BOTANICALS IN RIBES NIGRUM L. BUD-PREPARATIONS: AN ANALYTICAL FINGERPRINTING TO EVALUATE THE BIOACTIVE CONTRIBUTION TO TOTAL PHYTOCOMPLEX

Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino

Via Leonardo da Vinci 44, 10095 - Grugliasco (TO), ITALY

BOTANICALS IN *RIBES NIGRUM* L. BUD-PREPARATIONS: AN ANALYTICAL FINGERPRINTING TO EVALUATE THE BIOACTIVE CONTRIBUTION TO TOTAL PHYTOCOMPLEX

Running head: BOTANICAL CHARACTERIZATION IN *RIBES NIGRUM* BUD-PREPARATIONS

Donno D.*, Beccaro G.L., Mellano M.G., Cerutti A.K., Marconi V., Bounous G.

* Corresponding author: e-mail: dario.donno@unito.it

Number of Figures: 6

Number of Tables: 6

ABSTRACT

Context: *Ribes nigrum* L. (Family: *Grossulariaceae*) is among the most commonly used herbal medicines and it is popularized for its alleged tonic effect and curative and restorative properties. The current practice of identifying herbal extracts is by measuring the concentration

of the main botanicals; their concentrations are used to characterize the herbal preparations and fingerprinting is recommended by the main Pharmacopeias as a potential and reliable strategy for the quality control of complex mixtures.

Objective: The aim of this research was to perform an analytical study of *Ribes nigrum* bud-preparations, in order to identify and quantify the main bioactive compounds, obtaining a specific chemical fingerprint to evaluate the single class contribution to herbal preparation phytocomplex.

Materials and methods: The same analyses were performed using a High Performance Liquid Chromatograph-Diode Array Detector both on University lab preparations and on commercial preparations from different Italian locations: different chromatographic methods were used to analyse the macerated samples, two for polyphenols and one for terpenic compounds.

Results: *Ribes nigrum* was identified as a rich source of anti-inflammatory and antioxidant compounds: the observed analytical firgerprint demonstrated that these bud-preparations represent a rich source of terpenic and polyphenolic compounds, especially catechins and phenolic acids.

Discussion and conclusion: Analytical fingerprinting could be an important tool to study the assessment of chemical composition and bioactivities of the plant-derived products, helping in find out new sources of natural health-promoting compounds: this study allowed to develop an effective tool for the quality control through the botanical fingerprinting of bud preparations.

Keywords: blackcurrant, flavonoids, phenolic acids, monoterpenes, bioactive profile, HPLC

INTRODUCTION

Ribes nigrum L. (Family: *Grossulariaceae*) is commonly used as herbal medicine and it is popularized for its alleged tonic effect and possible curative and restorative properties (Tabart et al., 2011; Tabart et al., 2012); *Ribes nigrum* is a shrub spontaneously growing in the cold and temperate climate zones and today many orchards with different genetic materials are realized in order to produce fruit, leaves and buds. The most important industrial product of *Ribes nigrum* is fruit; however, due to their particular chemical composition and excellent flavor, leaves and buds are also used in some applications as a raw material for the herbal and cosmetic industries: many people use the buds as medicinal preparation for their anti-inflammatory activity and anti-dermal diseases (eczema and psoriasis) (Dvaranauskaite et al., 2008).

For this reason, bud-preparations, derived from embryonic fresh plant tissues, are important therapeutic remedies, prescribed in hepatic, respiratory, circulatory and inflammatory disorders, but data on their chemical composition are lacking as, until now, phytochemical studies have principally been performed on barks, roots and root exudates, leaves, fruit and seeds (Peev et al., 2007; Donno et al., 2012a).

Polyphenols and terpenes are the dominant majority of biologically active plant compounds with antioxidative and anti-inflammatory properties: these secondary plant metabolites may be nutritionally important and play critical roles in human health in the prevention of chronic diseases such as pulmonary inflammation, cancer, cardiovascular and neurodegenerative diseases (Zhang et al., 2009; Komes et al., 2011; Mattila et al., 2011; Donno et al., 2012b; Tabart et al., 2012).

By nature herbal preparations are complex matrices, comprising a multitude of compounds, which are prone to variation due to environmental factors and manufacturing conditions (Komes et al., 2011; Steinmann & Ganzera, 2011; Donno et al., 2012a; Edwards et al.,

2012). The analysis of plant and herbal preparation secondary metabolites is a challenging task because of their chemical diversity: low variability is usually observed even within the same species and an herbal preparation detailed chemical profile is certainly necessary also to ensure the reliability and repeatability of clinical and pharmacological studies (Mok & Chau, 2006).

It is estimated that 100,000–200,000 metabolites occur in the plant kingdom (Oksman-Caldentey & Inze, 2004), and only highly selective and sensitive methods will be suitable for controlling their composition and quality because many traditional herbal preparations contain several medicinal plants, (Steinmann & Ganzera, 2011): the most important chromatographic or electrophoretic techniques coupled to different detectors are employed for this purpose. High Performance Liquid Chromatography (HPLC) is still the preferred separation technique for the analysis of natural products (Gray et al., 2010).

The current practice for herbal extract identification is by measuring the concentration of the main bioactive compounds, called “markers”: the concentrations of the main chemical components are used to characterize the herbal preparation (Mok & Chau, 2006) and referred to as the “fingerprint”: indeed, some studies showed that synergistic or additive biological effects of different phytochemicals (phytocomplex) contribute to disease prevention better than a single compound or a group of compounds (Jia et al., 2012).

Chromatographic fingerprinting is recommended by the main national and international Pharmacopoeias as a potential and reliable strategy for the quality control of complex mixtures like herbal medicines: however, it should be noted that many traditional preparations are composed of multiple herbs, so that the analysis of selected constituents might not reflect their overall quality or efficacy (Zhou et al., 2008; Zhao et al., 2009; Qiao et al., 2010; Steinmann & Ganzera, 2011). Different kind of features can be selected to characterize the herbal

preparations, and referred to as the overall fingerprint: genetic, quality, sensory or morphological features could be used to create a fingerprint as showed in other studies (Canterino et al., 2012; Mellano et al., 2012): in this study, polyphenolic and terpenic composition was referred to as a chemical fingerprint.

The aim of this research was to perform an analytical study of *Ribes nigrum* bud-preparations, in order to identify and quantify the main bioactive polyphenolic and terpenic compounds, obtaining a specific profile of the main polyphenols and terpenes and the total bioactive compound content; the same analyses were performed using an HPLC-DAD both on University lab preparations and on commercial preparations in order to obtain a chemical fingerprint for the assessment of the single bioactive class contribution to total bud-preparation phytocomplex.

MATERIAL AND METHODS

Plant material

University lab preparations and commercial preparations were evaluated. In February 2012, samples of *Ribes nigrum* L. buds were picked up in a germplasm repository in San Secondo di Pinerolo, Turin Province (Italy): two different varieties (Rozenthal and Daniels) were sampled, in order to test the genotype effect on the chemical composition of the final product. Buds were used fresh to prepare herbal preparations.

Commercial products from five different Italian herbal companies were also considered: the companies are located in San Gregorio di Catania (Catania Province), Predappio (Forlì-Cesena Province), Collepardo (Frosinone Province), Cambiasca (Verbania Province) and Binasco

(Milano Province). University lab and commercial preparations were labelled with a code (Table 1).

Macerated sample preparation protocol

The protocol of bud-preparations is detailed in the monograph "Homeopathic preparations", quoted in the French Pharmacopoeia, 8th edition, 1965 (Pharmaciens, 1965). Bioactive compounds were extracted through a cold maceration process for 21 days, in a solution of ethanol (95%) and glycerol, followed by a first filtration (Whatman Filter Paper, Hardened Ashless Circles, 185 mm Ø), a manual pressing and, after two days of decanting, a second filtration (Whatman Filter Paper, Hardened Ashless Circles, 185 mm Ø). Macerated samples were then stored at N.A., at 4°C and 95% R.H.

Solvents and chemicals

The maceration solvents, ethanol and glycerol, were purchased from Fluka Biochemika (Switzerland) and Sigma Aldrich (USA) respectively. Analytic HPLC grade solvents, methanol and formic acid, were purchased from Sigma Aldrich (USA) and Fluka Biochemika (Switzerland) respectively; potassium dihydrogen phosphate was also purchased from Sigma Aldrich (USA). Milli – Q ultrapure water was produced by using Sartorius Stedium Biotech mod. Arium.

All calibration standards were purchased from Sigma Aldrich (USA): caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, hyperoside, isoquercitrin, quercetin, quercitrin,

rutin, gallic acid, ellagic acid, catechin, epicatechin, limonene, phellandrene, sabinene, γ-terpinene and terpinolene.

Standard preparation

Chemical structures of all the compounds are showed in Fig. 1.

Stock solutions of cinnamic acids and flavonols with a concentration of 1.0 mg/mL were prepared in methanol: from these solutions, four calibration standards were prepared by dilution with methanol; stock solutions of benzoic acids and catechins with a concentration of 1.0 mg/mL were prepared in 95% methanol and 5% water: from these solutions, four calibration standards were prepared by dilution with 50% methanol–water.

Stock solutions of monoterpenes with a concentration of 1.0 mg/mL were prepared in methanol: from these solutions, four calibration standards were prepared by dilution with methanol.

HPLC sample preparation and storage

Macerated University lab and commercial preparations were filtered with circular pre-injection filters (0.45 µm, polytetrafluoroethylene membrane, PTFE) and then stored for a few days at N.A., 4°C and 95% R.H..

Apparatus and chromatographic conditions

An Agilent 1200 High Performance Liquid Chromatograph, equipped with a G1311A quaternary pump, a manual injection valve and a 20 µl sample loop, coupled to an Agilent GI315D UV-Vis diode array detector, was used for the analysis.

Three different chromatographic methods were used to analyse the macerated samples, two for polyphenols and one for terpenic compounds. The first method (A) was used for the analysis of cinnamic acids and flavonols; bioactive compound separation was achieved on a ZORBAX Eclipse XDB – C18 column (4.6 x 150 mm, 5 µm), while the mobile phase consisted of methanol and a solution of 40 mM potassium dihydrogen phosphate in water. The flow rate was 1.0 mL min^{-1} (gradient analysis, 60 minutes) and the detector wavelength was 330 nm (Peev et al., 2007; Donno et al., 2012a). The second method (B) was used for the analysis of benzoic acids and catechins; bioactive molecules were separated on a ZORBAX Eclipse XDB – C18 column (4.6 x 150 mm, 5 µm), while the mobile phase consisted of a solution of methanol/water/formic acid (5:95:0,1 v/v/v) and a mix of methanol/formic acid (100:0,1 v/v). The flow rate was 1.0 mL min^{-1} (gradient analysis, 35 minutes) and the detector wavelengths were 250, 280 and 320 nm (Moller et al., 2009; Donno et al., 2012a).

The third method (C) was used for the analysis of monoterpenes; chromatographic separation was performed using a ZORBAX Eclipse XDB – C18 column (4.6 x 150 mm, 5 µm). The

liquid flow rate was 1.0 mL min^{-1} using water and methanol as mobile phase with a linear gradient of 75 minutes; UV spectra were recorded at 220 and 235 nm (Zhang et al., 2009).

Identification and quantification of bioactive compounds

All single compounds were identified in samples by comparison of their retention times and UV spectra with those of standards in the same chromatographic conditions. Quantitative determinations were performed using an external standard method. Calibration curves in the 125 – 1000 mg/L range with good linearity for a four point plot were used to determine the concentration of polyphenolic and terpenic compounds in bud–preparation samples: the linearity for each compound was established by plotting the peak area (y) versus the concentration (x) of each analyte. The limit of detection (LOD) and the limit of quantification (LOQ) of the three chromatographic methods were defined as the lowest amount of analyte that gives a reproducible peak with a signal-to-noise ratio (S/N) of 3 and 10, respectively. Calibration curve equations, linearity (R^2), LOD and LOQ for all of the compounds are summarized in (Table 2).

All samples were analysed in triplicate (three repetitions for three plants for each University lab sample and three repetitions for three products for each commercial sample), and all data are given in order to assess the repeatability of the used methods (standard deviation). Accuracy was checked by spiking samples with a solution containing each bioactive compound in a concentration of 10 mg/mL.

Examples of *Ribes nigrum* bud–preparation chromatographic profiles are reported in Fig. 2 and Fig. 3. Total bioactive compound content (TBCC) were determined as the sum of the most

important classes of polyphenols and terpenic compounds present in the samples. Four polyphenolic classes were considered: benzoic acids (gallic acid and ellagic acid), catechins (catechin and epicatechin), cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid and ferulic acid) and flavonols (hyperoside, isoquercitrin, quercetin, quercitrin and rutin); one terpenic class was considered: monoterpenes (limonene, phellandrene, sabinene, γ-terpinene, terpinolene). All results were expressed as mg per 100 g of buds fresh weight (FW).

Statistical Analysis

Results were subjected to ANOVA and t Student Test for mean comparison (SPSS 18.0 Software) and HSD Tukey multiple range test ($P<0.05$). Principal Component Analysis (PCA) was performed on the single botanical concentration data.

RESULTS

Total bioactive compound content (TBCC)

The content of total bioactive compounds in the evaluated bud-preparations is reported in Figure 4. Statistically significant differences were observed among the analysed samples, with a lower TBCC value of 3478.95 mg/100 g_{FW} (sample C1) and an higher value of 6507.29 mg/100 g_{FW} (sample UL2).

Principal Component Analysis was performed on all samples and it reduced the initial variables (single bioactive compound concentration) into four principal components (83.15% of total variance) and the initial seven groups into four groups, confirming the statistically

significant differences in TBCC (ANOVA Test): the new groups were called A (UL1), B (UL2), C (C1, C2, C3, C5) and D (C4) (Fig. 5). PCA variable graph (Fig. 6) showed a correlation between the most of polyphenols and PC1 (32.62% of total variance) and a correlation between monoterpenes, except limonene and sabinene, and PC2 (24.77% of total variance).

Single bioactive compound profile

All data are reported in Table 3 (method A), 4 (method B) and 5 (method C).

Ribes nigrum bud-preparations showed the following botanical composition: four cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid, ferulic acid), one flavonol (quercetin), one benzoic acid (gallic acid), two catechins (catechin, epicatechin) and five monoterpenes (limonene, phellandrene, sabinene, γ-terpinene, terpinolene); hyperoside, isoquercitrin, quercitrin, rutin and gallic acid were not detected. Single bioactive compound concentration ranged from 0.84 mg/100 g_{FW} (chlorogenic acid, C1 sample) to 1309.19 mg/100 g_{FW} (γ-terpinene, UL2 sample).

Statistically significant differences were observed both in the University lab bud–preparations and in commercial bud–preparations: the most important differences were observed in the concentration of catechin, limonene and terpinolene.

Fingerprinting

Chemical fingerprint of *Ribes nigrum* bud-preparations was reported: in total, 13 botanicals were identified by HPLC/DAD. By single bioactive compound profile, botanicals were

grouped into polyphenolic and terpenic classes to evaluate the contribution of each class to total phytocomplex composition.

Chemical fingerprint showed the prevalence of monoterpenes and catechins in chemical composition of the all analyzed samples (mean values were considered): the most important class was monoterpenes (82.94%), followed by catechins (9.46%), cinnamic acids (3.64%), flavonols (2.67%) and benzoic acids (1.29%) (Tab. 6).

Therefore, monoterpenes and catechins were two main groups of bioactive compounds in the evaluated bud-preparations: monoterpene contribution ranged from 77.75% in C4 sample to 87.01% in UL2 sample, while catechins contributed to total phytocomplex in a different range, from 6.67% (UL2) to 13.52% (C2).

DISCUSSION

The HPLC analysis of botanicals is nowadays a widespread and well developed characterization tool and some analytical reports were found in literature. These compounds are very interesting because of their wide structural variability (5,000 derivatives are known up to now), which explains their broad spectrum of pharmacological effects and medicinal uses (Ganzera, 2008): in most reports comparable analytical conditions were described, which are based on reverse-phase (RP) stationary phases and acid mobile phases (Matsui et al., 2007; Guo et al., 2008).

Reports on the analysis of phenolic acids (e.g. caffeic acid and its derivatives) by HPLC coupled to diode array or mass detectors have been published. They describe phenolic acid determination in medicinal plants and preparations, as *Ribes nigrum* bud-preparations (Urpi-

Sarda et al., 2009; Castro et al., 2010), according to single bioactive compound concentrations showed in this research. Among other identified classes, flavonols and catechins were also selected for quantitative studies (Huang et al., 2008; Yi et al., 2009; Surveswaran et al., 2010). Based on the obtained results, the most of researches pointed out that the identified polyphenolic compounds significantly contribute to the total phytocomplex of these herbal preparations: the obtained fingerprints were useful for authentication and quality control purposes (Amaral et al., 2009; Dugo et al., 2009); present study confirmed these results, adding as well as the terpenic compounds also significantly contributed to the *Ribes nigrum* bud-preparation phytocomplex, such as anti-inflammatory constituents in herbal preparations (Zhang et al., 2009): few studies emphasized on the identification of single terpenoids in plant material by HPLC analysis (Steinmann & Ganzera, 2011).

It is well-known that chemical composition of secondary plant metabolites highly depends on some factors such as climate conditions, harvesting time and plant genotype (Dvaranauskaite et al., 2008; Donno et al., 2012a), and the results of this research confirmed this hypothesis: ANOVA and PCA results showed that the *Ribes nigrum* bud-preparation composition (different locations) was similar in all the samples but the single compound concentrations were different; moreover, observing the chemical composition, results showed that few compounds were not detected in herbal medicines: chromatographic fingerprinting could be applied in the differentiation of *Ribes nigrum* bud-preparations by other species (Zhao et al., 2009; Donno et al., 2012a).

In this study, effective HPLC–DAD methods were developed for fingerprint analysis and component identification of *Ribes nigrum* bud-preparations from different locations. Comparing with other analytical studies (Tsao & Yang, 2003; Dugo et al., 2009), the chromatographic

conditions were optimized to obtain an effective fingerprint containing enough information of constituents with good resolution and reasonable analysis time. For optimizing the elute conditions, linear gradients in different slope were used for the compound separation, because some constituents were similar in the structure with each other. In the macerated samples, most constituents was also weakly acid, so adding formic acid was necessary for enhancing the resolution and eliminating peak tailing (Zhao et al., 2009). The choice of detection wavelength was a crucial step for developing a reliable fingerprint (Zhou et al., 2008; Zhao et al., 2009). A full-scan on the chromatogram from 190 to 400 nm was performed and only selected wavelengths were suitable to achieve more specific peaks as well as a smooth baseline.

The methods showed a good resolution for most peaks and could be routinely used to evaluate bud-preparation overall quality. The results indicated that the developed methods were feasible for comprehensive authentication and quality control of *Ribes nigrum* bud-preparations. Knowledge of molecular structure, composition and quantity is necessary to understand botanical role in determining potential health effects, because many traditional preparations contain multiple herbs; moreover, pretending to have a natural origin, these preparations sometimes contain a mixture of synthetic adulterants (e.g. sildenafil, diazepam, captopril and amoxicillin), which explains their (unexpected) power but is also responsible for side effects of “unknown” reason (Liang et al., 2006; Uchiyama et al., 2009; Kesting et al., 2010): so that only highly selective, sensitive and versatile analytical techniques will be suitable for quality control purposes (Hager et al., 2008).

This study is only a preliminary research about *Ribes nigrum* bud–preparation chemical composition; by hyphenating High Performance Liquid Chromatography and mass spectrometry,

the high quality demand of the consumer is fulfilled, providing the lab technicians with a multitude of technical options and applications (Gray et al., 2010; Steinmann & Ganzera, 2011).

CONCLUSIONS

Regarding the bud-preparations evaluated in this study, *Ribes nigrum* was identified as a rich source of anti-inflammatory and antioxidant compounds: the observed analytical fingerprint demonstrated that these bud-preparations represent a rich source of terpenes and polyphenolic compounds, especially catechins; this research suggested that identified botanicals might contribute to the total phytocomplex of these herbal preparations.

With gaining popularity of herbal remedies worldwide, the need of assuring safety and efficacy of these products increases as well. Analytical fingerprinting could be an important tool to assess the chemical composition and bioactivities of the plant-derived products, helping in find out new sources of natural health-promoting compounds: only in this way it will be possible to develop a new generation of standardized products which fulfill today's standards for quality, safety and efficiency of herbal preparations.

REFERENCES

Amaral S, Mira L, Nogueira JMF, Da Silva AP, Florencio MH. (2009). Plant extracts with anti-inflammatory properties-A new approach for characterization of their bioactive compounds and establishment of structure-antioxidant activity relationships. *Bioorganic & Medicinal Chemistry,* 17**,** 1876-1883.

Canterino S, Donno D, Mellano MG, Beccaro GL, Bounous G. (2012). Nutritional and sensory survey of Citrus sinensis (L.) cultivars grown at the most Northern limit of the Mediterranean latitude. *Journal of Food Quality,* 35**,** 108-118.

Castro J, Krishna MVB, Choiniere JR, Marcus RK. (2010). Analysis of caffeic acid derivatives in echinacea extracts by liquid chromatography particle beam mass spectrometry (LC-PB/MS) employing electron impact and glow discharge ionization sources. *Analytical and Bioanalytical Chemistry,* 397**,** 1259-1271.

Donno D, Beccaro GL, Mellano GM, Cerutti AK, Canterino S, Bounous G. (2012a). Effect of Agronomic And Environmental Conditions on Chemical Composition of Tree-species Buds Used For Herbal Preparations. *International journal of plant research (VEGETOS),* 25**,** 21-29.

Donno D, Beccaro GL, Mellano MG, Torello Marinoni D, Cerutti AK, Canterino S, Bounous G. (2012b). Application of sensory, nutraceutical and genetic techniques to create a quality profile of ancient apple cultivars. *Journal of Food Quality,* 35**,** 169-181.

Dugo P, Cacciola F, Donato P, Jacques RA, Caramao EB, Mondello L. (2009). High efficiency liquid chromatography techniques coupled to mass spectrometry for the characterization of mate extracts. *Journal of Chromatography A,* 1216**,** 7213-7221.

Dvaranauskaite A, Venskutonis PR, Raynaud C, Talou T, Viškelis P, Dambrauskiene E. (2008). Characterization of Steam Volatiles in the Essential Oil of Black Currant Buds and the Antioxidant Properties of Different Bud Extracts. *Journal of Agricultural and Food Chemistry,* 56**,** 3279-3286.

Edwards JE, Brown PN, Talent N, Dickinson TA, Shipley PR. (2012). A review of the chemistry of the genus Crataegus. *Phytochemistry,* 79**,** 5-26.

Ganzera M. (2008). Quality control of herbal medicines by capillary electrophoresis: Potential, requirements and applications. *Electrophoresis,* 29**,** 3489-3503.

Gray MJ, Chang D, Zhang Y, Liu J, Bensoussan A. (2010). Development of liquid chromatography/mass spectrometry methods for the quantitative analysis of herbal medicine in biological fluids: a review. *Biomedical Chromatography,* 24**,** 91-103.

Guo XR, Chen XH, Li L, Shen ZD, Wang XL, Zheng P, Duan FX, Ma YF, Bi KS. (2008). LC-MS determination and pharmacokinetic study of six phenolic components in rat plasma after taking traditional Chinese medicinal-preparation: Guanxinning lyophilized powder for injection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* 873**,** 51-58.

Hager TJ, Howard LR, Liyanage R, Lay JO, Prior RL. (2008). Ellagitannin Composition of Blackberry As Determined by HPLC-ESI-MS and MALDI-TOF-MS. *Journal of Agricultural and Food Chemistry,* 56**,** 661-669.

Huang WY, Cai YZ, Xing J, Corke H, Sun M. (2008). Comparative analysis of bioactivities of four Polygonum species. *Planta Medica,* 74**,** 43-49.

Jia N, Xiong YL, Kong B, Liu Q, Xia X. (2012). Radical scavenging activity of black currant (Ribes nigrum L.) extract and its inhibitory effect on gastric cancer cell proliferation via induction of apoptosis. *Journal of Functional Foods,* 4**,** 382-390.

Kesting JR, Huang J, Sorensen D. (2010). Identification of adulterants in a Chinese herbal medicine by LC-HRMS and LC-MS-SPE/NMR and comparative in vivo study with

standards in a hypertensive rat model. *Journal of Pharmaceutical and Biomedical Analysis,* 51**,** 705-711.

Komes D, Belščak-Cvitanović A, Horžić D, Rusak G, Likić S, Berendika M. (2011). Phenolic composition and antioxidant properties of some traditionally used medicinal plants affected by the extraction time and hydrolysis. *Phytochemical Analysis,* 22**,** 172-180.

Liang QL, Qu J, Luo GA, Wang YM. (2006). Rapid and reliable determination of illegal adulterant in herbal medicines and dietary supplements by LC/MS/MS. *Journal of Pharmaceutical and Biomedical Analysis,* 40**,** 305-311.

Matsui Y, Nakamura S, Kondou N, Takasu Y, Ochiai R, Masukawa Y. (2007). Liquid chromatography-electrospray ionization-tandem mass spectrometry for simultaneous analysis of chlorogenic acids and their metabolites in human plasma. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* 858**,** 96-105.

Mattila PH, Hellström J, Mcdougall G, Dobson G, Pihlava J-M, Tiirikka T, Stewart D, Karjalainen R. (2011). Polyphenol and vitamin C contents in European commercial blackcurrant juice products. *Food Chemistry,* 127**,** 1216-1223.

Mellano MG, Beccaro GL, Donno D, Marinoni DT, Boccacci P, Canterino S, Cerutti AK, Bounous G. (2012). Castanea spp. biodiversity conservation: collection and characterization of the genetic diversity of an endangered species. *Genetic Resources and Crop Evolution,* 59**,** 1727-1741.

Mok DKW, Chau FT. (2006). Chemical information of Chinese medicines: A challenge to chemist. *Chemometrics and Intelligent Laboratory Systems,* 82**,** 210-217.

Moller C, Hansen SH, Cornett C. (2009). Characterisation of Tannin-Containing Herbal Drugs by HPLC. *Phytochemical Analysis,* 20**,** 231-239.

Oksman-Caldentey KM, Inze D. (2004). Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends in Plant Science,* 9**,** 433-440.

Peev CI, Vlase L, Antal DS, Dehelean CA, Szabadai Z. (2007). Determination of some polyphenolic compounds in buds of Alnus and Corylus species by HPLC. *Chemistry of Natural Compounds,* 43**,** 259-262.

Pharmaciens OND 1965. Pharmacopée Française, Codex Medicamentarius Gallicus, Codex Français: Monographie, Préparations Homéopathiques. *In:* Population MDLSPEDL (ed.) VIII ed. Paris.

Qiao X, Han J, He WN, Wu T, Wang BR, Ye M, Guo DA. (2010). Chemical fingerprint of commercial Radix Echinopsis and quantitative analysis of alpha-terthienyl. *Journal of Separation Science,* 33**,** 530-538.

Steinmann D, Ganzera M. (2011). Recent advances on HPLC/MS in medicinal plant analysis. *Journal of Pharmaceutical and Biomedical Analysis,* 55**,** 744-757.

Surveswaran S, Cai YZ, Xing J, Corke H, Sun M. (2010). Antioxidant properties and principal phenolic phytochemicals of Indian medicinal plants from Asclepiadoideae and Periplocoideae. *Natural Product Research,* 24**,** 206-221.

Tabart J, Franck T, Kevers C, Pincemail J, Serteyn D, Defraigne J-O, Dommes J. (2012). Antioxidant and anti-inflammatory activities of Ribes nigrum extracts. *Food Chemistry,* 131**,** 1116-1122.

Tabart J, Kevers C, Evers D, Dommes J. (2011). Ascorbic Acid, Phenolic Acid, Flavonoid, and Carotenoid Profiles of Selected Extracts from Ribes nigrum. *Journal of Agricultural and Food Chemistry,* 59**,** 4763-4770.

Tsao R, Yang R. (2003). Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. *Journal of Chromatography A,* 1018**,** 29-40.

Uchiyama N, Kikura-Hanajiri R, Kawahara N, Goda Y. (2009). Identification of a cannabimimetic indole as a designer drug in a herbal product. *Forensic Toxicology,* 27**,** 61-66.

Urpi-Sarda M, Monagas M, Khan N, Llorach R, Lamuela-Raventos RM, Jauregui O, Estruch R, Izquierdo-Pulido M, Andres-Lacueva C. (2009). Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A,* 1216**,** 7258-7267.

Yi T, Chen HB, Zhao ZZ, Jiang ZH, Cai SQ, Wang TM. (2009). Identification and Determination of the Major Constituents in the Traditional Uighur Medicinal Plant Saussurea involucrata by LC-DAD-MS. *Chromatographia,* 69**,** 537-542.

Zhang L, Shu X, Ding A, Yu L, Tang Y, Duan J-A, Shang E, Shen X. (2009). LC-DAD-ESI-MS-MS Separation and Chemical Characterization of the Inflammatory Fraction of the Roots of Euphorbia kansui. *Chromatographia,* 70**,** 805-810.

Zhao ZY, Dong LL, Lin F. (2009). Fingerprint Analysis of Euonymus alatus (Thuhb) siebold by LC-DAD and LC-ESI-MS. *Chromatographia,* 69**,** 429-436.

Zhou Y, Jiang SY, Ding LS, Cheng SW, Xu HX, But PPH, Shaw PC. (2008). Chemical Fingerprinting of Medicinal Plants "Gui-jiu" by LC-ESI Multiple-Stage MS. *Chromatographia,* 68**,** 781-789.

Tables

Tab. 1. Source and identification code of the analysed samples.

Sample	City	Province	Region	Identification code
University lab 1	San Secondo di Pinerolo	Torino	Piemonte	UL1
University lab 2	San Secondo di Pinerolo	Torino	Piemonte	UL2
Company 1	San Gregorio di Catania	Catania	Sicilia	C1
Company 2	Predappio	Forlì-Cesena	Emilia-Romagna	C2
Company 3	Collepardo	Frosinone	Lazio	C3
Company 4	Cambiasca	Verbania	Piemonte	C4
Company 5	Binasco	Milano	Lombardia	C5

Tab. 2. Calibration curve equations, R^2, LOD and LOQ of the used chromatographic methods for each calibration standard.

Class	Standard	Method	Calibration curve equations (peak area = y; concentration = x)	R^2	LOD (mg/L)	LOQ (mg/L)
Cinnamic acids	caffeic acid	A	y = 10.155x + 13.008	0.985	1.232	4.107
	chlorogenic acid	A	y = 7.165x + 95.749	0.995	0.627	2.091
	coumaric acid	A	y = 10.904x + 187.144	0.999	1.037	3.456
	ferulic acid	A	y = 6.181x - 273.562	1.000	1.012	3.373
Flavonols	hyperoside	A	y = 14.315x - 262.753	1.000	0.549	1.829
	isoquercitrin	A	y = 11.437x + 100.974	0.998	0.475	1.585
	quercetin	A	y = 5.505x - 418.512	0.996	1.897	6.323
	quercitrin	A	y = 5.162x - 168.272	0.996	1.072	3.575
	rutin	A	y = 8.213x + 105.923	0.999	0.672	2.241
Benzoic acids	gallic acid	B	y = 10.703x + 59.149	0.998	0.283	0.944
	ellagic acid	B	y = 5.766x + 281.063	0.988	1.881	6.271
Catechins	catechin	B	y = 6.567x - 178.554	0.999	1.755	5.850
	epicatechin	B	y = 6.104x - 172.263	0.997	1.749	5.829
Monoterpenes	limonene	C	y = 1.347x + 30.797	0.997	2.108	7.026
	phellandrene	C	y = 4.488x - 39.986	1.000	1.312	4.374
	sabinene	C	y = 29.237x - 296.283	1.000	0.026	0.087
	γ-terpinene	C	y = 2.461x + 205.211	0.993	2.758	9.194
	terpinolene	C	y = 0.056x - 1.809	0.995	7.479	24.930

1

Tab. 3. Single polyphenolic profile of University bud–preparations and commercial bud–preparations (method A). Different letters for each sample indicate the statistically significant differences at $P<0.05$.

METHOD A	*cinnamic acids (mg/100 g FW)*											
sample name	***caffeic acid***			***chlorogenic acid***			***coumaric acid***			***ferulic acid***		
	mean	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*
UL1	5.986	a	1.145	4.013	a	1.754	2.143	a	1.486	126.939	b	33.288
UL2	8.468	a	0.689	1.960	a	2.336	8.243	b	1.983	205.919	c	16.170
C1	5.818	a	0.435	0.844	a	0.646	2.982	a	1.038	133.842	b	12.331
C2	6.151	a	0.813	1.177	a	0.636	3.315	a	0.822	134.176	b	10.132
C3	22.869	b	2.472	0.864	a	0.075	4.152	a	0.209	42.244	a	4.207
C4	85.039	d	1.565	13.127	b	2.493	39.817	c	1.735	153.000	b	0.899
C5	37.987	c	2.234	24.406	c	3.158	7.757	b	0.805	69.421	a	0.723

METHOD A	*Flavonols (mg/100 g FW)*														
sample name	***hyperoside***			***isoquercitrin***			***quercetin***			***quercitrin***			***rutin***		
	mean	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*
UL1	n.d.	/	/	n.d.	/	/	106.813	ab	15.691	n.d.	/	/	n.d.	/	/
UL2	n.d.	/	/	n.d.	/	/	125.270	b	13.023	n.d.	/	/	n.d.	/	/
C1	n.d.	/	/	n.d.	/	/	93.399	a	6.247	n.d.	/	/	n.d.	/	/
C2	n.d.	/	/	n.d.	/	/	94.399	a	2.290	n.d.	/	/	n.d.	/	/
C3	n.d.	/	/	n.d.	/	/	165.806	c	4.678	n.d.	/	/	n.d.	/	/
C4	n.d.	/	/	n.d.	/	/	105.961	ab	2.259	n.d.	/	/	n.d.	/	/
C5	n.d.	/	/	n.d.	/	/	117.957	b	1.081	n.d.	/	/	n.d.	/	/

Tab. 4. Single polyphenolic profile of University bud–preparations and commercial bud–preparations (method B). Different letters for each sample indicate the statistically significant differences at $P<0.05$.

METHOD B	*Benzoic acids (mg/100 g FW)*						*Catechins (mg/100 g FW)*					
sample name	***ellagic acid***			***gallic acid***			***catechin***			***epicatechin***		
	mean	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*
UL1	n.d.	/	/	20.424	a	3.857	249.537	ab	75.127	104.801	ab	32.633
UL2	n.d.	/	/	61.105	b	8.400	292.256	ab	63.608	141.965	bc	20.902
C1	n.d.	/	/	55.036	b	4.420	157.306	a	11.401	101.777	ab	6.074
C2	n.d.	/	/	46.440	b	21.191	329.133	b	88.014	191.961	d	3.608
C3	n.d.	/	/	56.262	b	1.670	238.588	ab	2.420	132.134	bc	1.827
C4	n.d.	/	/	106.575	c	0.503	490.686	c	7.120	154.602	cd	4.388
C5	n.d.	/	/	54.121	b	3.836	274.767	ab	1.388	67.504	a	3.824

Tab. 5. Single terpenic profile of University bud–preparations and commercial bud–preparations (method C). Different letters for each sample indicate the statistically significant differences at $P<0.05$.

METHOD C	*Monoterpenes (mg/100 g FW)*														
sample name	***limonene***			***phellandrene***			***sabinene***			***γ-terpinene***			***terpinolene***		
	mean	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*	*mean*	*Tukey HSD*	*SD*
UL1	380.473	ab	100.139	64.732	a	13.187	30.970	a	16.271	28.497	a	15.760	3382.533	d	290.008
UL2	1298.200	d	310.905	74.571	a	17.809	196.103	b	39.423	1309.192	b	370.441	2784.041	c	171.753
C1	649.515	bc	31.710	504.298	d	7.209	27.498	a	2.690	48.770	a	1.631	1697.864	a	70.449
C2	224.658	a	6.342	149.762	b	8.001	217.870	b	5.883	60.147	a	0.349	2395.201	bc	57.017
C3	859.927	c	7.222	149.573	b	6.283	39.484	a	5.896	143.154	a	1.132	2151.387	b	18.609
C4	911.788	c	8.498	327.717	c	6.748	182.977	b	5.296	65.508	a	2.910	2527.106	bc	118.068
C5	644.830	bc	3.576	528.999	d	5.669	222.978	b	4.146	164.950	a	4.373	1611.298	a	134.242

Tab. 6. Contribution (%) of botanical classes to the phytocomplex in analysed *Ribes nigrum* bud–preparations.

Sample	cinnamic acids	flavonols	benzoic acids	catechins	monoterpenes
UL1	3.09%	2.37%	0.45%	7.86%	86.23%
UL2	3.45%	1.93%	0.94%	6.67%	87.01%
C1	4.12%	2.68%	1.58%	7.45%	84.16%
C2	3.76%	2.45%	1.20%	13.52%	79.07%
C3	1.75%	4.14%	1.40%	9.25%	83.45%
C4	5.63%	2.05%	2.06%	12.50%	77.75%
C5	3.65%	3.08%	1.41%	8.94%	82.91%
mean value	3.64%	2.67%	1.29%	9.46%	82.94%

Figures

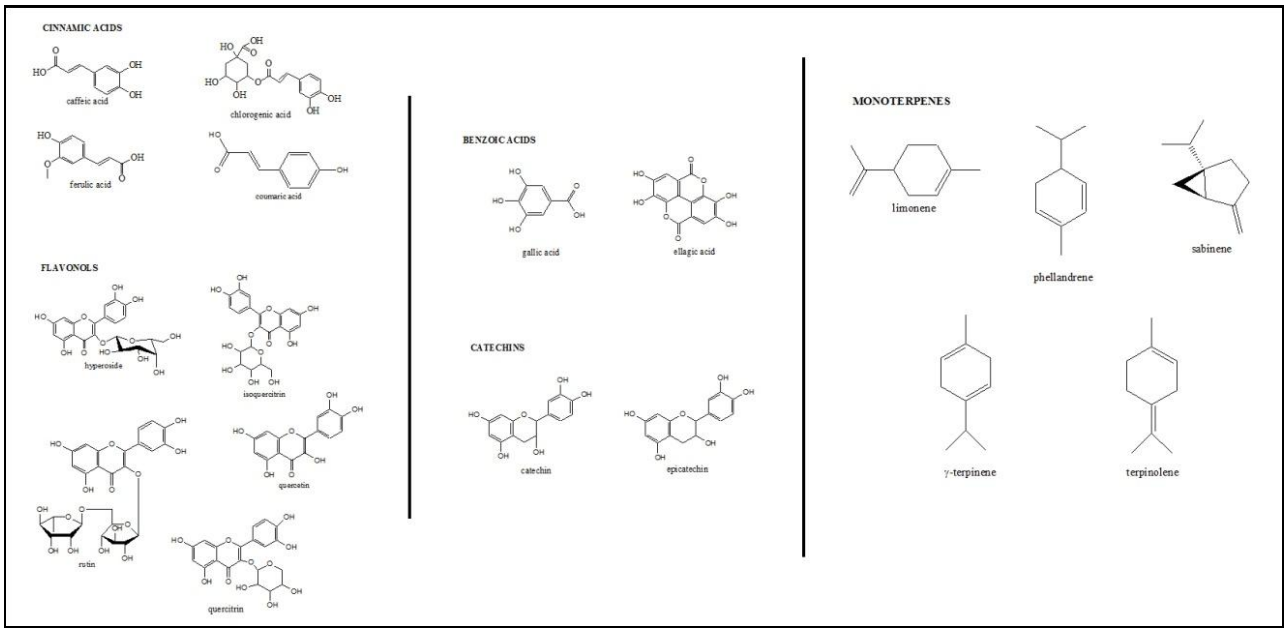

Fig. 1. Chemical structures of the detected bioactive compounds.

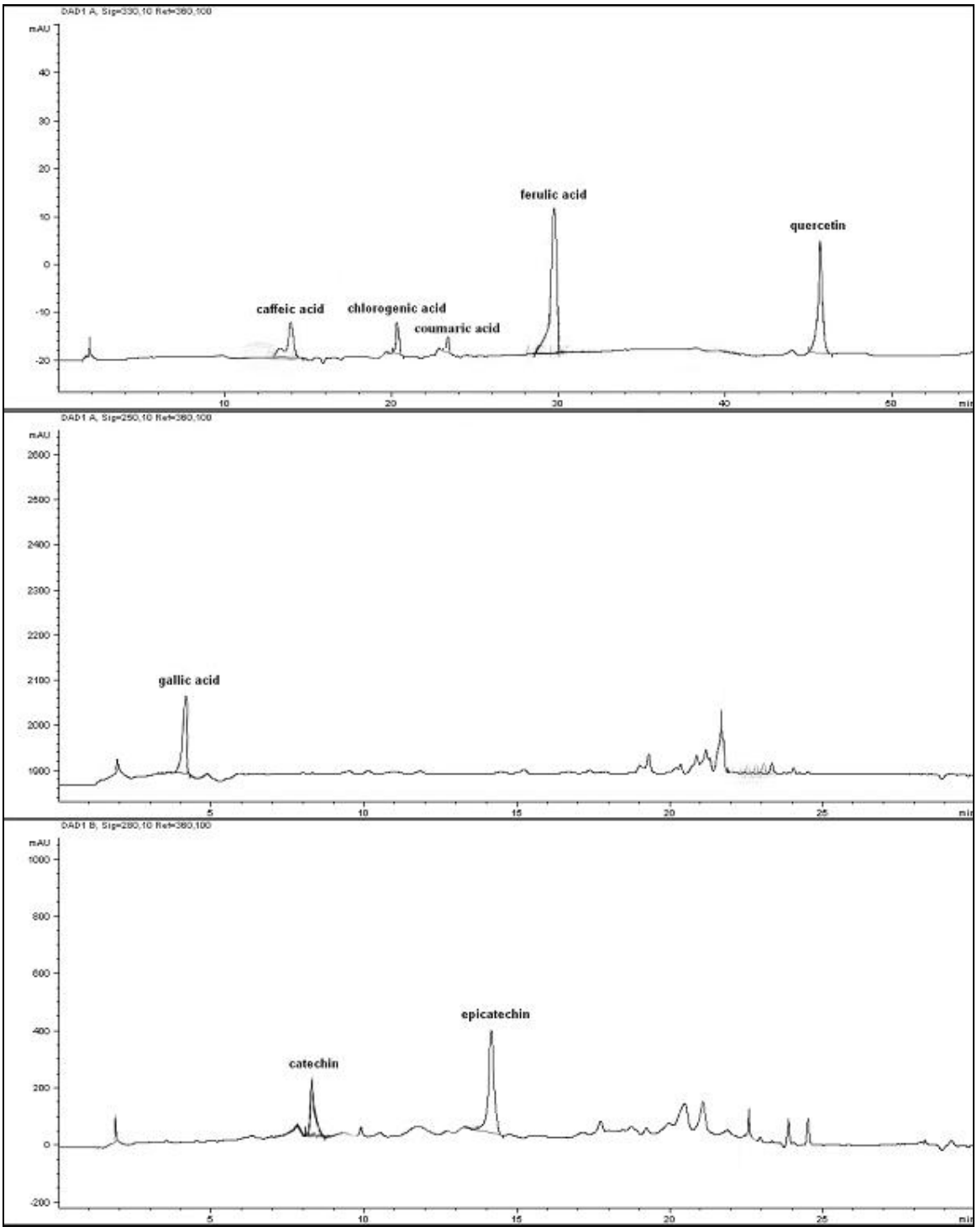

Fig. 2. HPLC/DAD *Ribes nigrum* bud–preparation polyphenolic profile.

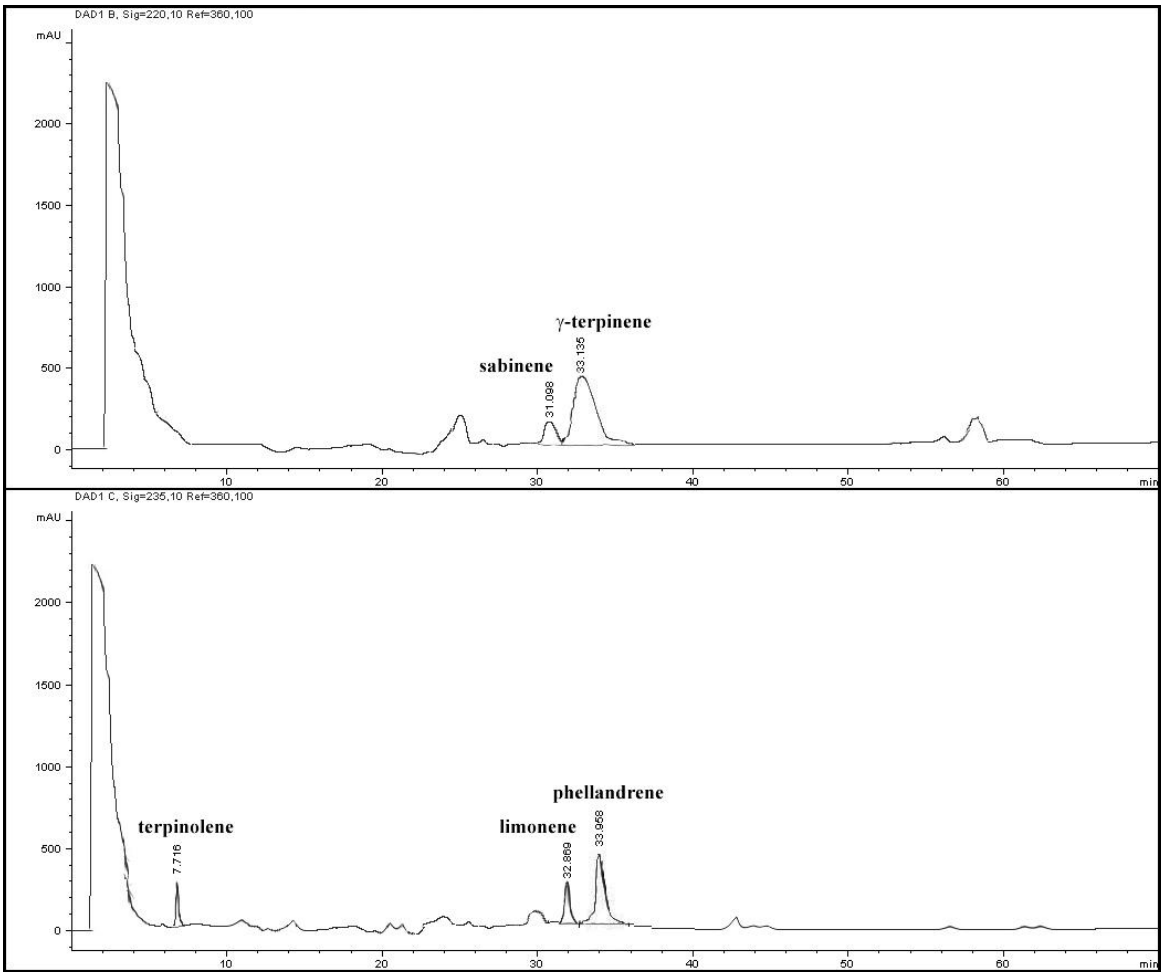

Fig. 3. HPLC/DAD *Ribes nigrum* bud–preparation terpenic profile.

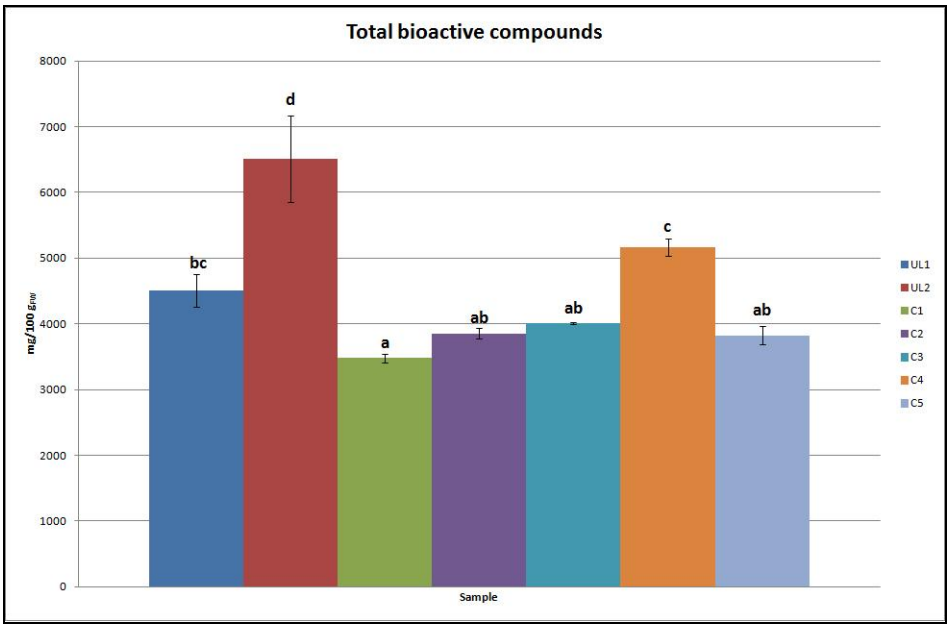

Fig. 4. TBCC in University lab and commercial bud–preparations. Different letters for each sample indicate the significant differences at $P<0.05$.

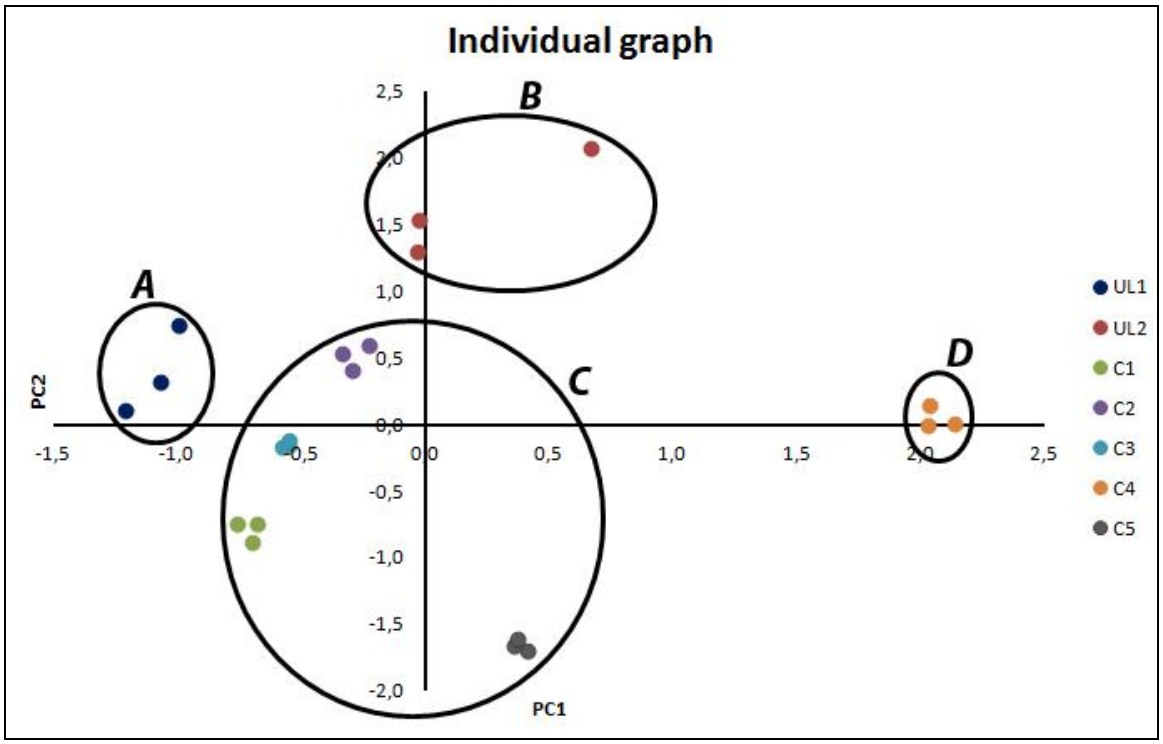

Fig. 5. PCA individual graph of bud–preparation samples.

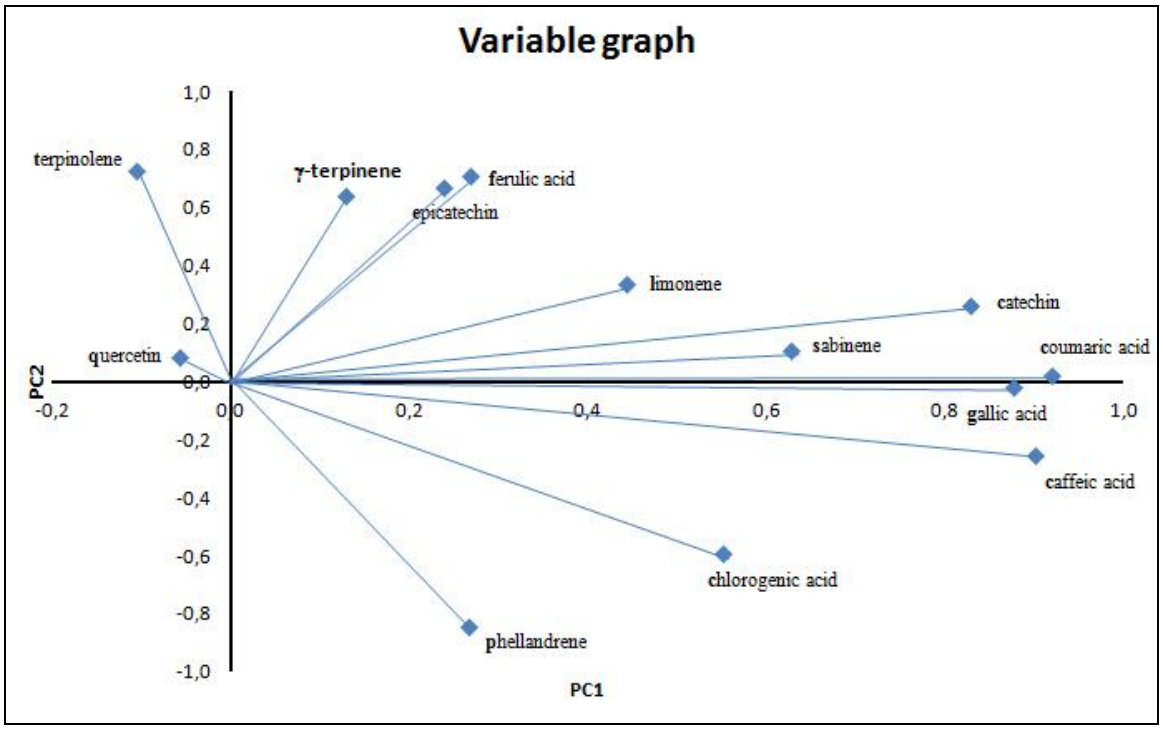

Fig. 6. PCA variable graph of bud–preparation samples.