts through a Michael-addition with free sulfhydryl or amino groups in proteins and alkylates them. Due to the fact that most sesquiterpene lactones are thermolabile, less volatile compounds, they present no specific chromophores in the molecule and are sensitive to acidic and basic mediums, and their identification and quantification represent a difficult task for the analyst. Another problematic aspect is represented by the complexity of vegetal samples, which may contain compounds that can interfere with the analysis. Therefore, this paper proposes an overview of the methods used for the identification and quantification of sesquiterpene lactones found in *Artemisia* genus, as well as the optimal conditions for their extraction and separation.

1. Introduction

Sesquiterpene lactones (SLs) are probably the largest class of secondary metabolites in plants, with over 5000 structures reported to date [1–4]. They are fifteen carbon compounds formed from condensation of three isoprene units, followed by cyclization and oxidative transformation to make a *cis* or *trans*-fused lactone. The γ-lactone ring, usually with an α-methylene group, is a significant characteristic of SLs. Their molecule may present hydroxyls, esterified hydroxyls, or epoxide groups, some SLs occur in glycosylated form, and few contain halogen or sulfur atoms [5]. Sesquiterpene lactones are bitter, colourless substances, with lipophilic character and a variety of structural arrangements. They are classified depending on their carboxylic skeleton into the following main groups: germacranolides (10-membered rings), the largest group and biogenetic precursors of the majority of sesquiterpene lactones; eudesmanolides and eremophilanolides (6/6-bicyclic compounds); and guaianolides, pseudoguaianolides, and hypocretenolides (all 5/7-bicyclic compounds) [6, 7]. Sesquiterpene lactones play an important role in communication between plants and interaction with insects, microorganism, and animals acting as attractants, deterrents, and antifeedants [1, 8, 9]. One plant species usually produces one type of sesquiterpene lactones, found chiefly in leaves and flowers in concentrations of 0.01% to 8% dry weight [5, 10].

Although SLs are present in approximately 16 plant families, they are prevalent in *Asteraceae* family where they can be found in almost all genera, notably in *Artemisia*,

Arnica, *Ambrosia*, *Helenium*, *Tanacetum*, and *Vernonia* [1, 11]. Within this family, genus *Artemisia* is very well represented having approximately 500 species, distributed worldwide and thriving in various habitats. *Artemisia* species are aromatic plants exploited for their volatile oil [12] and many of them are used all over the world in traditional medicine in order to treat conditions such as fever, malaria, inflammation, ulcer, diabetes, and intestinal worms. Morphological and phytochemical variability characterises this genus and also polyploidy is commonly reported, so different chemotypes and cytotypes will synthesize diverse metabolites [13]. SLs are produced in large amounts in glandular trichomes in response to biotic stresses but are also found in secretory canals of underground plant organs [2]. The most common SLs in *Artemisia* species are guaianolides, eudesmanolides, and germacranolides. Probably, the best known compound in this group is an endoperoxide SL isolated from *Artemisia annua*, artemisinin, a modern antimalarial used in artemisinin combination therapies that also displays anticancer activity.

The biological activity of SLs is mainly attributed to the α-methylene-γ-lactone group (αMγL) in their structure. The αMγL acts as a Michael acceptor and reacts with nucleophiles (sulfhydryl or amino groups) in enzymes, transcription factors, and other proteins, alkylating them irreversibly [8, 14]. The alkylation will disrupt the proper function of the biological macromolecule due to steric and chemical changes. This is considered to be the primary mechanism of action of SLs that underlies their cytotoxicity. It also explains cell wall damage in microbes and prevalence of contact dermatitis in humans. Yet, other factors can influence the potency of SLs: number of alkylating groups, lipophilicity, molecular geometry and size, chemical environment, other functional groups neighboring the αMγL, and the target sulfhydryl [2, 5].

Considering the increasing importance of SLs from *Artemisia* genus and potential applications in medicine and agriculture, this paper aims to review the recent information relative to biological activities and analysis methods of these molecules. The knowledge of different types of analysis methods is necessary for the analyst that must choose the most appropriate method for the sample, taking into account the available equipment. The most common methods applied to SLs are chromatographic techniques, particularly HPLC with different detection methods, followed by GC. Since these methods are difficult, time consuming, and expensive, we have also chosen to present some analysis methods that are cheaper and available to all laboratories, such as spectrophotometric techniques (UV-Vis) and TLC.

2. Biological Activities of Sesquiterpene Lactones

2.1. Antitumor Activity. The antimalarial drug artemisinin and its derivatives are very potent anticancer compounds, highly selective on cancer cells with almost no side effects on normal cells and a broad spectrum of action: leukaemia, colon, melanoma, osteosarcoma, pancreas, breast, ovarian, prostate, hepatic, renal, central nervous system, and lung cancer cells [15–18]. Some disadvantages of artemisinin, such as low solubility, short plasma half-life, and poor bioavailability [19], were surpassed by the semisynthetic or fully synthetic derivatives, such as artesunate, artemether, dihydroartemisinin, and artemisone.

Artemisinin (Figure 1) is a cadinanolide with a 1,2,4-trioxane ring system, found most importantly in *Artemisia annua* L. and in minor quantities in *A. apiaceae* Hance and *A. lancea* Vaniot [20]. The presence of artemisinin in *Artemisia sieberi* and *Artemisia scoparia* in small quantities was also reported [21, 22]. However, other bioactive compounds in *Artemisia annua* contribute to the overall activity of extracts: SLs arteannuin B and artemisitene, but also scopoletin and 1,8-cineole [23]. The flavonoids present in *Artemisia annua* act synergically with artemisinin against malaria and cancer: they modify the absorption and metabolism of artemisinin in the body and exhibit beneficial immunomodulatory activity in cancer patients [24].

The antitumor mechanism of artemisinin is based on cleavage of its endoperoxide bridge by the iron in cancer cells and formation of free radicals. Free radicals will produce cell alterations such as apoptosis, deoxyribonucleic acid (DNA) damage, modulation of nuclear receptor responsiveness, arrest of growth, inhibition of angiogenesis, inhibition of tumour invasion, migration, and metastasis. These pleiotropic effects can account for effectiveness of artemisinin compounds in multidrug resistant types of cancer [25].

Some artemisinin derivatives reached the phase of clinical trials: the efficacy of artesunate combination therapy was evaluated in advanced breast cancer and another trial assessed the activity and tolerability of artesunate in colorectal adenocarcinoma [6]. A clinical trial in 120 patients with advanced non-small cell lung cancer tested the effect of artesunate in combination with traditional chemotherapeutic drugs [26]. A pilot study in ten patients with advanced cervix carcinoma proved the efficiency of dihydroartemisinin [27]. For some compounds, individual clinical cases were reported: artemether oral treatment was used in a patient with pituitary macroadenoma [28], and artesunate was used in laryngeal squamous cell carcinoma [29] and metastatic uveal melanoma [30] with good results and lack of side effects.

In the early 1980s, arglabin (Figure 1) was isolated from the Kazakhstan endemic plant *Artemisia glabella* Kar. et Kir. and was approved for use for cancer treatment in 1996 in the same country. The compound prevents farnesylation of cell proteins, killing both normal and cancer cells, with a 50–100 times increased toxicity for tumor cells [31]. Arglabin is found in all plant organs and throughout the entire period of vegetation in concentrations of 0,08–0,6% [32]. The compound was also identified in *A. myriantha* [33].

The aerial parts of *Artemisia amygdalina* Decne produce significant amounts of ludartin, a highly cytotoxic guaianolide, also found in *Artemisia indica* [34]. Ludartin displays IC_{50} values of 6.6 μM and 19.0 μM against mouse melanoma (B16F10) and human epidermoid carcinoma (A-431) in MTT assay [35]. Ludartin is a position isomer of arglabin and can be easily converted into clinically important antitumor arglabin [36].

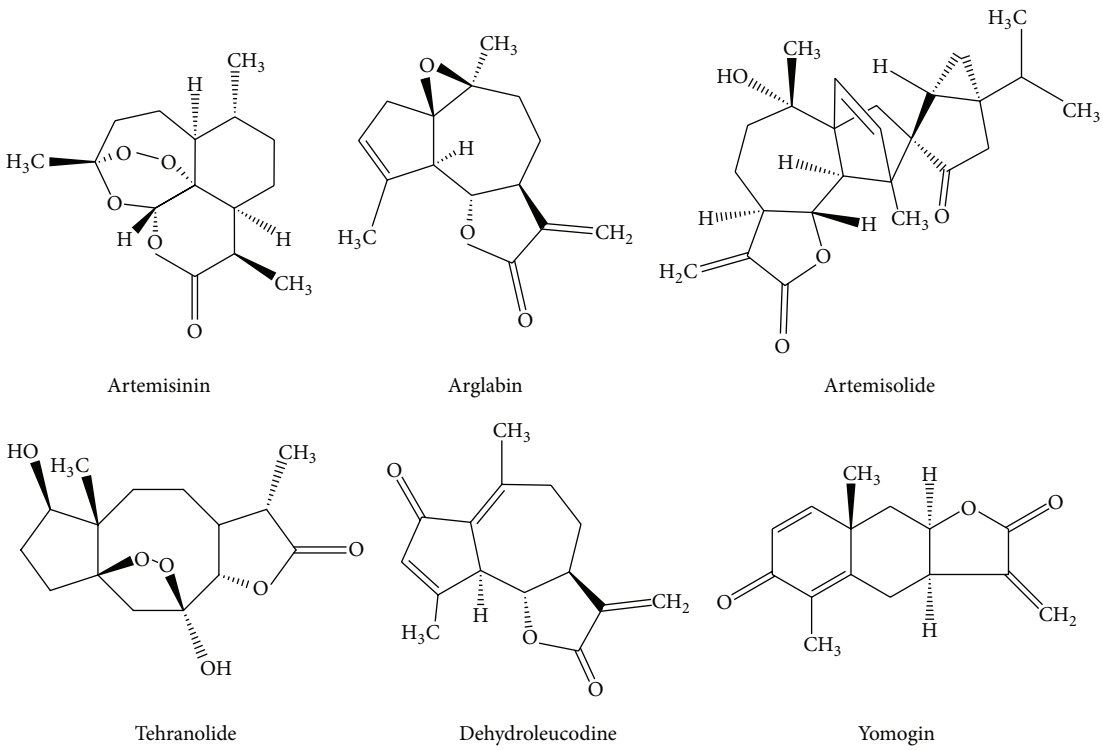

Figure 1: Structures of bioactive sesquiterpene lactones from *Artemisia* genus.

Arteminolides A–D, sesquiterpene lactones extracted from the aerial parts of *Artemisia argyi,* are potent farnesylprotein transferase (FPTase) inhibitors with IC_{50} values of 0.7–1 μM. They inhibit tumor growth in mouse xenograft models and in human tumour xenograft [37]. Another cytotoxic compound produced by *Artemisia argyi* is artemisolide (Figure 1), sesquiterpene lactone with a cyclopropane ring which exhibits *in vitro* activity against human acute lymphoblastic leukaemia Molt-4, promyelocytic leukaemia HL-60, and SW620 colon cancer cell lines [38].

Yomogin (Figure 1), a eudesmane sesquiterpene lactone isolated from *Artemisia princeps,* has been shown to inhibit tumor cell proliferation [39]. Yomogin synergistically increased differentiation of human promyelocytic leukemia HL-60 cells when combined with 1,25-dihydroxyvitamin D or all-trans-retinoic acid and stimulated differentiation to monocytes, respectively, granulocytes. So, these combinations can be used in therapy of myeloid leukemias [40]. Moreover, yomogin induces apoptosis in human promyelocytic leukemia HL-60 cells through caspase-8 activation, Bid cleavage, and Bax translocation to mitochondria, followed by release of cytochrome c into the cytoplasm [41].

Eight highly oxygenated guaianolides, named artemdubolides A–H, were isolated from *Artemisia dubia* and two of them manifested reduced cytotoxicity on human colon carcinoma Colo205 and human melanoma MDA-MB-435 cells *in vitro* [42].

A new antitumor sesquiterpene lactone with an endoperoxide moiety, tehranolide (Figure 1), was isolated from *Artemisia diffusa*. Tehranolide selectively inhibits proliferation of breast cancer cells through cell cycle arrest and apoptosis [43] and also modifies the immune responses and increases antitumor immunity [44].

2.2. Anti-Inflammatory and Immunomodulatory Effect. SLs also exhibit anti-inflammatory and immunomodulatory actions, properties that can be beneficial in tumour treatment or chronic diseases and can enhance the success of therapy. The main mechanism of anti-inflammatory activity is by inhibiting the expression of nuclear factor κB (NF-κB). NF-κB is a ubiquitous protein that regulates over 150 inflammatory genes and mediates immune response in humans. NF-κB controls the response of other effectors such as cytokines, inflammatory molecules, and cell adhesion molecules [2]. Therefore, inhibition of NF-κB decreases inflammatory response and suppresses cancer growth. In an extensive study comprising over 100 sesquiterpene lactones, researchers established that guaianolides are most potent inhibitors of NF-κB and their efficacy is due mostly to the α,β-unsaturated carbonyl group [45].

Artemisinin inhibits the secretion of tumour necrosis factor (TNF)-α, interleukin- (IL-) 1β, and IL-6 in a dose-dependent manner, thus exerting an anti-inflammatory effect on phorbol myristate acetate- (PMA-) induced THP-1 human monocytes [46]. Moreover, in a mouse model of contact hypersensitivity, topical administration of artemisinin produced anti-inflammatory and immunomodulatory effects [47].

Dihydroartemisinin inhibits phorbol 12-myristate 13-acetate- (PMA-) induced COX-2 expression in murine macrophage RAW 264.7 cells via downregulation of AKT and MAPK kinase signaling pathways. Dihydroartemisinin

decreased PMA-induced COX-2 expression and PGE 2 production, as well as COX-2 promoter-driven luciferase activity in a dose-dependent manner [48].

Both artemisinin and dihydroartemisinin suppress delayed hypersensitivity to sheep blood cells in mice, manifesting immunosuppressive action [49, 50]. Dihydroartemisinin also impaired growth of ductal carcinoma in mice and decreased the levels of interleukin IL-4 [50]. Artemisinin diminishes the number of regulatory T cells in murine breast cancer model [51].

Artesunate is therapeutically relevant to inflammatory responses of microglial cells [52] and inhibits production of interleukin IL-1β, IL-6, and IL-8 in human rheumatoid arthritis through NF-κB inhibition [53].

SLs artemisinin, dihydroartemisinin, artemisinic acid, and arteannuin B significantly reduce LPS-activated production of prostaglandin E2 (PGE2). Arteannuin B also inhibited lipopolysaccharide- (LPS-) induced *in vitro* production of nitric oxide (NO) and secretion of cytokines (VEGF, IL-1*b*, IL-6, and TNF-α) [54].

One study evaluated the enriched sesquiterpene lactone fraction from *Artemisia annua* on different nociceptive and inflammatory animal models. The sesquiterpene lactones fraction containing artemisinin (1.72%) and deoxyartemisinin (0.31%) demonstrated pain relief on chemical-induced nociception assays in mice. The i.p. treatment produced a relevant reduction in the reaction time of the animals in both phases of the formalin test, significantly reduced the sensitivity to mechanical allodynia stimulus, reduced the paw edema caused by carrageenan injection, and promoted high antinociceptive activity in tail flick model suggesting relationship with the opioid system [55].

Another NF-κB inhibitor, artemisolide, was isolated from *Artemisia asiatica* by activity-guided fractionation using the NF-κB mediated reporter gene assay [56, 57]. Artemisolide suppresses production of prostaglandin E_2 and nitric oxide (NO) in macrophages. In the same way, other bioactive sesquiterpene lactones were isolated from *Artemisia sylvatica*: arteminolides B and D, moxartenolide, deacetyllaurebiolide, 3α,4α-epoxyrupicolins C–E, and 3-methoxytanapartholide. All separated compounds also inhibited NO and TNF-α production [58].

Nitric oxide (NO) is synthesized in the body through oxidation of L-arginine by a family of synthases that can be constitutive (cNOS) or inducible (iNOS). iNOS induction in tissues increases the concentration of NO and can cause inflammatory effects including vasodilation, edema, and cytotoxicity. The induction of the enzyme is mediated by proinflammatory cytokines such as γ-interferon, tumor necrosis factor (TNF), IL-1, and IL-6. Thus, iNOS enzyme has become a new target for pharmacological research to find new substances useful in the treatment of chronic inflammatory diseases.

The anti-inflammatory effect of dehydroleucodine (Figure 1) isolated from *A. douglasiana* was investigated in arthritis induced by Freund's adjuvant carrageenan-induced and cotton pellet-induced granuloma. Dehydroleucodine inhibited both chronic and acute carrageenan-induced inflammations but was most efficient in the chronic phase. The sesquiterpene lactone also inhibited inflammation in the granuloma test, probably by interfering with transcription factors, such as NF-κB and cytokines [59].

Yomogin, an eudesmane sesquiterpene isolated from *Artemisia princeps*, exhibits intense anti-inflammatory activity. It has been shown that yomogin inhibits NO production in LPS-activated RAW 264.7 cells by suppressing i-NOS enzyme expression [60] and blocks the degranulation of mast cells by inhibiting the release of beta-hexosaminidase from the cultured RBL-2H3 cells in a dose-dependent manner [61]. Also, yomogin exhibited a novel histamine H_1 receptor antagonism in the guinea pig ileum [62].

Arglabin, a sesquiterpene lactone isolated from *Artemisia myriantha* Wall, manifests immunomodulating properties. Arglabin triggered the production of cytokines involved in host defence mechanisms: IL-1, TNF-alpha, and IL-2. Lower concentrations of arglabin were the most effective in inducing cytokines secretion [63]. Furthermore, arglabin exhibits antiexudative and antiproliferative properties on the models of acute inflammation caused by formalin, carrageenan, and histamine and on the model of proliferative inflammation accompanying cotton-pellet granuloma [64]. It has been shown that arglabin effectively attenuates the high glucose-stimulated activation of NF-κB, the degradation of IκBα, and the expression of MCP-1, TGF-β1, and FN in rat mesangial cells [65]. A recent study proposes that arglabin could be a promising new drug to treat inflammation and atherosclerosis, based on its pharmacological actions: it reduces inflammation and plasma lipids, increases autophagy, and orients tissue macrophages into an anti-inflammatory phenotype in ApoE2.Ki mice fed a high-fat diet [66].

Other anti-inflammatory sesquiterpene lactones mentioned in the literature are dimeric guaianolides from *Artemisia anomala* [67], and those isolated from *Artemisia khorassanica* Podl., which inhibits iNOS and COX-2 expression through the inactivation of NF-κB [68]. SLs barrelierin, artemalin, barrelin, and desoxyvulgarin from *Artemisia barrelieri* also exhibited anti-inflammatory activities [69].

2.3. Antiulcer Activity. Sesquiterpene lactones of the guaianolide and eudesmanolide types are considered to be of interest in treatment of gastric and peptic ulcers because they have an effect in the regulation and prevention of oxidative damage and inflammation-mediated biological damage [70]. Dehydroleucodine, a sesquiterpene lactone isolated from the aerial parts of *Artemisia douglasiana* Besser, exerts *in vivo* cytoprotective actions against ethanol-induced gastric mucosal injury.

Several related guaianolides and pseudoguaianolides were also found to exhibit cytoprotection: ludartin, 8-angeloyloxy-3-hydroxyguaia-3(15),10(14),11(13)-trien-6,12-olide, hymenin, mexicanin I, helenanin, and 9-O-desacetylsparthulin-2-O-angelate. Desacetoxymatricarin did not show cytoprotective activity, suggesting that the presence of the alpha-methylene-gamma-lactone moiety is a requirement for the antiulcerogenic activity [71].

Dehydroleucodine exhibits anti-inflammatory and gastrointestinal cytoprotective action [59]. The compound

stimulates mucus production and inhibits histamine and serotonin release from intestinal mast cells [72] and could act as a selective mast cell stabilizer by releasing cytoprotective factors and inhibiting proinflammatory mast cell mediators. Gastrointestinal mast cells are involved in pathologic effects but also play a protective role in defense against parasitic and microbial infections. Thus, it is believed that stabilization of mast cells may be a key mechanism in the protection of gastrointestinal tract from injury [73, 74].

The crude ethanol extract and the enriched sesquiterpene lactone fraction of *Artemisia annua* aerial parts exhibited antiulcerogenic activity on the indomethacin induced ulcer in rats. The sesquiterpene lactone fraction yielded three different polarity fractions on column chromatography. For the medium polarity fraction, it was demonstrated that the active compounds of *Artemisia annua* act by increasing the prostaglandin levels in the gastric mucosa [75].

Three SLs isolated from the ethanol extract of *Artemisia annua*—artemisinin, dihydro-epideoxyarteannuin B, and deoxyartemisinin—were tested on ethanol and indomethacin-induced ulcers in rats. Both dihydro-epideoxyarteannuin B and deoxyartemisinin reduced the ulcerative lesion index produced by ethanol and indomethacin, while artemisinin did not manifest cytoprotection. Previous treatment with indomethacin, a cyclooxygenase inhibitor, blocked the antiulcerogenic activity of compounds on ethanol-induced ulcer, suggesting that the activity is the consequence of an increase in prostaglandin synthesis [76].

Furthermore, SLs may exhibit another benefic effect in ulcer through their antimicrobial activity. Thus, artemisinin and its analogues manifested remarkably strong activity against *Helicobacter pylori*, the pathogen responsible for peptic ulcer diseases [77]. Both dehydroleucodine and *Artemisia douglasiana* extract showed *in vitro* activity against six clinical isolates of *Helicobacter pylori*, with MICs between 1–8 and 60–120 mg/L, respectively [78].

2.4. Antimicrobial Activity

2.4.1. Antiparasitic. Artemisinin and its analogues show marked activity against *Plasmodium* species *in vivo* and *in vitro*. It is effective even against multidrug resistant strains of the malaria parasite and in cases of cerebral malaria. Nowadays, artemisinin and its derivatives are recommended by the World Health Organisation to be used as first choice therapy in the treatment of malaria as part of ACT (artemisinin combination therapy).

Artemisinin has an endoperoxide bridge to which its antimalarial properties are attributed. The proposed mechanism of action involves the formation of free-radical intermediates, resulting from the direct interaction of the endoperoxide group with the intraparasitic iron, and the alkylation of malarial-specific proteins by the artemisinin-derived free radicals, thus damaging the microorganelles and membranes of the parasite. This radical will damage the infected blood cell, which will lead to the disposal of the cell by the hosts own immune system [79]. Artemisinin also targets the parasite mitochondria or the translationally controlled tumour protein and PfATP6, a parasite-encoded sarcoplasmic-endoplasmic reticulum calcium ATPase, which is crucial for the development of the parasite [80].

Other sesquiterpene lactones isolated from *Artemisia* species also showed antimalarial properties. From the leaves and flowers of *Artemisia gorgonum* several sesquiterpene lactones were isolated and evaluated for antiplasmodial activity. Compounds ridentin and hanphyllin had an inhibitory concentration 50 (IC_{50}) of 5.4 and 2.3 μg/mL against *Plasmodium falciparum*, respectively. The antimalarial activity may be attributed to the exomethylene group of the lactone function [81].

Dihydroartemisinin, the main metabolite of artemisinin, is a broad-spectrum antiparasitic drug, being active against *Plasmodium, Schistosoma, Toxoplasma, Trichomonas vaginalis, Leishmania,* and *Giardia lamblia* [82].

Dehydroleucodine induces programmed cell death in both the replicative epimastigote form and the infective trypomastigote form of *Trypanosoma cruzi*, which is a different mechanism of action than the conventional drugs to kill the parasite. A combination of DhL with conventional anticha-gasic drugs showed synergic activity on decreasing parasite viability. Chagas disease or American Trypanosomiasis is caused by the flagellated protozoan parasite *Trypanosoma cruzi* and is one of the world's neglected tropical diseases [83].

Visceral leishmaniasis, caused by the protozoan *Leishmania* sp., affects 500,000 people annually and emerging resistance to conventional antimony therapy has underlined the need for safer yet effective antileishmanial drugs. Artemisinin exhibited antipromastigote activity with IC_{50} ranging from 100 to 120 μM in *Leishmania donovani*, *Leishmania infantum*, *Leishmania tropica*, *Leishmania mexicana*, *Leishmania amazonensis,* and *Leishmania braziliensis*. It was demonstrated that artemisinin exerted a direct parasiticidal activity, while also inducing a host protective response. For *in vivo* studies, the BALB/c mouse model meets eligibility requirements such as the chronic infection pattern, which resembles human visceral leishmaniasis. In *in vivo* studies on mouse model, treatment with artemisinin led to a significant reduction in splenic weight, a significant inhibition of parasites and a restoration of cytokines such as interferon-γ and interleukin-2 (IL-2) [84].

Santonin, a sesquiterpene lactone isolated from *Artemisia cina* or other santonin-containing species of *Artemisia,* was widely used in the past as an anthelminthic, a drug that expels parasitic worms from the body, by either killing or stunning them. Due to the severe side effects, the need for a purgative, and the development of many safer anthelmintic drugs, santonin has largely fallen out of use [85].

2.4.2. Antibacterial. Sesquiterpene lactones are one of the main mechanisms of plants defense against microbial attacks. They act by disruption of a microbe's cell membrane, an effect attributable to the polar groups on these antimicrobial compounds disrupting the phospholipid membrane [2].

In an attempt to isolate antibacterial constituents from *Artemisia princeps* var. *orientalis*, secotanapartholides A and B were identified as bioactive compounds. These sesquiterpene lactones produced a clear inhibitory effect against

Clostridium perfringens, Bacteroides fragilis, and *Staphylococcus aureus* and had no effect on the growth of lactic acid-producing bacteria (*Bifidobacterium adolescentis, Bif. breve, Lactobacillus acidophilus,* and *Lact. casei*) and *Escherichia coli* [86].

Vulgarone B, a component of *Artemisia iwayomogi* essential oil, exhibited significant inhibitory activity against some antibiotic-susceptible and antibiotic-resistant human pathogens. Furthermore, the combination with oxacillin resulted in synergism against antibiotic-resistant *Staphylococcus aureus*. The antibiotic mechanism may involve bacterial DNA cleavage [87].

As mentioned earlier, artemisinin and dehydroleucodine show strong antimicrobial activity against *Helicobacter pylori*, the major cause of chronic gastritis and peptic ulcer [77, 78].

2.4.3. Antifungal. Vulgarone B, a sesquiterpene ketone isolated from the volatile fraction of *Artemisia douglasiana*, exhibited antifungal activity against *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides,* and *Botrytis cinerea*. Structure-activity studies revealed that the α,β-unsaturated carbonyl function is a prerequisite for the antifungal activity, so vulgarone B may act as Michael-type acceptor for biological nucleophiles [88].

Artemisinin and its derivatives showed antifungal properties against *Pneumocystis carinii in vitro* [89, 90].

While investigating the action of various sesquiterpene lactones on the growth patterns of four fungal genera, *Colletotrichum*, *Fusarium*, *Botrytis*, and *Phomopsis,* Wedge et al. noticed that the most effective compounds are those that contain an αMγL group but lack bulky sterically inhibitory groups, which limit access to the αMγL. Also, nonpolar or weakly polar compounds were more bioactive and sesquiterpene lactones of a guaianolide structure had the greatest antifungal potency [91].

2.4.4. Antiviral. Several *in vitro* studies showed that artemisinin has antiviral effect on hepatitis B and C viruses [92, 93], a range of human herpes viruses (human cytomegalovirus, herpes simplex virus type 1, and Epstein-Barr virus) [94–96], influenza virus A [97], and a bovine viral diarrhea virus [98] in the low micromolar range. Artesunate was used successfully for reducing the number of CMV (human herpes virus 5) in an immunosuppressed child without traceable toxicity [99].

3. Methods of Analysis of Sesquiterpene Lactones

3.1. Extraction and Isolation. The extraction methods of SLs may include common procedures, such as extraction using shaker, sonication process, reflux extraction, or Soxhlet extraction, but also less handy methods, like supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE). The extraction solvents found in the literature are n-hexane, petroleum ether, methanol, acetonitrile, chloroform, toluene, and combinations of them, at different concentrations and different periods of time (from several seconds to days). Isolation of SLs is achieved by further purifying the obtained extracts through repeated column chromatography using different stationary phases (usually normal-phase silica gel) and eluents of increasing polarity (usually hexane-ethyl acetate mixture). The resulting fractions are monitored by TLC in order to separate the compounds of interest. For exemplification, some methods of extraction for well-known SLs in *Artemisia* genus will be described hereafter.

Dehydroleucodine was extracted from *A. douglassiana* after soaking the plant material in chloroform at room temperature, evaporating to dryness and dissolving the extract in ethanol 95%. After removing impurities by treatment with lead tetraacetate solution, the filtrate was extracted three times with chloroform and the resulting extract was chromatographed with ethyl acetate-hexane (1 : 9) to yield dehydroleucodine [71, 100].

Dehydroleucodine and a new SL, named dehydroparishin-B, were identified in the chloroform extract from the aerial parts of *A. douglasiana*. After exhaustively boiling the plant material with chloroform, the extract was partitioned with aqueous 5% $NaHCO_3$. The organic phase was subjected to repeated column chromatography on silica gel with hexane-ethyl acetate mixtures to afford dehydroleucodine. The aqueous $NaHCO_3$ phase was acidified and extracted with ethyl acetate. The organic phase was evaporated to dryness and subjected to column chromatography, while monitoring the resulting fractions by TLC to give pure dehydroparishin-B [101].

Tehranolide was extracted from *A. diffusa* by maceration 24 hours with a mixture of n-hexane/ethyl acetate/methanol (1 : 1 : 1). The concentrated extract was run through a silica gel column with n-hexane/ethyl acetate mixtures of increasing polarities and further with n-hexane/ethyl acetate/methanol mixtures to produce higher polarities. TLC was used to monitor the fractions and tehranolide was identified by ^{13}C-NMR spectra [102].

Yomogin was extracted from the aerial parts of *A. princeps* with methanol at room temperature for 7 days. The concentrated extract was suspended in water and partitioned with dichloromethane and ethyl acetate. Dichloromethane fraction was column chromatographed over silica gel using a gradient elution of methanol and dichloromethane. The bioactive fraction was further purified through repeated column chromatography with hexane and ethyl acetate mixture to give yomogin [60].

Bioactivity guided fractionation was used to isolate yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide from *A. vulgaris* leaves. The plant material was defatted by soaking in hexane for 24 hours, and then the chloroform extract was obtained and chromatographed on a Sephadex LH-20 column with 80% methanol : 20% chloroform. Each subfraction was tested on the guinea pig ileum in order to assess the histamine antagonist activity. The active ones were combined and further purified by repeated preparative TLC, giving a mixture of the two compounds. Alternatively, to increase the yield of active compounds, the crude chloroform extract was defatted repeatedly with petroleum ether and run through silica gel column chromatography using gradient

elution of ethyl acetate and dichloromethane to afford a mixture of the same two components. Yomogin was separated through repeated recrystallization with methanol and its structure was confirmed by X-ray crystallography [62].

As a result of impressive hypoglycemic effects *in vivo*, an infusion from the aerial parts of *A. ludoviciana* was subjected to column chromatography in order to identify the active compounds. The dried infusion was partitioned between ethyl acetate and water and the organic phase was chromatographed repeatedly on normal-phase silica gel with ethyl acetate and hexane, yielding six compounds. One of the compounds was the known guaianolide ludartin which manifested significant hypoglycemic effect on its own [103]. Ludartin was also isolated from the crude hexane extract of *A. amygdalina* shoots using a similar procedure [35].

Bioactivity guided fractionation led to the isolation of leucodin from *A. iwayomogi* as moderate antioxidant and antimicrobial compound. The 80% ethanol extract was partitioned successively with n-hexane, chloroform, ethyl acetate, and n-butanol and the fractions were tested for biological activity. The active ethyl acetate-soluble fraction was purified trough repeated column chromatography to yield five compounds, characterized based on EI-MS, UV, IR, and NMR spectral data [104].

Santonin is usually isolated from its primary sources, *A. cina* and *A. maritima*, through chloroform extraction, formation of a barium salt, precipitation of the lactone by acidification, and crystallization from ethanol : water [105]. A new method for santonin extraction from the flowering tops of *A. caerulescens* ssp. *cretacea* involves maceration and percolation over aluminium oxide column with 5% methanol in chloroform. The concentrated extract is exhaustively extracted with boiling water and the aqueous solutions are extracted with chloroform to yield santonin [106]. Santonin was also extracted from aerial parts of *A. pallens* with acetone or acetone : methanol, followed by extract fractionation on silica gel using n-hexane and hexane : acetone [107, 108].

For artemisinin extraction, the most applied technique is liquid solvent extraction with toluene, n-hexane, chloroform, or petroleum ether and extraction times ranging from a few minutes to several hours. The first published laboratory method for artemisinin isolation consisted in the extraction of *A. annua* leaves with petroleum ether followed by column chromatography of the extract over silica gel and elution with a chloroform-ethyl acetate mixture [109].

Artemisinin and its precursors, arteannuin B and artemisinic acid, were isolated from *A. annua* leaves after 100% ethanol extraction at room temperature and fractionation with ethyl acetate and column chromatography of ethyl acetate phase on silica gel with a mixture of petroleum ether and ethyl acetate of increasing polarity. The fractions were monitored through TLC, combined and purified by crystallization to afford the SLs [54].

Rhianna Briars and Larysa Paniwnyk compared a conventional method of extraction of artemisinin from *Artemisia annua* leaf with hexane in a water bath at temperature of 25°C, 35°C, and 45°C with an ultrasonic extraction at the same temperature. After HPLC analysis it was observed that ultrasonic extraction at lower temperature is better than at a higher temperature, also improving the purity [110].

An efficient and fast method with low consumption of solvents is the microwave-assisted extraction (MAE) [111]. Extraction of artemisinin by microwave-assisted extraction was performed in a closed vessel apparatus allowing temperature control and programmable heating power. Extractions were carried out with water, ethanol, toluene, or n-hexane, at 60°C temperature, except for hexane (35°C). Artemisinin recovery was similar with ethanol, toluene, and n-hexane. Water extraction did not succeed as the plant extract degraded in this solvent. Optimal extraction conditions were extraction time 12 minutes, vegetal particles diameter 0,125 mm, and solvent/plant ratio higher than 11 [112]. Liu et al. compared four methods of artemisinin extraction from leaves, flower buds, stems, and roots of *Artemisia annua*: an extraction method at room temperature, heat-reflux extraction at 50°C, a Soxhlet extraction at 50°C, and microwave-assisted extraction at the same temperature of 50°C. They demonstrated that after MAE extraction a high recovery of artemisinin is obtained in less time and with less consumption of reagents [113].

Pressurized solvent extraction (PSE) uses conventional solvents at elevated temperatures and pressures which bring about liquid extraction above the boiling point of the solvent. This technique was applied to powdered *Artemisia annua* leaves loaded into an extraction cell and placed in a thermostated oven. The selected extraction solvent (water or ethanol) was pumped through the extraction cell at a flow-rate of 0,5 mL/min for 20 minutes. Pressure had no noticeable influence on the recovery of artemisinin, whatever the solvent used, but a higher temperature significantly favoured artemisinin extraction, particularly in water [114].

In recent years, supercritical fluid extraction (SFE) has become the method of choice for the extraction of secondary metabolites from plant material. Thus, using a supercritical fluid composed of CO_2 and 3% methanol at 50°C temperature, 15 MPa pressure, and 2 mL/min flow-rate, artemisinin was quantitatively extracted from the aerial parts of the plant. These mild conditions avoid the degradation of the analytes and allowed us to obtain clean plant extract that does not need further purification. By adding 16.25% ethanol as cosolvent to the supercritical fluid extraction with CO_2, the artemisinin extraction yields were substantially improved [115, 116].

In order to analyze artemisinin and artemisinic acid, Kohler et al. associated supercritical fluid extraction (SFE) with supercritical fluid chromatography (SFC) coupled with flame ionization detector (FID), which allowed the determination of compounds without a precleaning step [117].

Two SLs from *A. princeps*, artecanin and canin, were isolated through chromatographic separation of the methanol extract and identified by MS and NMR data analysis. After partitioning the methanol extract with hexane and dichloromethane, the latter fraction was subjected to repeated column chromatography on silica gel with dichloromethane-methanol and ethyl acetate-hexane mixtures. The selected fraction was further chromatographed on Sephadex LH-20 column with dichloromethane-methanol (1 : 1) and subfractions subjected to flash-chromatography on RP-18 column

with methanol-water mixtures to afford artecanin and canin [118].

Using preparative chromatographic techniques, Martins et al. isolated from the chloroform extract of *A. gorgonum* 11 compounds that included SLs arborescin, arglabin, deacetylglobicin, 2α-hydroxyarborescin, sanchillin, and hanphyllin. The extract was run over silica gel columns and eluted with ethyl acetate/n-hexane, ethyl acetate/toluene, and dichloromethane/methanol mixtures of increasing polarity [119].

Two new guaianolides were isolated from *A. argyi* leaves after extraction with 95% ethanol and partition of the concentrated extract between petroleum ether and chloroform. The chloroform extract was repeatedly fractioned and the resulting subfractions were purified by semipreparative HPLC to afford artemisinin A and isoartemisolide [120].

3.2. Detection and Quantification

3.2.1. UV-Vis Spectrophotometry. When applying a cheap, simple, and handy method to all laboratories, such as spectrophotometry, a problem can occur in case of analytes that do not have specific chromophore groups in molecule [121] and thus have no significant absorption in the UV-Vis work domain and also do not possess specific chemical groups able to react with certain compounds to form colored products [122]. For these reasons, analysis of sesquiterpene lactones through UV-Vis spectrophotometry is not an easy task.

One of the specific methods for determination of artemisinin in UV domain has the next principle: absorbance measurement of a reaction product of artemisinin in strong alkaline solution. The reaction is completed in 15 minutes and the reaction product is stable for 5 hours [123]. For dissolution of artemisinin different solvents can be used like DMSO, methanol, ethanol, ethyl acetate, and sodium hydroxide and as alkaline reagents potassium hydroxide, calcium hydroxide, sodium carbonate, and sodium bicarbonate. The interaction between artemisinin and the alkaline medium produces a homogenous electronic transition band at 250–330 nm, with a maximum absorbance at 291 nm, and the resulting product is monotype, as shown by the Gaussian curve (bell shape curve). All solvents used show similar spectral resolutions, but peak intensity is decreasing in the order: DMSO, methanol, ethanol, and ethyl acetate. Also, the best reactivity was recorded in the case of sodium hydroxide and potassium hydroxide, with the peak transition varying with concentration.

Just like artemisinin, determination of artesunate is challenging because it has not a distinct chromophore and presents a peroxide bridge which absorbs at lower wavelengths [114, 124]. In order to determine artesunate in tablets, a very simple and sensitive method can be used: artesunate tablets are dissolved in simulated intestinal fluid (monobasic potassium phosphate and sodium hydroxide, pH 6.8) and the absorbance is measured. The pH 6.8 protects the basic chemical nucleus without breaking the lactone ring. The maximum reproducible absorbance is reached at 287 nm with good values of detection limit and quantification limit. Excipients do not interfere with the determination [125].

Other methods approach the endoperoxide ring destruction and introduction in the molecule of at least one double bond. For this purpose, a method comprising two steps has been developed and validated: ethanol solution of artesunate was subjected to alkaline hydrolysis with sodium hydroxide at 50 ± 0.1°C for 60 minutes. After cooling, the solution was treated with acetic acid in ethanol, and the reaction product had an absorbance maximum at 242 nm, yielding apparently furanose acetal, which presents conjugated double bonds, a chromophore with UV absorption [126].

Direct determination of artemisinin and its derivatives in the visible domain, by treatment with certain reagents in order to obtain colored compounds, is not possible, because these artemisinins do not have chemical groups that react easily. Thus, it is necessary to have an intermediate step in which by treatment with acids or bases they are transformed into more reactive compounds, such as enolate/carboxylates or α,β-unsaturated decalones, and then reaction with certain reagents to form colored compounds or to bleach, depending on concentration [122, 127, 128].

Thus, for determination of artemisinin and its derivatives accurate, simple, and fast spectrophotometric methods have been developed and validated. They are based on the cleavage of endoperoxide linkage in acidic medium (hydrochloric acid), releasing hydrogen peroxide (H_2O_2), which reacts with potassium iodide and releases iodine in equivalent amount. Further, the released iodine reacts with various chromogenic agents. As chromogenic agent safranin O can be used. A constant and maximum absorbance is obtained in the range of pH 4-5, registered at 521 nm, with the system being stable for a period of 2 hours. There is a bleach of red colored safranin O, which is transformed in leuco form proportional to the concentration of the analyte. In case of artemisinin determination from tablets, the interferences level was considered acceptable and the excipients used did not hinder the determination [129]. The same method can be applied in the same conditions for artesunate determination [130].

Other chromogenic agents used for determination of both artemisinin and artesunate from tablets are methylene blue and soluble starch. In the first case, the released iodine from the same reaction bleaches methylene blue proportional to concentration and the absorbance is recorded at 665.6 nm. In the second case, the released iodine will form a violet colour with starch, the colour is proportional to the concentration of the analyte, and the absorbance is recorded at 445.6 nm. In case of artesunate, the method which uses methylene blue is more sensitive and more selective, and in case of artemisinin the one who uses soluble starch [121].

Determination of artesunate can also be performed using variamine blue as chromogenic agent. The released iodine oxidizes the leuco form of variamine blue and forms a purple colour compound, the colour being proportional to the concentration of the analyte and is recorded at 556 nm.

Another accurate and precise method for determination of artemisinin and its derivatives is based on a decomposition process in acidic medium at elevated temperatures. The resulted compound presents reactive methylene centres that have the ability to quickly release protons and reduce

an acidic solution of p-dimethylaminobenzaldehyde to [4-(dimethylamino) phenyl] methanol, at an optimal temperature of 60°C for 25 minutes. The purple colored product is stable for 4 hours in the laboratory environment and has a maximum absorbance at 540 nm, proportional to the analyte concentration [122].

Artesunate analysis can be done through a simple and inexpensive method after alkaline decomposition of the compound and the reaction between decomposition product with a diazonium salt, 1,5-naphthalene disulfonate salt (Fast Red TR salt). The reaction is pH dependent and positive for artesunate at pH 4. A yellow coloration will be obtained, proportional to the concentration of the analyte, with maximum of absorbance at 420 nm [131, 132]. This test cannot be applied for artemether and therefore Green et al. have developed a method to determine artemether, artesunate, and dihydroartemisinin. In that case decomposition was performed in acidic medium, and after an incubation period of 4 hours, α,β-unsaturated decalone was obtained. This compound was diazo-coupled with the same diazonium Fast Red TR salt, producing a yellow colour in 5 minutes [132].

For analysis of dihydroartemisinin from tablets, a derivatization reaction can be used, after decomposition in acidic medium, at high-temperature (90°C) with formation of a carbonyl compound. The carbonyl compound reacts with p-nitroaniline, yielding a yellow coloured adduct which shows peaks at 205, 230, and 380 nm [133].

In case of artemisinins analysis from tablets, the extraction of interest analytes is unnecessary because the excipients do not influence the methods described until now, in case analysis from plants is necessary to separate them from the vegetal product and then perform spectrophotometric analysis. An example is that of artemisinin extracted with toluene from the vegetal product in [134] and subjected to a process of alkaline hydrolysis at 50°C for 45 minutes. A mixture of ethanol and trifluoroacetic acid was used as solvent for determination of absorbance at 218 nm [135].

Another example of artemisinin analysis from *Artemisia annua* plant implies Soxhlet extraction with petroleum ether and n-hexane prior to derivatization. Derivatization is achieved by treatment with 0.25% NaOH solution at 50°C for 1 and 1.5 hours and then neutralization with acetic acid 0.2 M. UV spectra are recorded at 203 nm prior to and at 258 nm after derivatization. It was observed that after derivatization the extinction coefficient increased 30–40 times [136].

3.2.2. High Performance Liquid Chromatography. HPLC is the most commonly used technique for the quantification of artemisinin and its derivatives in plants, using detection methods like UV detection (HPLC-UV) or diode array detection (HPLC-DAD), mass spectrometry (HPLC-MS), HPLC/tandem mass spectrometry (LC/MS/MS), evaporative light scattering detector (HPLC-ELSD), and electrochemical detection (HPLC-ECD).

The HPLC-UV and HPLC-DAD analysis requires a pre- or postcolumn derivatization [137], process which in case of HPLC-ELSD is not necessary, which means an advantage of the latter method. On the other hand, its sensitivity is lower than other detection methods, such as ECD and MS [113, 138, 139]. A disadvantage of HPLC-ECD is the requirement of elimination of oxygen from system [117]. HPLC analysis of artemisinin and its derivatives is influenced by the extraction method from vegetal products, mobile phase, column, and detector used.

Since artemisinin detection is difficult, in most cases, after extraction the compound is derivatized using NaOH solutions of different concentrations (0.2%, 0.25%), at different temperatures, the optimum being 50°C, for a period of time ranging from 30 minutes to 1 hour, followed by neutralization with acetic acid of different concentrations (0.08 M or 0.2 M).

The HPLC columns used are suitable for determining the sesquiterpene lactones, with most authors using a normal phase C18 or a reversed-phase C18, but also LC-CN column or silica gel RP-60, with different dimensions: length 50–250 mm, 2.1–4.6 mm ID, and 1.8–5 μm particle size. Generally the column temperature is 30°C, but analyses can be conducted at room temperature, too.

As elution methods, both gradient and isocratic elution were used. Mobile phases for isocratic elution consist of methanol : water/sodium phosphate buffer/acetonitrile. In case of gradient elution, the mobile phases contain different combinations: phosphate buffer : acetonitrile/methanol and methanol or water modified with trifluoroacetic acid to adjust the pH to 3.0–3.5 or phosphoric acid-methanol/acetonitrile.

Highly used detection techniques include UV/DAD from 254 to 350 nm depending on the compound, MS positive ESI mode, ELSD, and the association LC-DAD-MS that has a great specificity [140]. Table 1 contains an overview of HPLC methods applied to sesquiterpene lactones analysis in *Artemisia* species. The majority of HPLC analyses described in the literature are for artemisinin and related compounds.

3.2.3. Gas Chromatography. Sesquiterpene lactones analysis by gas chromatography (GC) is difficult because on one hand the majority of them are thermolabile substances and on the other hand they are less volatile compounds. For this reason, derivatization or transformation into stable degradation products is needed. Detection methods applied in sesquiterpene lactones analysis include GC-MS [141], GC-ECD, and GC-FID [139].

For example, *Artemisia pallens* extract was analysed by GC with MS detection (impact ionization), showing the presence of compounds, such as alpha-santonin, diisobutyl phthalate, tetradecane, and hexadecane [107].

Liu et al. have developed and validated a sensitive method of artemisinin quantification by gas chromatography with ECD detection (electron-capture detection), although not analyzing artemisinin as a whole molecule due to its thermal instability. This method uses small quantities of plant (*Artemisia annua*) and intermediate steps in processing the samples such as extraction, centrifugation, and evaporation have been removed. The samples were analyzed directly after a single solvent one-step extraction, with 97% recovery or more and a limit of detection and quantification less than 3 μg/mL and 9 μg/mL, respectively [113].

GC with flame ionization detection was also applied in analysis of SLs. A retention time of 7.57 minutes was recorded

Table 1: Summary of HPLC conditions for sesquiterpene lactones.

Sample	Extraction, derivatization	Detected sesquiterpene lactones	Detection	Chromatographic conditions	Reference
Sandy, clayey, and humic soil	(i) Supercritical fluid extraction (SFE) (ii) Supercritical fluid: CO_2 (iii) Extraction time: 20 minutes (iv) Precolumn derivatization (0.2% NaOH, 50°C, 30 min, then acidified 0.08 M acetic acid)	Artemisinin	UV, 260 nm	(i) Column: C18 Bio Wide Pore (25 cm × 4.6 mm, 5 μm) (ii) Column temperature: 30°C (iii) Elution type: isocratic (iv) The mobile phase: methanol/acetonitrile/0.9 mM Na_2HPO_4–3.6 mM NaH_2PO_4 buffer (pH 7.76) solution (45/10/45 v/v/v) (v) Injection volume: 20 μL (vi) Flow rate: 1 mL/min (vii) Retention time: 7.5 min	[154]
A. santonicum L., *A. taurica* Willd., *A. spicigera* K. Koch, *A. herba-alba* Asso, *A. haussknechtii* Boiss., *A. campestris* L., *A. araratica* Krasch., *A. armeniaca* Lam., *A. austriaca* Jacq., and *A. abrotanum* L.	(i) Extraction with n-hexane at room temperature for 2 days with a laboratory-scale shaker (ii) Precolumn derivatization (0.2% NaOH, 50°C, 30 min, then acidified 0.08 M acetic acid)	Artemisinin	DAD, 254 nm	(i) Column: ACE-5 C18 column (250 × 4.6 mm, 5 μm) (ii) Column temperature: 30°C (iii) Elution type: isocratic (iv) The mobile phase: formic acid (0.2% v/v) : acetonitrile (50 : 50 v/v) (v) Flow rate: 1 mL/min. (vi) Retention time: 5.58 min	[155]
Artemisia annua L	(i) Extraction in Soxhlet extractor with petroleum ether (30–60°C) for 6 h (ii) Precolumn derivatization (0.2% NaOH, 45°C, 30 min, and then acidified 0.08 M acetic acid)	Artemisinin	UV, 260 nm	(i) Column: RP-C18 silica column (250 × 4.6 mm, 5 μm) (ii) Column temperature: 30°C (iii) Elution type: isocratic (iv) The mobile phase: methanol/acetonitrile/0.9 mM Na_2HPO_4–3.6 mM NaH_2PO_4 buffer (pH 7.76) solution (45/10/45 v/v/v) (v) Injection volume: 10 μL (vi) Flow rate: 0.5 mL/min (vii) Retention time: 16.85 min	[156]
A. absinthium leaves	(i) Extraction with various solvent types: 100% methanol, 75% methanol, 50% methanol, 25% methanol, 75% acetonitrile in a thermostatic rotary shaker, and various temperatures (30–60°C) for various time intervals (ii) Precolumn derivatization (0.25% NaOH, 50°C, 1 hour and then being acidified with 0.2 M acetic acid solution)	Anabsinthin and derivatized artemisinin	DAD, 205 and 258 nm	(i) Column: C8 (250 mm × 4.6 mm, 5 μm) (ii) Column temperature: 30°C (iii) Flow rate: 1 mL/min (iv) Elution type: gradient elution: 90% A/10% B, hold for 5 min, to 60% B in the next 13 min (v) The mobile phase: A: methanol/0.1% trifluoroacetic acid (TFA) (15/85), B: methanol/0.1% TFA (85/15) (vi) Artemisinin not detected	[157]

Table 1: Continued.

Sample	Extraction, derivatization	Detected sesquiterpene lactones	Detection	Chromatographic conditions	Reference
A. annua leaves	(i) Dipping dried leaves in 100% chloroform, 8 s (ii) Precolumn derivatization (0.2% NaOH and then acidified with 0.08 M acetic acid solution)	Artemisinin	260 nm	(i) Column: RP-C18 silica column (250 × 4.6 mm) (ii) Elution type: isocratic (iii) The mobile phase: methanol/acetonitrile/0.9 mM Na_2HPO_4–3.6 mM NaH_2PO_4 buffer (pH 7.76) solution (45/10/45 v/v/v) (iv) injection volume: 1 μL	[158]
A. annua leaves	(i) Solid phase extraction (ii) Liquid-liquid extraction method (iii) Purification procedures	Artemisinin	IR	(i) Column: LC-CN column (25 mm × 4 mm × 5 μm) (ii) Column temperature: 35°C (iii) Elution type: isocratic (iv) The mobile phase: methanol : water (60 : 40 v/v) (v) Injection volume: 20 μL (vi) Flow rate: of 1 mL/min (vii) Retention time: 6.932 min	[159]
A. annua	Extraction by refluxing with hexane at 75°C for 1 hour	Artemisinin	ELSD	(i) Column: C18-RP (250 mm × 4.0 mm, 5 μm) (ii) Column temperature: room temperature (iii) Elution type: isocratic (iv) The mobile phase: water adjusted to pH 3.0–3.5 with trifluoroacetic acid (TFA) : acetonitrile (65 : 35) (v) Flow rate: 1.0 mL/min (vi) Retention time: 7.63 min	[138]
A. herba alba and *A. monosperma* aerial parts	Extraction in Soxhlet extractor with methanol at 60°C	α, β-Dihydroartemisinin, dihydroartemisinic aldehyde, arteannuin B, dihydroartemisinic acid, dihydroartemisinic alcohol, artemisitene, artemisinin, and artemisinic acid	HPLC-DAD (215, 254, 294, and 334 nm), LC-positive mode ESI-MSn	(i) Column: C18 column (50 mm × 2.1 mm, 1.8 μm) (ii) Column temperature: 30°C (iii) Elution type: gradient, 0 min—A : B 10 : 90; 36 min—A : B 100 : 0; 40 min—A : B 100 : 0 (iv) The mobile phase: A: methanol, B: 0.2% formic acid (v) Injection volume: 10 μL (vi) Flow rate: 0.2 mL/min (vii) Retention time: 12.4 min α-dihydroartemisinin, 12.8 min β-dihydroartemisinin, 15.2 min dihydroartemisinic aldehyde, 15.5 min arteannuin B, 15.7 min dihydroartemisinic acid, 18.8 min dihydroartemisinic alcohol, 36.6 min artemisitene, 37.7 min artemisinin, and 23.7 min artemisinic acid	[160]

Table 1: Continued.

Sample	Extraction, derivatization	Detected sesquiterpene lactones	Detection	Chromatographic conditions	Reference
A. annua	(i) Extracted twice with scintanalyzed toluene in a ultrasonic bath, in ice-cold water, for 30 minutes (ii) Precolumn derivatization	Artemisinin	260 nm	(i) Column: C-18 column (15 cm × 4.6 mm, 5 μm) (ii) Elution type: isocratic (iii) The mobile phase: 0.01 M sodium phosphate buffer: methanol [55 : 45 (v/v)] pH 7.0 (iv) Flow rate: 1 mL/min (v) Retention time: 12.0 min	[161]
A. annua leaves	Extraction in Soxhlet extractor with petroleum ether : n-hexane (2 : 1) for 4 hours	Artemisinin	DAD, 258 nm	(i) Column: C8 (250 × 4.6 mm, 5 μm) (ii) Column temperature: 30°C (iii) Elution type: gradient, 5 min—70% A: 30% B to 60% B in the next 13 min (iv) The mobile phase: A: 0.9 mM Na_2HPO_4, 3.6 mM NaH_2PO_4 buffer (pH 7.76); B: acetonitrile (v) Injection volume: 20 μL (vi) Flow rate: 1 mL/min (vii) Retention time: 6.476 min	[136]
A. annua leaves	(i) Sonication with toluene (ii) Precolumn derivatization (0.2% NaOH, 50°C, 45 min and then acidified 0.2 M acetic acid)	Artemisinin	DAD, 258 nm	(i) Column SB C18 column (150 × 4.6 mm) 5 μm (ii) Elution type: isocratic (iii) The mobile phase: 45% (v/v) methanol and 55% 0.01 M sodium phosphate buffer (pH7.0) (iv) Flow rate: 1 mL/min (v) Retention time: 12 min	[162]
A. annua L leaves, flower buds, stems, and roots	(i) Room temperature extraction (ii) Heat-reflux extraction at 50°C (iii) Soxhlet extraction at 50°C (iv) MAE (microwave-assisted extraction) (v) Solvent: petroleum ether : acetone (4 : 1, v/v)	Artemisinin	ELSD	(i) Column: RP-C18 column (150 mm × 4.6 mm i.d., 5 μm) (ii) Column temperature: 30°C (iii) Elution type: isocratic (iv) The mobile phase: water : acetonitrile (40 : 60 v/v) (v) Injection volume: 10 μL (vi) Flow rate: 1 mL/min (vii) Retention time: 9 min	[113]
A. annua L	Extraction with methanol by sonication, 45 minutes	Artemisinin	LC-MS with SIM	(i) Column: ODS3 column (250 × 4.6 mm, 5 μm) (ii) Elution type: gradient, 72% B for 6 min, and it was then increased to 100% B in 1 min (iii) The mobile phase: water (0.1% formic acid) and (B) acetonitrile (iv) Injection volume: 2 μL (v) Flow rate: 1.2 mL/min (vi) m/z 265.3	[163]

Table 1: Continued.

Sample	Extraction, derivatization	Detected sesquiterpene lactones	Detection	Chromatographic conditions	Reference
A. annua seeds, aerial parts	Extraction in Soxhlet extractor with methanol, 60°C	Artemisinin	HPLC/DAD 214, 217, 280, and 290 nm HPLC-positive mode ESI-MS	(i) Column: SB-C18 column (150 mm × 4.6 mm i.d., 1.8 μm) (ii) Column temperature: 30°C (iii) Elution type: gradient, 0 min, A : B 10 : 90; 36 min, A : B 70 : 30; 50 min, A : B 100 : 0; 60 min (iv) The mobile phase: (A) methanol and (B) 0.2% formic acid (v) Flow rate: 0.8 mL/min (vi) Retention time: 35.2 min	[140]
A. annua L	Maceration with dichloromethane or hexane, at room temperature, for 72 hours	Artemisinin	HPLC-DAD (210 nm), HPLC-MS (API electrospray)	(i) Column: RP-18 column (250 mm × 4.6 mm i.d., 5 μm) (ii) Column temperature: 26°C (iii) Elution type: isocratic (iv) The mobile phase: water adjusted to pH 3.2 by formic acid (A), and acetonitrile (B) 50% A : 50% B (v) Injection volume: 20 μL (vi) Flow rate: 1.3 mL/min (vii) Retention time: 15.1 min	[153]
A. annua L	Extraction with different solvents and mixtures: n-hexane, isopropyl alcohol, ethanol, toluene by maceration, percolation, or decoction, at low temperatures and vigorous shaking	Artemisinin, arteannuin, and artemisitone	RP-HPLC/refraction index	(i) Column: RP-C18 column (100 mm × 4 mm i.d., 3 μm) (ii) Column temperature: room temperature (iii) Elution type: isocratic (iv) The mobile phase: methanol (80–90%), water (v) Injection volume: 100 μL (vi) Flow rate: 0.5 mL/min (vii) Retention time: 1.7 min (arteannuin), 2 min (artemisinin), and 5.3 min (artemisitone)	[142]

for artemisinin, but other peaks were observed at around 2.35 minutes (artemisinic acid), 4.3 minutes (deoxyartemisinin), and 4.59 minutes (arteannuin B) and their identity has to be confirmed by mass spectrometric detection. The analysis of artemisinin by GC-FID is made via major degradation products, unlike HPLC-ELSD [138]. Another GC-FID method applied to determine artemisinin from *Artemisia annua* extract in hexane led to a retention time of 24.6/34,4 minutes and 30.6/32.2 minutes for arteannuin B [142].

3.2.4. Thin Layer Chromatography. For fast and simple analysis, TLC-densitometric technique can be used. This is based on the transformation of artemisinin after treatment with ammonia vapors in a compound containing chromophore group, 10-azadesoxyartemisinin, detected by UV based TLC densitometry [143].

Simultaneously, determination of artemisinin, arteannuin-B, and artemisinic acid at nanograms levels from *Artemisia annua* can be achieved by using RP-18 F_{254S} thin-layer chromatographic plates, mobile phase containing 0.2% trifluoroacetic acid in water/acetonitrile (35 : 65, v/v), derivatization with anisaldehyde reagent in acidic medium, and densitometric determination at 426 nm in absorption-reflectance mode [144].

Widmer et al. developed an extract from *Artemisia annua* leaves (obtained by sonication in toluene) on silica gel 60 plates with mobile phase cyclohexane : ethyl acetate : acetic acid (20 : 10 : 1), derivatization with anisaldehyde reagent in ethanol : water (10 : 8), heated at 100°C, for 12 minutes, and densitometric evaluation of fluorescence at 520 nm [145].

TLC evaluation of artemisinin can be carried out on silica gel RP-18 60 F_{254} plates, with mobile phase methanol : acetonitrile : ethyl acetate : acetic acid (30 : 20 : 2 : 1) and densitometric evaluation at 254 nm. The limits of detection and quantification were 4 μg/mL and 10 μg/mL, respectively.

Rimada et al. chose for TLC analysis silica gel with fluorescent indicator, at 254 nm, followed by derivatization with 1% vanillin in sulphuric acid, at 105–110°C. The results demonstrated the presence of arteannuin B, artemisinin, and artemisitone [142].

In order to analyze santonin, an accurate, reproducible, simple, and rapid high performance thin layer chromatography method (HPTLC) has been developed. Precoated aluminum plates with silica gel 60 F_{254} were used, mobile phase hexanes : ethyl acetate (3 : 2), and densitometric detection at 258 nm [108].

3.2.5. Other Methods of Analysis. Another method used to analyze artemisinin and artemisinic acid is supercritical fluid chromatography (SFC) coupled with flame ionization detector (FID) [117].

Reys et al. developed a feasible alternative method that uses an amperometric detector based on hemin adsorbed on silica gel modified for quantification of artemisinin [146].

Also, ^{1}HNMR is a suitable and valid method applied by Rimada et al. for artemisinin analysis in purified *Artemisia annua* extract in presence of N, N-dimethyl-formamide as internal standard [142].

3.3. Structure Identification. The complete elucidation of a compound structure by a single method is an impossible mission, this being achieved by a combination of several methods of analysis, among which IR, NMR, and MS.

In IR spectroscopy, due to the complexity of the spectra, specific bands are attributable accurately only when these are intense and correspond to groups such as carbonyl, hydroxyl, C–H, and aromatic rings. It is important to remember that there are no two different substances with the same IR spectrum, especially when using the area under the 1500 cm^{-1}, which is considered the fingerprinting area.

Through magnetic resonance imaging the following characteristics can be established: the structural data of the organic compounds, the dynamic properties of the molecules, the quantitative analysis of the compounds as such or in mixtures, the percentage of hydrogen in an unknown sample, the number and type of carbon atoms in the structure, and the position of carbon atoms, with or without protons.

Mass spectroscopy is an instrumental method of analysis which is based on the fragmentation of the molecules of organic substances by radiation with high energy, up to 100 eV, and the analysis of the number, the charge, and mass of the resulting fragments to obtain information on the structure and identity of researched substances. Due to energy accumulation, fragmentation of molecules occurs with breaking of interatomic bonds, a process resulting mostly in positive ions (seldom negative), radicals, radical ions, and neutral molecules. These fragments constitute important parts in the recreation of the molecular structure.

Next, the spectra of well-known sesquiterpene lactones from *Artemisia* genus are discussed comparatively : artemisinin, dihydroartemisinin, artesunate, and artemether (Figure 2). Artesunate is obtained from the reduction of artemisinin to dihydroartemisinin and esterification of the latter with succinic anhydride and artemether is obtained by treatment of dihydroartemisinin with methanol and an acid catalyst [147].

In the case of IR spectra, the common elements are attributed to stretching vibrations of C=O (1420–1300 cm^{-1}), C–O (1380–1370 cm^{-1}, 1235 cm^{-1}, and 1093 cm^{-1}), C–O–O–C (890–820 cm^{-1}, 1121.62 cm^{-1}), O–O (825 cm^{-1}), C–O–C (1023.89 cm^{-1} and 1277.83 cm^{-1}), C–H bending vibrations (1225–950 cm^{-1}), C–H stretching vibrations (2844.99, 2873.61, 2914.58, and 2936.97 cm^{-1}), rocking vibration of CH_2 (700 cm^{-1}) and CH_3 (2947 cm^{-1}), and aromatic ring vibrations (1650–1400 cm^{-1} and 2000–1620 cm^{-1}) [121, 148, 149].

Differences occur in the region 1750–1725 cm^{-1} and 1005–925 cm^{-1}, for vibrations of C–O–C=O and CH_2–CH_2 bonds in artesunate and the appearance of vibrations that prove transformation of C=O in C–O (1034.14 cm^{-1}) and the presence of OH group (3371.57 cm^{-1}) in dihydroartemisinin [148].

The spectrum of artemisinin and dihydroartemisinin contains each 15 carbon atoms, which consists of 3 methyl groups (CH_3) 4 methylene groups (CH_2), 5 methine groups (CH), and 3 quaternary carbon atoms [148]. The artemether

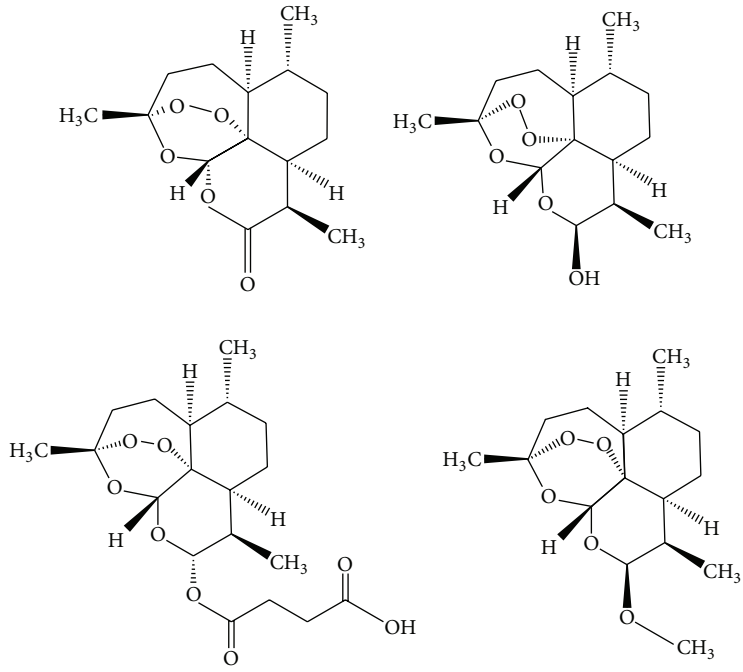

Figure 2: Artemisinin, dihydroartemisinin, artesunate, and artemether.

spectrum contains 16 carbon atoms, having an extra methyl group (CH_3) in position 10, and artesunate contains 19 carbon atoms, with the addition of a succinyl group in position 10.

For the methyl groups in positions 3, 6, and 9, the chemical shifts in ^{13}C NMR spectrum recorded the values 13.37, 20.56, and 26.26 ppm. For C-10 atom, if artemisinin corresponds to a chemical shift of 172.24 ppm, the transformation of carbonyl group in OH group is demonstrated by a change in the value to 96.60 ppm [148].

The ^{1}H NMR spectra showed the presence of the hydroxyl group at 2.77 ppm (singlet 1H). There were also observed chemical shifts for the protons of the methyl groups at positions 3, 6, and 9 (singlet, 1.43 ppm, and 0.96 ppm doublet) and of methylene groups in positions 4, 5, 7, and 8, multiplet signal.

Another example of structure elucidation is illustrated by the work of Tian et al. who identified three new eudesmane sesquiterpene lactones called artemivestinolides A-C [150] and three rare sesquiterpenes called arvestolide A-C [151] from *Artemisia vestita* (Figure 3).

The IR spectra of the 6 compounds highlight the presence of OH group in artemivestinolides and arvestolide A at 3478 cm^{-1} and 3354 cm^{-1}, respectively. The carbonyl group is present in all cases at 1766, 1726 cm^{-1}/1783, 1733 cm^{-1}/1773, 1734 cm^{-1}/1778, and 1734 cm^{-1}. The double bond is only observed in arvestolides B and C, at 1641 and 1673 cm^{-1}.

In the ^{1}H NMR analysis, the 6 compounds presented signals corresponding to a secondary methyl group (δ_H 1.20/1.21/1.22/1.27/1.26/1.26), H-13; 2 tertiary methyl groups (δ_H 1.12/0.88/0.89/0.95/1.31/1.13), H-14, and (δ_H 2.04/2.13/-/2.08/2.05/2.01), H-17; 2 oxymethine protons (δ_H 5.13/4.71/4.15/4.69/4.97/5.10), H-1, and (δ_H 4.60/4.23/4.49/5.13/5.02/4.68), H-6; 2 olefinic protons in artemivestinolide (δ_H 5.10/5.20/5.03), H-15; and just 1 olefinic proton in both arvestolides B and C (δ_H 6.11/3.27), H-15.

In the case of ^{13}C NMR analysis, all six compounds, with the exception of artemivestinolide C (15 carbon atoms), have 17 carbon atoms, the skeleton being of type 6/6/3 for artemivestinolides, 6/8/3 for arvestolide A, and 5/8/3 for arvestolides B and C. In all compounds, the lactone carbonyl is highlighted, δ_C 179.1/179.7/180/177.13/176.7/186.4. In five compounds, the acetyl group was observed at C1 (δ_C 170.6/170.5) and for arvestolide A, the OH group at C4 (δ_C 73.7) [150, 151].

In the case of MS analysis, generally using ESI positive ionisation mode, the pseudo-molecular ions are in the form $[M + Na]^+$, $[M + H]^+$, $[2M + Na]^+$, $[2M + K]^+$, and $[M + NH4]^+$ [148, 152]. For example, $[M + Na]^+$ is observed in case of arvestolide A *m/z* 347.1465 [151] and artemisinin *m/z* 305 [153], $[M + H]^+$ in case of artecanin *m/z* 279.1235 [118].

4. Conclusions

Sesquiterpene lactones are large and structurally divers group of natural products, found almost ubiquitously in plants of *Asteraceae* family. Genus *Artemisia*, one of the largest in this family and with worldwide distribution, contains numerous valuable sesquiterpene lactones. They present a broad spectrum of biological activities, such as antitumor, antimalarial, anti-inflammatory, immunomodulatory, antiulcerogenic, antibacterial, antifungal, and antiviral. In most cases, the documented mechanism of action involves the presence of α-methylene-γ-lactones and α,β-unsaturated cyclopentenone ring. The present paper proposes an overview of biological activities and of methods used for the identification and quantification of sesquiterpene lactones found in *Artemisia*

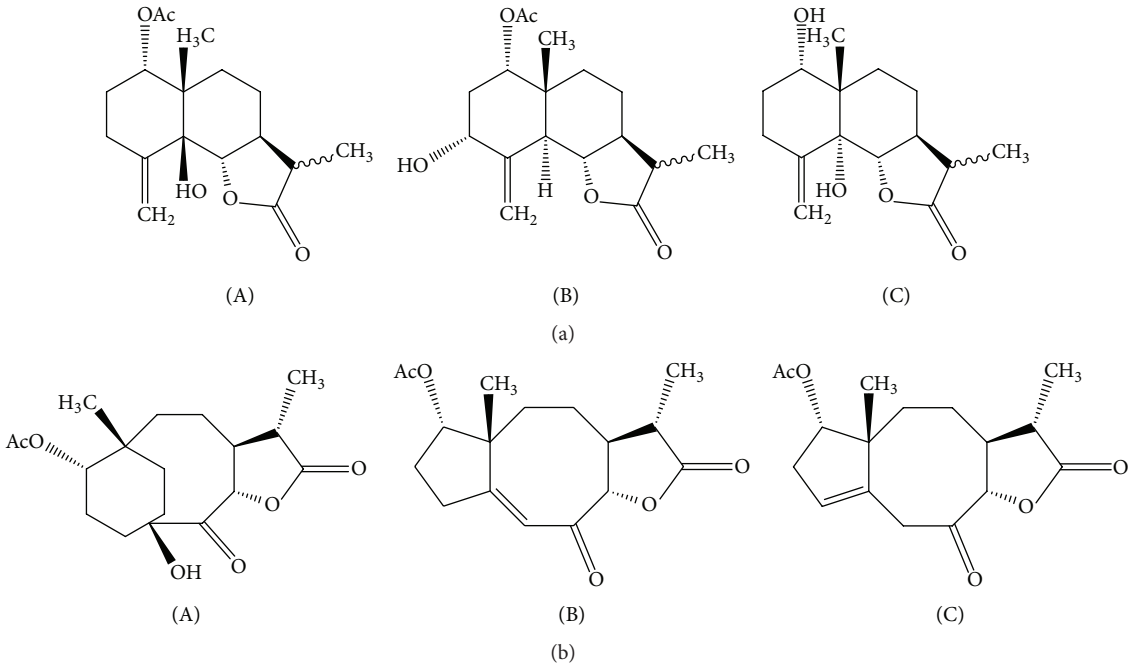

Figure 3: Artemivestinolides (A)–(C) (a) and arvestolide (A)–(C) (b) from *A. vestita*.

genus. The potential for drug development from *Artemisia* species continues to grow, particularly in the area of parasitic diseases and cancer treatment. The information summarized here is intended to serve as a reference tool to people in all fields of natural products chemistry.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007–2013, Project no. POSDRU/159/1.5/S/136893.

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