Research Article

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Analytical fingerprint and chemometrics as phytochemical composition control tools in food supplement analysis: characterization of raspberry bud preparations of different cultivars

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Abstract

BACKGROUND: The raspberry, *Rubus idaeus* L., provides several plant parts (as buds) used for food supplements. The aim of this research was to establish a technique for chemical composition control of *R. idaeus* herbal preparations, using chromatographic methods. These methods allowed us to identify and quantify the main phytochemicals, obtaining a specific phytochemical fingerprint (phytocomplex). Combined with two different chemometric methods – clustering analysis and principal component analysis – the raspberry bud extracts of the different cultivars were efficiently characterized.

RESULTS: *Rubus idaeus* buds were identified as a rich source of anti-inflammatory and antioxidant compounds: organic acids, vitamins and catechins were found to be the most discriminating variables by chemometric techniques to differentiate raspberry cultivars. In particular, catechins (13.25%) and flavonols (8.71%) were the most important polyphenolic classes, followed by cinnamic and benzoic acids.

CONCLUSION: This study developed a useful tool for *R. idaeus* extract phytochemical characterization that could be applied also for differentiation and composition control of other herbal preparations.

Keywords: *Rubus idaeus*; bioactive compounds; traceability; chemometrics; HPLC

INTRODUCTION

Rubus is one of a 100 genera in the family Rosaceae, subfamily Rosoideae, tribe Potentilleae; there are 250 species of *Rubus* established worldwide, especially in the northern temperate zone, with the majority being indigenous to Western and Central Europe.[1] Many *Rubus* species are grown as ornamentals and for their fruits, while others, because of their accumulation of tannins – a characteristic of the family – are traditionally used to treat wounds, burns and inflammation.[2] The European red raspberry (*Rubus idaeus* L. subsp. *idaeus*), the North American red raspberry (*R. idaeus* subsp. *strigosus* Michx.) and the black raspberry (*R. occidentalis* L.) are the most commercially important species:[3] in particular, red raspberry (*R. idaeus* L.) is the most commercially grown raspberry, even if new cultivars are often hybrids of different genotypes.[4] However, because of their economic value, there is great interest also in growing raspberry crops in southern areas of Europe, such as Spain, Portugal and Italy;[5] for this reason, the sustainability of their production should also be evaluated.[6]

Besides edible fruits, *R. idaeus* provides several plant parts used for traditional folk medicine;[1] extracts of different raspberry plant parts have been used in several countries as natural remedies to treat various diseases, such as diabetes, many types of infections, colic and burns: for example, the leaves have been used as antispasmodic agents in traditional folk medicine.[7]

Therapeutic liquid preparations are poorly studied to date, but widely used in European countries: in particular, bud extracts must be exclusively obtained from fresh buds and young sprouts (meristematic fresh plant tissues), macerated and extracted with hydro-glycerol–alcoholic mixtures.[8] Research on these plant preparations is very limited or completely absent, to date;[9] in particular, no evaluation of the raw material (species, cultivars, growing conditions) has been conducted and no systematic chemical investigation has been reported on the bioactive compound content in herbal preparations obtained from raspberry buds.

Herbal preparations, derived from buds and sprouts, contain many different biologically active substances (botanicals).[10]

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Phenolic acids and flavonoids are phytochemicals that, although not essential for survival, over the long term may be one of the factors that contribute to the protective effects of plant products.[11] All these bioactive substances with physiological effects in humans are prone to variation due to genetic and environmental factors and manufacturing conditions.[12–14]

There is a demand for efficient quality control measures to ensure the authenticity of the botanical source and content of bioactive compounds in these products and to verify label claims;[15] however, for a long time, there has been no internationally approbated standard for the quality control of food supplements, such as bud preparations, which has seriously affected the development and exchange of plant material and its related medicinal products.

The most common method for analytical controls that is used in the herbal preparation industry is to spectrophotometrically quantify total bioactive compounds in these raw materials: spectrophotometric determination is a commonly adapted method because of its relatively mild conditions, rapidness and cost-effective nature, but this method does not provide any specificity regarding a botanical fingerprint for affording quality botanical supplements.[16]

Recently, the fingerprint approach was used for identification and direct analysis of plant extracts;[17] the best method of identifying preparations is by measuring the concentration of the main bioactive compounds, called 'markers'.[18] With the development of analytical techniques, chromatographic fingerprints have been widely used for the authentication and quality control of herbal products,[19] according to the US Food and Drug Administration, the European Medicines Evaluation Agency and the State Food and Drug Administration of China (SFDA). In particular, chromatographic fingerprints have been established using high-performance liquid chromatography (HPLC) as a rapid and reliable method for the characterization of plant extracts and herbal medicines.[20,21]

Fingerprint chromatograms consist of complex multivariate datasets due to the complexity of herbal medicines, so minor differences between very similar chromatograms might be missed.[22] Thus chemometric multivariate methods,[23] such as cluster analysis (CA) and principal component analysis (PCA), should be taken into consideration as a reasonable exploratory tool, and furthermore for quality control and standardization of these herbal medicines.[24]

The aim of this research was to establish an effective and combinational technique for chemical characterization and standardization of *R. idaeus* bud preparations using simple, sensitive and reliable HPLC–diode array detector (DAD) methods in order to identify and quantify the main phytochemicals (biomarkers) and to be able to obtain a specific botanical fingerprint for the assessment of the single bioactive class contribution to total bud preparation phytochemical profile. The influence of 'cultivar' factors on these bioactive substances in the raspberry bud extracts was analysed. The combination of two different chemometric methods – CA and PCA – could efficiently visualize the possible differences among bud extracts of the raspberry cultivars, and be an effective tool for food supplement characterization.

EXPERIMENTAL

Plant material

The field experiment was conducted during 2012–2013 at Agroscope in Conthey (longitude 7.3° E, latitude 46.2° N) at 500 m above sea level. Plantlets of different raspberry cultivars (*R. idaeus* L.) were transferred from small pots to 10 L pots at the beginning of May 2012. Red raspberry (*R. idaeus* L.) is the most commercially grown raspberry, even if new cultivars are often hybrids of different genotypes. Seven primocane-fruiting cultivars (Amira, Himbo Top, Imara, Joan J, Kwelli, Regina, Sugana) and the floricane-fruiting cultivar Tulameen were compared. These cultivars were selected because they are economically among the most important cultivars in the world market. The plants were grown under a polyethylene-coated shelter at a plant distance within the row of 0.3 m per pot. The distance between the north–south-oriented rows was 2.2 m. For each cultivar, eight plants for each biological replication (three biological replications in total) were installed, with one to two canes per plant. The substrate in the pots was 48% white peat and 52% wood bark and coconut fibre, with a pH of 5.5.

Table 1. Origin and identification code of the analysed samples

Species	Cultivar	Identification code
Rubus idaeus L.	Amira	Am
	Kwelli	Kw
	Imara	Im
	Himbo Top	Hi
	Sugana	Su
	Regina	Re
	Joan J	Jj
	Tulameen	Tu

Drip irrigation was used with two drippers per pot, together providing 4 L of nutrient solution per hour depending on maturity and conditions. The plants were fertigated with a balanced hydroponic nutrient solution in a closed system with drainage recycling. Water and nutrients were given by fertigation several times during the day according to the season and the duration of sunshine during a day. The nutrient solution consisted of (ppm): N (154), P (46), K (215), Ca (140), Mg (36), S (48), Fe (1.2), Mn (1.1), Cu (0.06), B (1.1), Zn (0.58), and Mo (0.05). The iron was in the form of Fe-EDDHA chelate. The solution pH was maintained at 5.8 (5.2–6.4) and EC at 1.2 mS cm^{-1} (0.8–1.6 mS cm^{-1}). The amount of nutrient solution given to the plants corresponded to obtaining a drainage amount of 20–30% of the total applied quantity of nutrient solution. This amount of drainage allowed any accumulation of nutrients in the substrate to be avoided. Insects and diseases were controlled using integrated pest management (IPM) practices. However, there were no pest and disease problems during the growth cycle until May 2012 up until March 2013.

All the buds from the sampled canes were picked and collected in March 2013 at bud break. Fifty buds were collected per cultivar and replication (3 g for each repetition). Three analytical replicates of three biological replicates of eight cultivars were considered. The collected buds were used fresh to manually produce herbal preparations in the lab. The buds of eight cultivars were sampled for three biological replications arranged in a randomized complete block, in order to test the cultivar effect on the chemical composition of the final product, and their preparations were labelled with a code. Cultivars and identification codes are shown in Table 1.

Solvents and chemicals

Ethanol, formic acid and organic acids were purchased from Fluka Biochemika (Buchs, Switzerland). Analytic HPLC-grade methanol,

Table 2. Main parameters of the chromatographic methods used

Method	Compounds of interest	Stationary phase	Mobile phase	Flow (mL min^{-1})	Time of analysis (min)	Gradient	Wavelength (nm)
A	Cinnamic acids, flavonols	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: 40 mmol L^{-1} KH_2PO_4/H_3PO_4, pH = 2.3 B: CH_3OH	1.0	60	Yes	330
B	Benzoic acids, catechins	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: H_2O/CH_3OH/HCOOH (5:95:0.1 v/v/v), pH = 2.5 B: CH_3OH/HCOOH (100:0.1 v/v)	1.0	35	Yes	250, 280, 320
C	Monoterpenes	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: H_2O/HCOOH (100:0.1 v/v/v), pH = 2.8 B: CH_3OH	1.0	75	Yes	220, 235
D	Organic acids	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: 50 mmol L^{-1} $(NH_4)H_2PO_4/H_3PO_4$, pH 2.8	0.5	20	No	214
E	Vitamins	KINETEX – C18 column (4.6 × 150 mm, 5 µm)	A: 5 mmol L^{-1} $C_{16}H_{33}N(CH_3)_3Br$/50 mmol L^{-1} KH_2PO_4, pH 2.5 B: CH_3OH	0.9	15	No	261, 348

[a] Mobile phase: A represents the aqueous buffer solution; B represents the organic solvent.

glycerol, all the polyphenolic and terpenic standards, potassium dihydrogen phosphate, ammonium dihydrogen phosphate, 1,2-phenylenediamine dihydrochloride (OPDA) and phosphoric acid were purchased from Sigma Aldrich. Milli-Q ultrapure water was produced by using the Arium system (Sartorius Stedim Biotech, Goettingen, Germany).

Cetyltrimethylammonium bromide (cetrimide), ascorbic acid and dehydroascorbic acid were purchased from Extrasynthése (Genay, France).

Macerated sample preparation protocol

The extraction solution was prepared based on the protocol of bud preparations detailed in the monograph 'Homeopathic preparations', quoted in the *French Pharmacopoeia*, 8th edition, 1965.[25]

Bioactive compounds were extracted through a cold maceration process for 21 days, in a solution of ethanol (95%) and glycerol (the solvent quantities are calculated in order to obtain a 1:20 macerate, so that the final product was 20 times the weight of the raw material in the dry state), followed by a first filtration (Whatman filter paper, hardened ashless, circles, 185 mm Ø), a manual pressing and, after 2 days of decanting, a second filtration (Whatman filter paper, as previously).[26] Macerated samples were prepared in the quality laboratory of the Agroscope Research Centre (Conthey, Sion, Switzerland) and then stored in dark bottles at normal atmosphere (NA), at 4 °C and 95% relative humidity. The HPLC analysis was carried out in the analytical laboratory of the University of Turin (DISAFA) in Italy.

Standard preparation

Linearity of the system was performed through the calibration curves of the stock standard solutions of all the considered bioactive compounds prepared by different stepwise dilution.

Stock solutions of cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid, ferulic acid), flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, rutin), monoterpenes (limonene, phellandrene, sabinene, γ-terpinene, terpinolene), ascorbic and dehydroascorbic acids with a concentration of 1.0 mg mL^{-1} were prepared in methanol; from these solutions, four calibration standards were prepared by dilution with methanol for each compound; stock solutions of benzoic acids (ellagic acid, gallic acid) and catechins (catechin, epicatechin) with a concentration of 1.0 mg mL^{-1} were prepared in 95% methanol and 5% water. In this case, four calibration standards were prepared by dilution with 50% methanol–water. Benzoic acids, in particular ellagic acid, and catechins were dissolved in a hydromethanol solution rather than in methanol as the other phenolic compounds, for better solubility.

Stock solutions of organic acids (citric, malic, oxalic, quinic, succinic and tartaric acids) with a concentration of 1.0 mg mL^{-1} were prepared in ultrapure water: four calibration standards were prepared by dilution with water.

HPLC sample preparation and storage

Macerated preparations were filtered with circular pre-injection filters (0.45 µm, polytetrafluoroethylene membrane, PTFE) and then stored for a few days at NA, 4 °C and 95% relative humidity (RH). All samples were analysed as such without dilution. For vitamin C analysis, 250 µL OPDA solution (18.8 mmol L^{-1}) was added to 750 µL of extracted samples for dehydroascorbic acid derivatization into the fluorophore 3-(1,2-dihydroxyethyl) furo(3,4-b)quinoxalina-1-one (DFQ). After 37 min in the dark the samples were analysed with an HPLC instrument coupled to a diode array detector (DAD).[27]

Apparatus and chromatographic conditions

An Agilent 1200 HPLC instrument, equipped with a G1311A quaternary pump and a manual injection valve (20 µL sample loop), coupled to an Agilent GI315D UV–visible DAD (Agilent Technologies, Santa Clara, CA, USA), was used for the analysis.

Five different chromatographic methods were used to analyse the samples: two for polyphenols and one for terpenic compounds, organic acids and vitamins, respectively. In this

Table 3. Calibration curve equations, R^2, LOD and LOQ of the chromatographic methods used for each calibration standard[16]

Class	Standard	Identification code	Method	Calibration curve equations	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Cinnamic acids	Caffeic acid	1	A	$y = 10.155x + 13.008$	0.985	1.232	4.107
	Chlorogenic acid	2	A	$y = 7.165x + 95.749$	0.995	0.627	2.091
	Coumaric acid	3	A	$y = 10.904x + 187.144$	0.999	1.037	3.456
	Ferulic acid	4	A	$y = 6.181x - 273.562$	1.000	1.012	3.373
Flavonols	Hyperoside	5	A	$y = 14.315x - 262.753$	1.000	0.549	1.829
	Isoquercitrin	6	A	$y = 11.437x + 100.974$	0.998	0.475	1.585
	Quercetin	7	A	$y = 5.505x - 418.512$	0.996	1.897	6.323
	Quercitrin	8	A	$y = 5.162x - 168.272$	0.996	1.072	3.575
	Rutin	9	A	$y = 8.213x + 105.923$	0.999	0.672	2.241
Benzoic acids	Ellagic acid	10	B	$y = 5.766x + 281.063$	0.988	1.881	6.271
	Gallic acid	11	B	$y = 10.703x + 59.149$	0.998	0.283	0.944
Catechins	Catechin	12	B	$y = 6.567x - 178.554$	0.999	1.207	4.024
	Epicatechin	13	B	$y = 6.104x - 172.263$	0.997	0.362	1.206
Monoterpenes	Limonene	14	C	$y = 1.347x + 30.797$	0.997	2.108	7.026
	Phellandrene	15	C	$y = 4.488x - 39.986$	1.000	1.312	4.374
	Sabinene	16	C	$y = 29.237x - 296.283$	1.000	0.026	0.087
	γ-Terpinene	17	C	$y = 2.461x + 205.211$	0.993	2.758	9.194
	Terpinolene	18	C	$y = 0.056x - 1.809$	0.995	7.479	24.930
Organic acids	Citric acid	19	D	$y = 1.695x + 16.075$	1.000	1.065	3.549
	Malic acid	20	D	$y = 1.962x - 16.921$	0.998	0.688	2.295
	Oxalic acid	21	D	$y = 20.034x + 287.523$	0.999	0.098	0.328
	Quinic acid	22	D	$y = 1.193x - 3.232$	1.000	2.054	6.845
	Succinic acid	23	D	$y = 0.845x + 47.492$	0.997	1.492	4.972
	Tartaric acid	24	D	$y = 4.609x - 73.283$	1.000	0.401	1.335
Vitamins	Ascorbic acid	25	E	$y = 40.541x - 798.702$	0.998	0.236	0.786
	Dehydroascorbic acid	26	E	$y = 5.844x + 197.332$	0.999	0.836	2.786

[a] The methods A–E are described in the text.
[b] Calibration curve equations: x represents the concentration; y represents the peak area.

research, these HPLC methods were developed for the comprehensive multi-component analysis of the bud preparations: multi-chromatographic fingerprinting, which consists of more than one chromatographic fingerprint and represents the whole chemical characteristics of the samples, is proposed as a strategy for phytochemical characterization of complex herbal medicines instead of reported single chromatographic fingerprinting.

In all of the used methods, bioactive compound separation was achieved on a Kinetex C18 column (4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA, USA).[28]

Different mobile phases were used for a specific and high-resolution compound identification (both linear gradient and isocratic analysis) and UV spectra were recorded at different wavelengths for better peak determination.[29] All method parameters (compound class of interest, stationary and mobile phase, flow rate, time of analysis, wavelengths) are reported in Table 2.

Identification and quantification of bioactive compounds

In the present study, 26 compounds were selected as the main bioactive markers of *R. idaeus* and identified in samples by comparison and combination of their retention times, chromatograms and UV spectra with those of authentic standards under the same chromatographic conditions and according to the literature. The biomarker compounds were selected on the basis of the similarity between the observed clinical effects of the considered extracts and the chemical composition of common drugs with the same therapeutic effects. Even if biomarkers are combined in bioactive classes, they can be considered discriminating variables because the effect of the entire class is due to the additive chemical properties of each single compound.

Quantitative determinations were performed using an external standard method: the calibration curves in the 125–1000 mg L^{-1} range were constructed by running four standards of different concentrations (125, 250, 500, 1000 ppm) in triplicate. The linearity for each compound was established by plotting the peak area (y) *versus* the concentration (x) of each analyte: three analyses were accomplished and the correlation coefficient was determined using a linear regression model. The limit of detection (LOD) and limit of quantification (LOQ) of all the methods for all the chemical markers were estimated at signal-to-noise ratios (S/N) of 3 and 10, respectively (the lowest amount of analyte with a reproducible peak), by injecting a series of dilute solutions with known concentration (Table 3), as reported by Betz *et al.*[30]

All samples were analysed in triplicate (three repetitions, each one from a different field replication, for each cultivar 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^{-1}.

Examples of chromatographic profiles of the raspberry bud preparation are reported in Fig. 1. Total bioactive compound content (TBCC) was determined as the sum of the most important classes of bioactive compounds present in the samples. All results were expressed as grams per kilogram of bud fresh weight (FW).

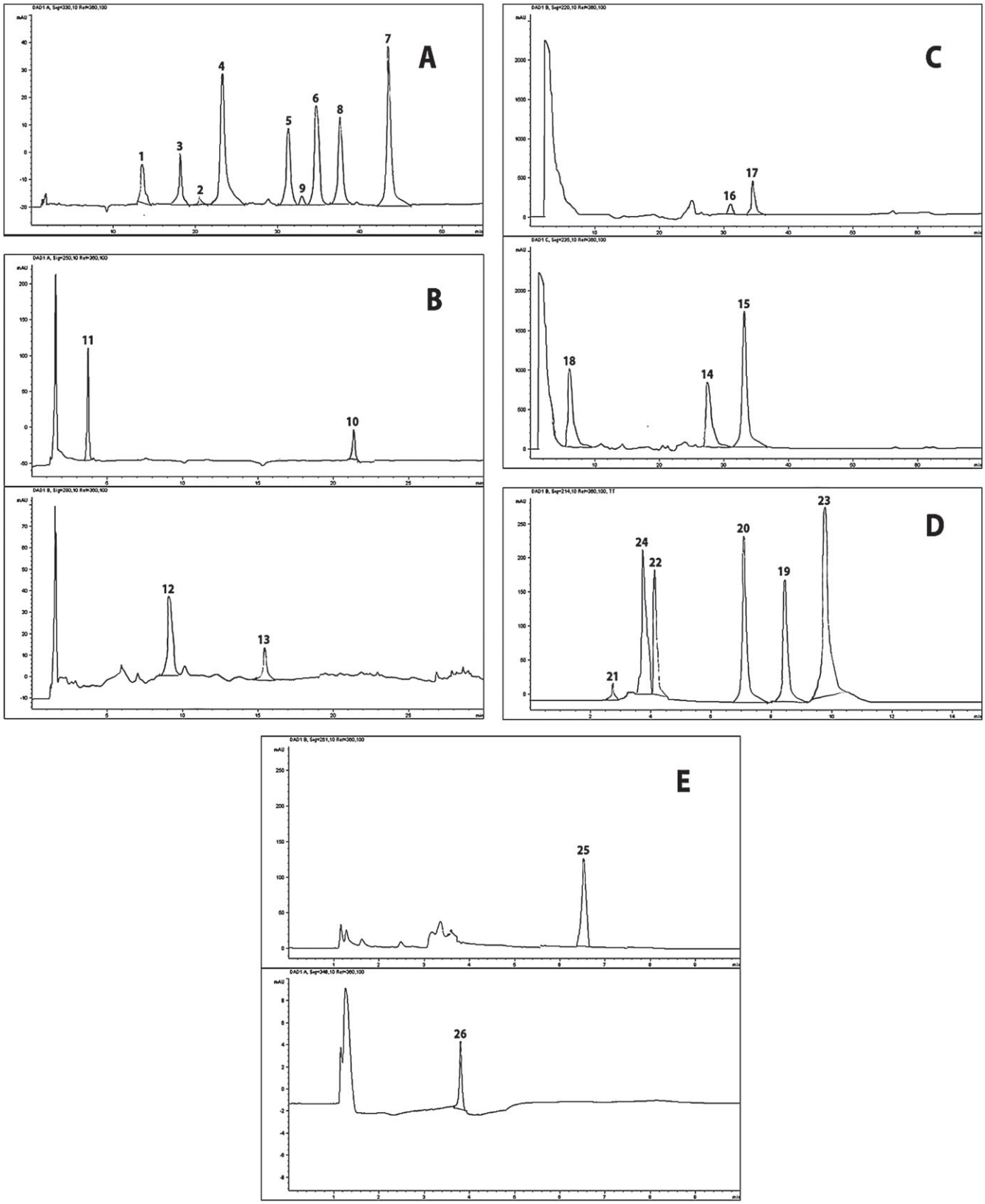

Figure 1. HPLC/DAD bioactive compound profile. Standard identification code was reported in Table 2. Letters A – E indicate the different chromatographic methods used for analytical fingerprint.

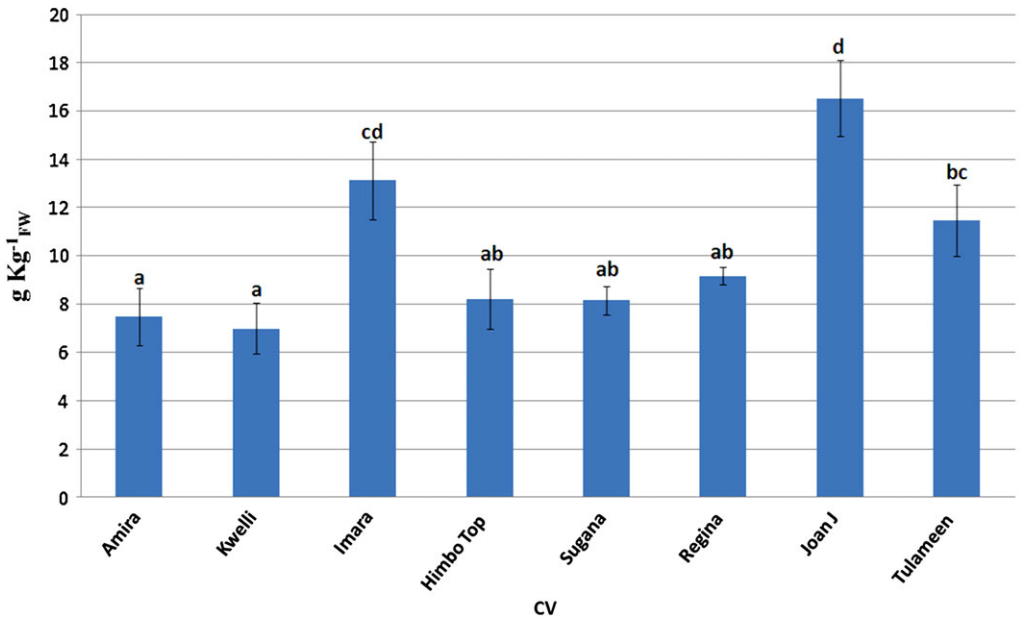

Figure 2. TBCC in eight raspberry cultivar bud preparations. Different letters for each sample indicate significant differences at $P < 0.05$ (three repetitions from three plants for each sample, $N = 9$).

Statistical analysis

Results were subjected to analysis of variance (ANOVA) test for mean comparison (SPSS 22.0 Software, Chicago, IL, USA) and Tukey's HSD multiple range test ($P < 0.05$).

Multivariate analysis was carried out on all of the samples. The data matrix was defined as 24 objects (three repetitions for eight samples) and seven variables (content of each bioactive class). Data were mean centred before MVA. Even if the original data were all concentration data in the same units, *Z*-score scaling was subsequently carried out in order to check possible differences in the results.

In order to evaluate the resemblance and differences in the samples, CA of macerated samples was performed using SPSS 22.0 software. Ward's method as the amalgamation rule and the squared Euclidean distance as metric were used to establish clusters.

For further discrimination of the investigated samples, principal component analysis (PCA) was performed on the column-centred data using SPSS 22.0 software.

RESULTS

TBCC and single bioactive compound profile

ANOVA test showed statistically significant differences among the considered cultivars both on the single bioactive compound concentrations and the total bioactive compound content.

The content of total bioactive compounds in the evaluated extracts is reported in Fig. 2. Statistically significant differences were observed among the analysed samples, with a lower TBCC value of 6.994 ± 1.043 g kg^{-1} FW for Kwelli and n higher value of 16.515 ± 1.575 g kg^{-1} FW for Joan J, followed by Imara and Tulameen.

All chemical composition data are reported in Table 4; all the considered phytochemical markers were detected in the raspberry bud preparations. For each bioactive compound, content mean value and standard deviation (SD), as an indication of the data variability (three analytical repetitions from three plants for each sample, $N = 9$), were reported. Some peaks were found in any of the samples and remained unidentified: they probably represent other bioactive markers with low therapeutic effects on human health, according to other studies.[10] Statistically significant differences were observed among the different cultivars for all single bioactive compounds; the most important differences were observed in the concentrations of catechin, oxalic acid, quinic acid and vitamin C.

Multivariate analysis

In herbal preparations, synergistic or additive therapeutic effects of several phytochemicals (phytocomplex), rather than a single compound, could contribute to disease prevention;[31] for this reason, single compounds belonging to the same chemical class were combined in bioactive classes for multivariate data handling. In order to better visualize the possible differences in the preparations and easily characterize the samples, PCA was performed on all the samples, and it reduced the initial variables (single bioactive class content) to three principal components (86.69% of total variance), placing the eight cultivars in the PCA score plot (Fig. 3) in relation to phytochemical composition. PC1 and PC2 well represent the system information (70.76% of total variance); the PCA gave rise to four groups, highlighted in Fig. 3 with circles, without statistical meaning, according to the phytochemical profile and CA results; the groups were named α (Amira, Kwelli, Himbo Top), β (Tulameen), χ (Sugana, Regina) and δ (Imara, Joan J). PCA loading plot showed a correlation between most of the polyphenolic classes (benzoic acids, cinnamic acids and flavonols) and PC1 (46.18% of total variance), and a correlation between vitamins/organic acids and PC2 (24.58% of total variance). Monoterpenes showed an intermediate position between PC1 and PC2 (Fig. 4). Organic acids, vitamins and catechins have been identified as bioactive classes with the most discriminating power among different cultivars; these three phytochemical classes included compounds with high statistical differences in their bioactive content.

In order to assess the resemblance and differences of analysed samples, a hierarchical agglomerative clustering analysis of

Table 4. Phytochemical profile of the eight raspberry cultivars. Data are expressed as g kg^{-1} FW. Samples are indicated with their identification code (Table 2)

	Cinnamic acids											
	Caffeic acid			Chlorogenic acid			Coumaric acid			Ferulic acid		
Sample	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test
Am	0.017	0.003	ab	0.022	0.005	d	0.002	0.001	a	0.133	0.018	ab
Kw	0.011	0.001	a	0.009	0.001	abc	0.010	0.002	b	0.110	0.017	a
Im	0.028	0.005	cd	0.014	0.005	c	0.008	0.003	ab	0.136	0.024	ab
Hi	0.034	0.003	d	0.011	0.001	bc	0.002	0.001	a	0.172	0.020	bc
Su	0.016	0.002	a	0.011	0.002	bc	0.023	0.003	c	0.149	0.013	ab
Re	0.032	0.002	cd	0.002	0.001	a	0.001	0.000	a	0.161	0.008	bc
Jj	0.015	0.002	a	0.002	0.002	a	0.021	0.002	c	0.123	0.013	ab
Tu	0.025	0.003	bc	0.003	0.002	ab	0.032	0.005	d	0.205	0.024	c

	Flavonols														
	Hyperoside			Isoquercitrin			Quercetin			Quercitrin			Rutin		
Sample	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test
Am	0.081	0.014	ab	0.259	0.033	bc	0.311	0.045	bc	0.212	0.034	bc	0.005	0.001	ab
Kw	0.049	0.007	a	0.189	0.027	b	0.190	0.026	a	0.145	0.019	ab	0.018	0.002	abc
Im	0.065	0.017	a	0.400	0.053	d	0.245	0.040	abc	0.197	0.040	bc	0.064	0.019	d
Hi	0.106	0.014	b	0.413	0.053	d	0.323	0.037	c	0.237	0.037	c	0.035	0.004	c
Su	0.073	0.006	ab	0.179	0.014	ab	0.249	0.024	abc	0.157	0.016	ab	0.001	0.000	a
Re	0.069	0.003	a	0.385	0.015	d	0.220	0.010	ab	0.175	0.008	bc	0.022	0.001	bc
Jj	0.054	0.006	a	0.087	0.007	a	0.186	0.019	a	0.094	0.010	a	0.005	0.001	ab
Tu	0.181	0.022	c	0.285	0.033	c	0.301	0.040	bc	0.202	0.025	bc	0.024	0.003	bc

	Benzoic acids						Catechins					
	Ellagic acid			Gallic acid			Catechin			Epicatechin		
Sample	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test
Am	0.043	0.004	abcd	0.088	0.011	d	0.855	0.123	c	0.915	0.122	c
Kw	0.040	0.008	abc	0.079	0.011	cd	0.710	0.102	bc	0.767	0.116	c
Im	0.072	0.025	bcd	0.039	0.009	ab	0.350	0.040	a	0.294	0.054	ab
Hi	0.090	0.015	d	0.121	0.018	ef	1.297	0.231	d	0.466	0.063	b
Su	0.025	0.003	ab	0.058	0.006	bc	0.335	0.028	a	0.270	0.027	ab
Re	0.076	0.005	cd	0.133	0.006	f	0.474	0.026	ab	0.264	0.014	a
Jj	0.005	0.002	a	0.101	0.005	de	0.640	0.049	abc	0.252	0.038	a
Tu	0.312	0.038	e	0.013	0.002	a	1.209	0.155	d	0.378	0.044	ab

	Monoterpenes															Vitamins		
	Limonene			Phellandrene			Sabinene			γ-Terpinene			Terpinolene			Vitamin c		
Sample	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test
Am	0.062	0.023	a	0.352	0.067	abc	0.076	0.014	b	0.127	0.020	a	0.201	0.065	a	0.072	0.008	cd
Kw	0.070	0.028	a	0.211	0.042	a	0.051	0.009	a	0.183	0.027	ab	0.194	0.052	a	0.032	0.003	ab
Im	0.067	0.024	a	0.311	0.037	ab	0.061	0.007	ab	0.117	0.019	a	0.236	0.054	a	0.046	0.017	bc
Hi	0.062	0.032	a	0.601	0.130	cd	0.055	0.009	ab	0.143	0.014	ab	0.314	0.074	a	0.089	0.019	d
Su	0.047	0.017	a	0.285	0.036	ab	0.055	0.005	ab	0.249	0.027	b	0.250	0.003	a	0.079	0.011	d
Re	0.120	0.006	ab	0.515	0.092	bc	0.071	0.009	ab	0.167	0.045	ab	0.232	0.030	a	0.020	0.003	ab
Jj	0.157	0.041	b	0.577	0.095	cd	0.054	0.006	ab	0.174	0.048	ab	0.220	0.031	a	0.038	0.006	ab
Tu	0.117	0.022	ab	0.781	0.142	d	0.052	0.009	ab	0.186	0.086	ab	0.224	0.043	a	0.012	0.003	a

Table 4. Continued

	Organic acids																	
	Citric acid			Malic acid			Oxalic acid			Quinic acid			Succinic acid			Tartaric acid		
Sample	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test	Mean value	SD	Tukey's test
Am	0.454	0.073	a	1.347	0.207	a	0.017	0.003	ab	0.486	0.086	a	1.213	0.213	ab	0.104	0.013	a
Kw	0.483	0.083	ab	1.286	0.150	a	0.010	0.001	a	0.408	0.049	a	1.639	0.267	b	0.099	0.013	a
Im	0.772	0.091	bc	4.326	0.402	c	0.054	0.011	cd	0.537	0.059	ab	4.407	0.616	d	0.272	0.046	ab
Hi	0.595	0.138	ab	1.537	0.187	a	0.037	0.005	bc	0.372	0.091	a	0.734	0.035	a	0.354	0.040	b
Su	0.525	0.078	ab	2.457	0.173	b	0.040	0.004	bc	0.779	0.066	bc	1.237	0.155	ab	0.598	0.048	c
Re	0.371	0.035	a	2.606	0.094	b	0.034	0.002	abc	0.564	0.073	ab	1.767	0.092	b	0.679	0.027	c
Jj	0.906	0.111	c	4.668	0.454	c	0.069	0.010	d	1.276	0.130	d	5.370	0.415	e	1.421	0.176	d
Tu	1.252	0.176	d	1.117	0.125	a	0.123	0.019	e	0.988	0.134	c	2.756	0.305	c	0.682	0.098	c

[a] For each bioactive compound, content mean value and standard deviation (SD), as indication of the data variability, were reported.
[b] In Tukey test column, for each compound, different letters indicate the significant differences at $P < 0.05$ among cultivars (three repetitions from three plants for each sample, $N = 9$, FW = fresh weight).
[c] Vitamin C content shows the sum of ascorbic acid and dehydroascorbic acid levels.

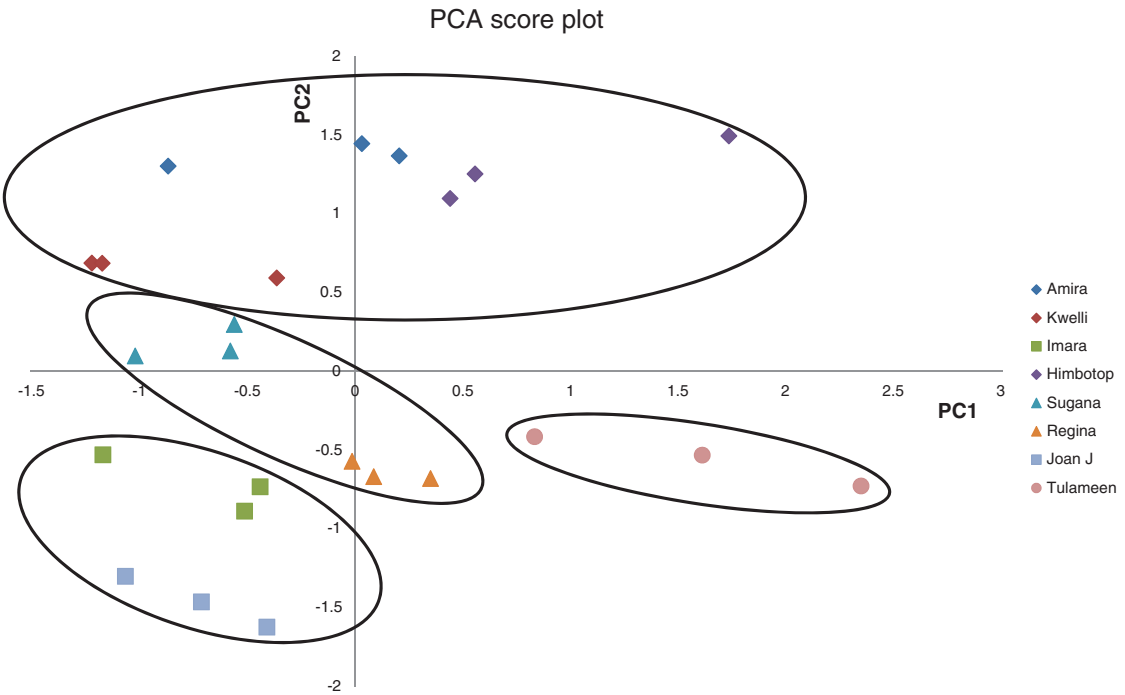

Figure 3. PCA score plot of bud preparation samples with for eight raspberry cultivars and three field replications per cultivar. The ellipses around each object group only indicate the position of a category in the plot without statistical meaning, based on the dendrogram results.

R. idaeus preparations was performed based on the concentration data of all seven considered bioactive classes. The results of CA are shown in Fig. 5; the obtained dendrogram more clearly reveals the differences among the cultivars. Using an appropriate distance level,[32] the samples have been classified into four clusters. The results of CA and PCA could validate each other and provided more references for the quality evaluation of raspberry bud extracts: the samples classified into the same group were associated with similar chemical composition and properties.

Fingerprint

The chemical fingerprint of *R. idaeus* bud preparations was reported: in total, 26 phytochemicals were identified by HPLC-DAD. By single bioactive compound profile, phytochemicals were grouped into single bioactive classes to evaluate the contribution of each class to the total phytocomplex composition.

The chemical profile showed the prevalence of organic acids and polyphenolic compounds in chemical composition of all the analysed preparations (Fig. 6): considering the mean value of all the cultivars, the most quantitatively important class was organic acids (63.31%), followed by polyphenolic compounds (25.70%), monoterpenes (10.45%) and vitamins (0.54%).

For each cultivar, the percentage ratio between each single class content and TBCC are reported (Fig. 7); in particular, among the polyphenolic compounds, the catechins (13.25%) and flavonols

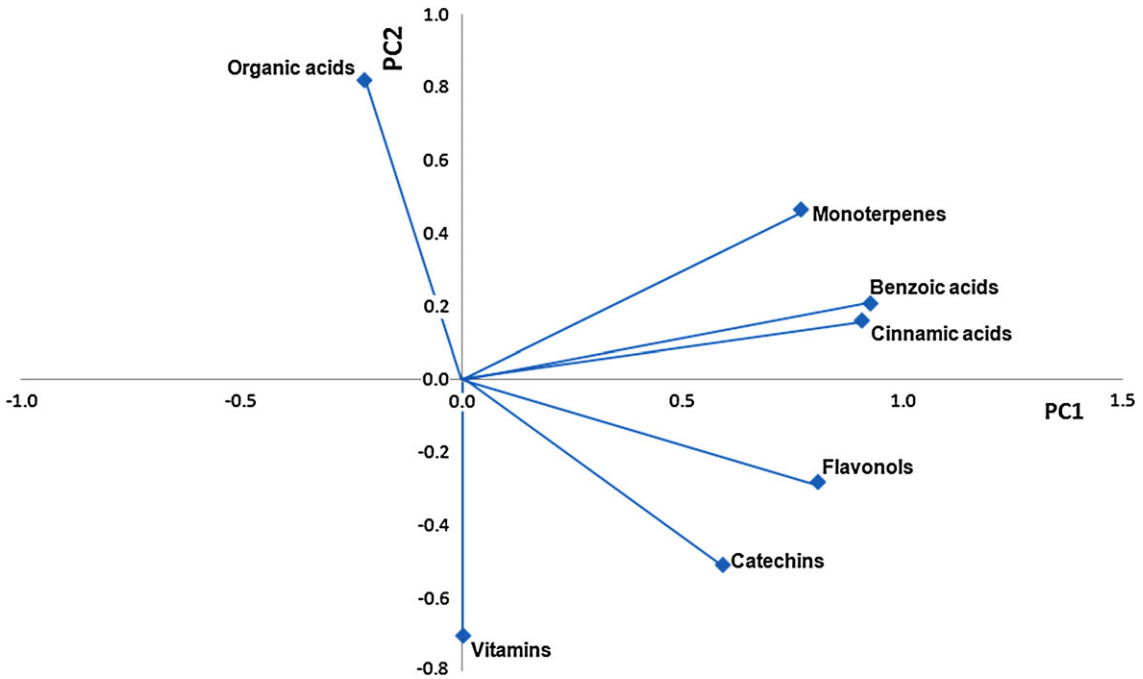

Figure 4. PCA loading plot of bud preparation samples showing correlation among bioactive compound classes and PCs.

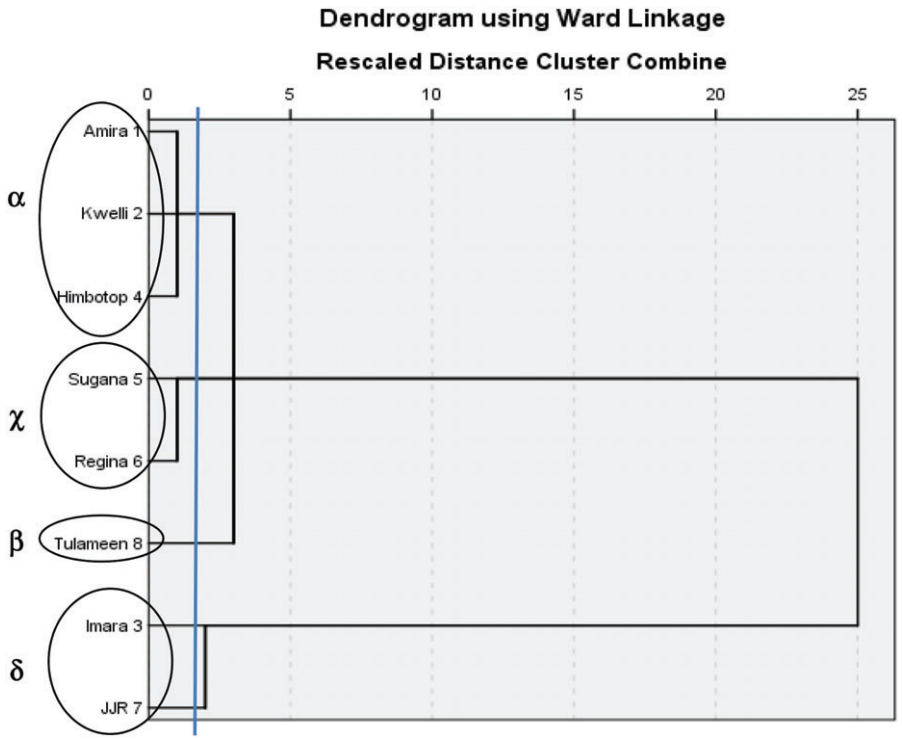

Figure 5. Dendrogram showing the four groups of eight raspberry cultivars analysed in this study. Ward's method as the amalgamation rule and the squared Euclidean distance as metric were used to establish clusters.

(8.71%) were the most quantitatively important, followed by cinnamic and benzoic acids (mean value of all the cultivars).

DISCUSSION

Owing to the increased use of herbal medicines worldwide, the safety and quality of medicinal plants has become a major concern for health authorities, and valuable sources for general analytical procedures are needed.[33]

In this context, the main aim of this research was to establish an effective and combinational method and unsupervised pattern recognition technique for phytochemical characterization of *R. idaeus* bud preparations, obtaining a botanical fingerprint of raspberry bud extracts by HPLC-DAD analyses. Compared with

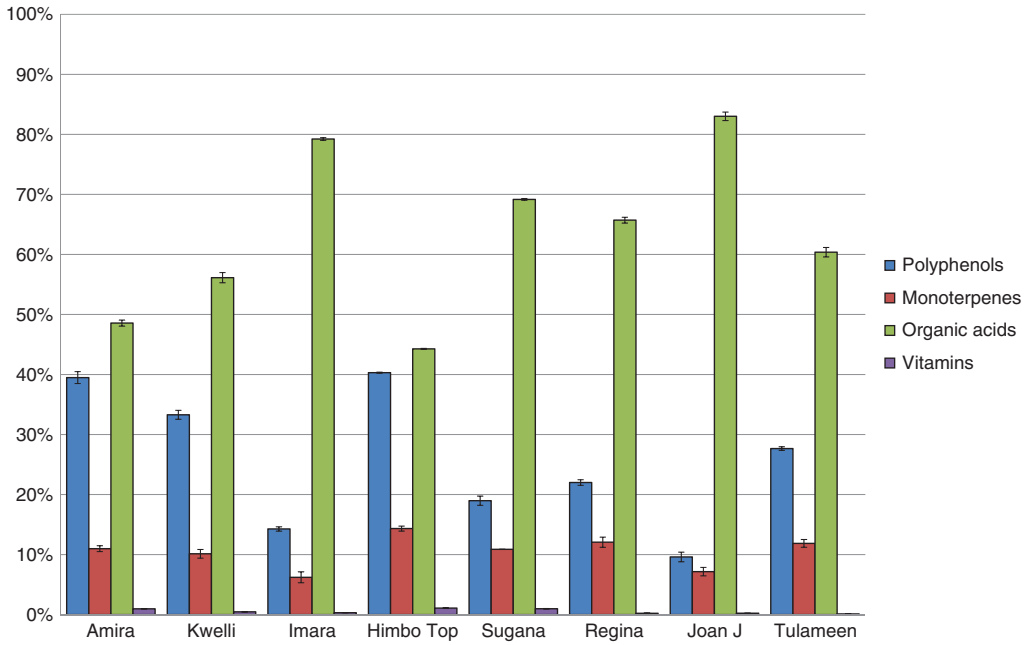

Figure 6. *Rubus idaeus* bud preparation percentage phytochemical composition (phytocomplex) in the eight analysed cultivars.

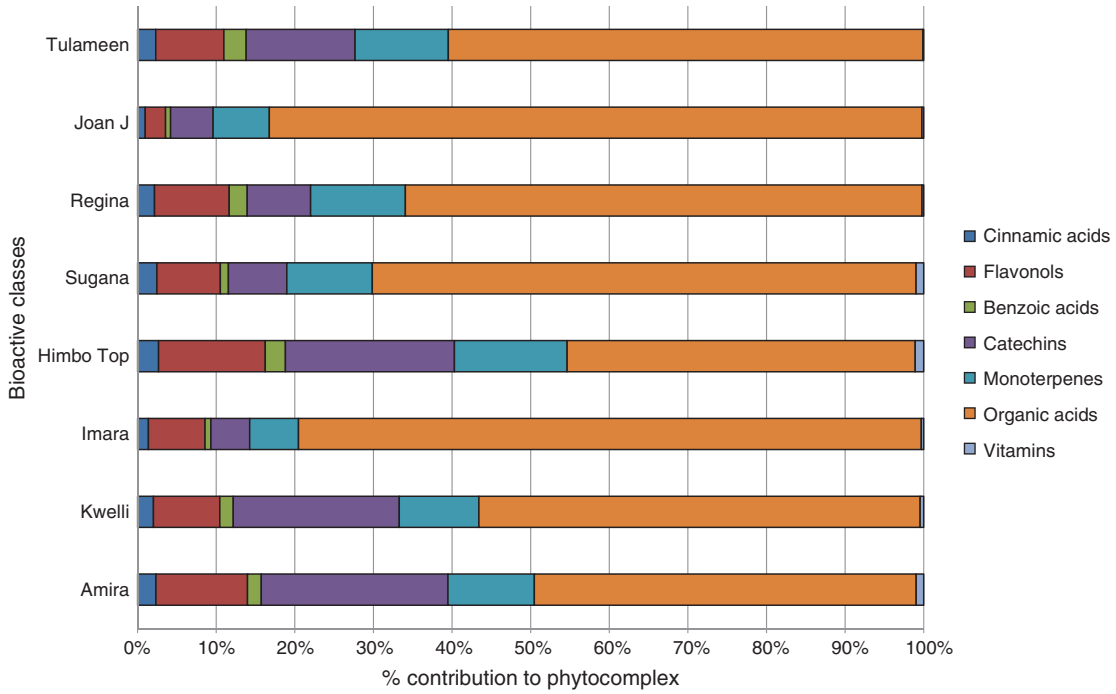

Figure 7. Contribution of each single bioactive class to the total phytocomplex (percentage ratio between each bioactive class content and TBCC) in analysed extracts for eight raspberry cultivars.

other analytical studies,[34] the chromatographic conditions were optimized to obtain information on bioactive compound composition with a good resolution and a reasonable analysis time in each analysis. Different linear gradients in different slopes were used, because some molecules were similar in the structure to each other in the same compound class. Moreover, most of compounds were also weakly acid, so adding formic (methods B and C) and phosphoric acid (methods A and D) was necessary for enhancing the resolution and eliminating peak tailing, as reported in other studies.[22] A full scan on the chromatograms from 190 to 400 nm was performed and DAD wavelengths have been selected in order to achieve more specific peaks as well as a smooth baseline, in accordance with other similar studies.[11]

Based on the obtained results, many studies only pointed out that the identified polyphenolic compounds significantly contribute to the total antioxidant activity;[3] there is still much confusion about the molecular mechanism of radical scavenging and the relationship between structure and activity of polyphenolic compounds,[35] because synergistic or additive biological effects of different phytochemicals, rather than a single compound or

a class of compounds, could contribute to disease prevention.[16] The present study confirmed these results; adding, as well as organic acids, terpenic compounds and vitamin C could also significantly contribute to the raspberry preparation phytocomplex; these bioactive classes could be considered antioxidant and anti-inflammatory health-promoting agents in food supplements.

It is well known that the chemical composition of secondary plant metabolites highly depends on factors such as pedoclimate, harvesting time and plant genotype,[36] and the results of this research confirmed this hypothesis for genotype condition; ANOVA test on TBCC and single bioactive compound profile showed that different raspberry cultivars presented different botanical concentrations in the corresponding bud preparations, but it might also be important to consider that bud phenological stage (bud harvesting time) and sampling site pedoclimatic characteristics could strongly influence the presence of these molecules, as reported in other studies.[37] In this case, this preliminary research did not focus on the sampling time and site effects because all the *R. idaeus* buds were picked in the same place (Conthey, Switzerland) and at the same phenological stage (bud break, March 2013).

A herbal medicine chromatographic fingerprint is a multivariate system, since in general it embraces most of the phytochemical constituents of a herbal product.[38] For this reason, it is very difficult to analyse the large number of complex multivariate data generated by a bud preparation chromatographic fingerprint;[34] several chemometric methods prove to be useful and versatile tools for the extraction, visualization and interpretation of the information. In this case, unsupervised pattern recognition methods such as PCA and CA allowed better visualization of the information that is included in the fingerprints. The results showed that PCA classification obtained from the seven main bioactive classes characterized the samples according to the different chemical compositions and provided information on the bioactive classes and chemical biomarkers with the most influence on the phytocomplex preparation, as shown in other similar studies.[21] The results of CA highlighted the similar chemical profile of cultivars in the same group.

In this study, HPLC fingerprint profiles of analysed *R. idaeus* bud preparations were similar by visual inspection, but there were many variations in chemical composition due to differences in the cultivars: chemometric methods should be applied with the HPLC fingerprint techniques for a better recognition of herbal extracts.[39] In this research, different biomarker compounds were found to be the most discriminating variables, which could be applied to accurate composition control of raspberry extracts; in particular, the phytocomplex graphical view showed that cultivars included in the α PCA group (Amira, Kwelli, Himbo Top) present the highest amount of antioxidant compound classes (polyphenols and vitamins) together with the highest amount of volatile molecules (organic acids and monoterpenes) most responsible for the *R. idaeus* bud preparation aroma. Catechin, oxalic and quinic acids and vitamin C represent the markers with the highest discriminating contribution on the preparation quality, based on the concentration differences found in this study.

The combination of chromatographic fingerprint and chemometric evaluation could be a powerful tool for herbal product quality control, in order to select the best cultivars depending on the desired traits and properties.[40] These hyphenated techniques could also be successfully used for the analysis and differentiation of several preparations commercially acquired in local markets. In this context, chemical fingerprint could be a useful tool for obtaining label certifications for the valorization of specific genotypes.

This is the first report on the identification and quantification of the main bioactive compounds selected as markers for *R. idaeus* bud preparations; it could be useful to guarantee the safety, efficacy and standardization of herbal products. the outcomes of this preliminary phytochemical investigation may provide a relevant contribution to the identification and quantification of lead compounds responsible for traditional therapeutic claims, but a further quantitative evaluation on the basis of their chemical structures with HPLC coupled to mass spectrometry is necessary.

CONCLUSIONS

In this study, simple, reliable and accurate analytical HPLC methods coupled to chemometrics were used for fingerprint analysis in order to detect and quantify bioactive compounds in raspberry bud preparations and for composition control of *R. idaeus* bud extracts; in this research, it has been shown that chemometric techniques such as CA and PCA were able to objectively and successfully visualize samples according to the cultivar origin.

Rubus idaeus buds were identified as a rich source of anti-inflammatory and antioxidant compounds, and the related bud preparations represent a rich source of organic acids and polyphenolic compounds, especially catechins and flavonols. Organic acids, vitamins and catechins were found to be the most discriminating variables among cultivars.

This study developed an important tool for the chemical evaluation of *R. idaeus* buds and its related products.

Finally, it also provided an important reference for the establishment of a preliminary method for characterization of the composition of plant-derived products and herbal preparations.

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