

Characterization of metabolites in different kiwifruit varieties by NMR and fluorescence spectroscopy

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Abstract

It is known from our previous studies that kiwifruits, which are used in common human diet, have preventive properties of coronary artery disease. This study describes a combination of ¹H NMR spectroscopy, multivariate data analyses and fluorescence measurements in differentiating of some kiwifruit varieties, their quenching and antioxidant properties. A total of 41 metabolites were identified by comparing with literature data Chenomx database and 2D NMR. The binding properties of the extracted polyphenols against HSA showed higher reactivity of studied two cultivars in comparison with the common Hayward. The results showed that the fluorescence of HSA was quenched by Bidan as much as twice than by other fruits. The correlation between the binding properties of polyphenols in the investigated fruits, their relative quantification and suggested metabolic pathway was established. These results can provide possible application of fruit extracts in pharmaceutical industry.

Keywords: NMR spectroscopy, Metabolites in kiwifruits, Fluorescence, Interaction of human serum albumin and polyphenols, Antioxidant and binding properties

1. Introduction

In our previous studies different kiwifruit varieties used as a remedy for protection of coronary artery disease were characterized by their antioxidant and nutritional properties [1–5]. Characterization was done by advanced analytical methods such as antioxidant assays, fluorescence and FTIR [2,3]. The ¹H NMR-based metabolomics approach has been applied in food chemistry with classification and evaluation of the antioxidant and binding properties [6,7]. Combination of ¹H NMR spectroscopy and multivariate data analyses were used in differentiating three closely related species of Apiaceae [8]. Principal component analysis (PCA),

partial least-squares analysis (PLS) and ¹H NMR was applied to distinguish variations among *C. caudatus* materials processed with various drying techniques [7]. ¹H NMR-based metabolomics data could be used to discriminate the different developmental stages and to predict the biological activities of *Ipomoea* leaves, monitoring the quality of fruits and vegetables [9]. ¹H high field NMR spectroscopy was applied to determine metabolic profiling of vegetables and fruits and their different bioactive changes in vitro and in vivo studies [10–13]. The popular consumption of kiwifruit is based on its health evaluation, as is shown in our recent studies, and partly depends on metabolites, particularly on polyphenols [14–18]. A full high field NMR study for the determination of the metabolic profiling of kiwifruit has been reported in the literature. Such method developed can be successfully applied and is suitable for high-throughput targeted metabolomics analysis related to nutritional intervention, or the study of the metabolic mechanism in response to a polyphenol-rich diet [19]. The changes in kiwifruit composition determined by classical methods [20] were in correspondence with the metabolic profiling of *Actinidia deli-*

ciosa, Hayward cultivar. The aqueous extracts and the water status of entire kiwifruits over the season (June–December) were determined by NMR methodologies [21]. Human serum albumin (HSA) is the major extracellular protein and functions as a carrier of various drugs. The flavonoids are a large class of polyphenolic compounds which occur naturally in plants and especially in fruits where they are widely distributed and exhibit antioxidant activity. The interaction of flavonoids was studied widely. There are some examples of such reactions of isovitexin, honokiol, magnolol, kaempferol, curcumin, genistein and caffeic acid phenethyl ester [22–26]. In our recent studies the binding interaction with HSA was investigated *in vitro* using fluorescence. The obtained results could explain the pharmacokinetic characteristics of polyphenols and flavonoids [2,3]. In the present study the metabolic profiling of kiwifruit extracts [*Actinidia deliciosa*, Hayward cultivar; *Actinidia arguta* (AM, selected *arguta* cultivar); *Actinidia eriantha* (Bidan cultivar)] was investigated by NMR and fluorescence spectroscopy. The correlation between the binding properties of polyphenols in the investigated fruits, their relative quantification and suggested metabolic pathway was established.

2. Materials and methods

2.1. Materials

Three varieties of kiwifruit were utilized in these studies which include *Actinidia arguta*, represented by AM (select *arguta*). AM is a mini kiwi [*A. arguta* (Siebold et. Zucc) Planch. ex. Miq.] was cultivated in 2013, on sandy loam soil, on the experimental field (ecological) of the Department of Environmental Protection, (SGGW). The sample assigned as KH (kiwifruit ‘Hayward’) belongs to *Actinidia deliciosa*, while BC (Bidan conventional) referred to *Actinidia eriantha*. Hayward’ and ‘Bidan’ were grown in an orchard in Heanang county in Jeonnam province in Korea, 2013.

2.2. Chemicals

All the NMR chemicals include 3-Trimethylsilylpropanoic acid (TSP), potassium phosphate monobasic (KH_2PO_4), methanol- d_4 (CD_3OD , 99.8%), sodium deuterium oxide (NaOD), and deuteriumoxide (D_2O , 99.9%), were purchased from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,9-dimethyl-1,10-phenanthroline (neocuproine), Folin–Ciocalteu reagent was purchased from Sigma Chemical Co., St. Louis, MO, USA. 2,4,6,-tripyrindyl-s-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade.

2.3. NMR sample preparation and measurement

The proton (^1H) and two dimensional (2D) J-resolved NMR procedure was carried out according to the previous reported protocols [6,7,11] with small modifications. The extraction for samples was done by transferring 100 mg of each sample to a 2 mL Eppendorf tube followed by the addition of 375 μL of both CD_3OD solvent and KH_2PO_4 buffer in D_2O (pH 6.0), containing 0.1% of TSP. The solution was then vortexed for 1 min before subjected to sonication for 15 min at controlled temperature. To get a clear supernatant, the mixture was afterward centrifuged at 13000 rpm for 10 min and 600 μL of it was pipetted to a NMR tube prior to analysis. The ^1H NMR analysis was performed at 25 °C on an INOVA 500 MHz spectrometer (Varian Inc., CA). For each sample, the required time was 3.53 min recording 64 scans with an acquisition time, a pulse width, and a relaxation delay of 220 s, 3.75 ms, and 1.0 s, respectively. These settings were for pre-saturation prior to ^1H NMR which is

required to suppress the water signal using low power selective irradiation. In addition, the spectral width of the recorded spectra was 20 ppm. The processing for all spectra, including phasing and baseline corrections was performed manually with Chenomx software (Version 6.2, Alberta, Canada). Moreover, the 2D J-resolved was conducted to endorse the metabolites identification.

2.4. Sample preparation for determination of bioactivity and total antioxidant capacity (TAC) of investigated fruits

The peeled fruits were weighed, chopped and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was lyophilized for 48 h (Virtis model 10–324), and the dry weight (DW) was determined. The samples were ground to pass through a 0.5 mm sieve and stored at -80°C [2,3,27]. Polyphenols were extracted twice from lyophilized fruits with methanol (concentration 25 mg/ml) at room temperature and were determined by Folin–Ciocalteu method [28] with measurements at 750 nm using a spectrophotometer (Hewlett–Packard, model 8452A, Rockville, USA). Flavonoids, extracted with 5% NaNO_2 , 10% $\text{AlCl}_3 \cdot x\text{H}_2\text{O}$ and 1 M NaOH, were measured at 510 nm. Total antioxidant capacity was determined by the following assays: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS $^{*+}$) was generated by the interaction of ABTS (7 mM) and $\text{K}_2\text{S}_2\text{O}_8$ (2.45 mM). This solution was diluted with methanol and measured at 734 nm [29]. Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe^{3+} -TPTZ) to a ferrous form (Fe^{2+}) [30]. Scavenging free radical potentials were tested in a methanolic solution (3.9 mL) of 1,1-diphenyl-2-picrylhydrazyl (DPPH) with the samples extracts in water or methanol (0.1 mL) [31]. Cupric reducing antioxidant (CUPRAC) assay is based on utilizing the copper (II) – neocuproine reagent as the chromogenic oxidizing agent [32].

2.5. Fluorometric studies

Profiles and properties of polyphenols in methanol extracts were determined by two (2D-FL) and three dimensional (3D-FL) fluorescence (model FP-6500, Jasco spectrofluorometer, serial N261332, Japan). Binding properties of kiwifruit extracts to human serum albumin (HSA) were evaluated by 2D and 3D-FL. For the fluorescence measurements, 3.0 mL of 2.0×10^{-6} M of HSA were prepared. The emission spectra were recorded in the range of 300–500 nm upon excitation at 280 nm. The 3D-FL was measured at the emission wavelengths between 200 and 795 nm, and the initial excitation wavelength at 200 nm. All solutions for protein interaction were prepared in 0.05 M Tris–HCl buffer (pH 7.4), containing 0.1 M NaCl [2,3,22,23].

2.6. Data analysis

The NMR data analysis was followed the reported procedure [8]. The conversion of ^1H NMR spectra to ASCII file using Chenomx software was done prior to multivariate data analysis (MVA), performed using SIMCA-P+ version 13.0 (Umetrics AB, Umeå, Sweden). This analysis consists of the exclusion of the residual water (4.70–4.90 ppm) and methanol (3.23–3.34 ppm) signals range. Next, all spectra were scaled to TSP and bucketed to bins with width 0.04 ppm, forming spectral region of 0.52–9.99 ppm. The binned integral of ^1H NMR data were then subjected to principal component analysis (PCA), which was applied to clearly differentiate the ^1H NMR spectra of the kiwifruit samples. Pareto method was also used for scaling purpose to give the same importance for all x variables in the analyses.

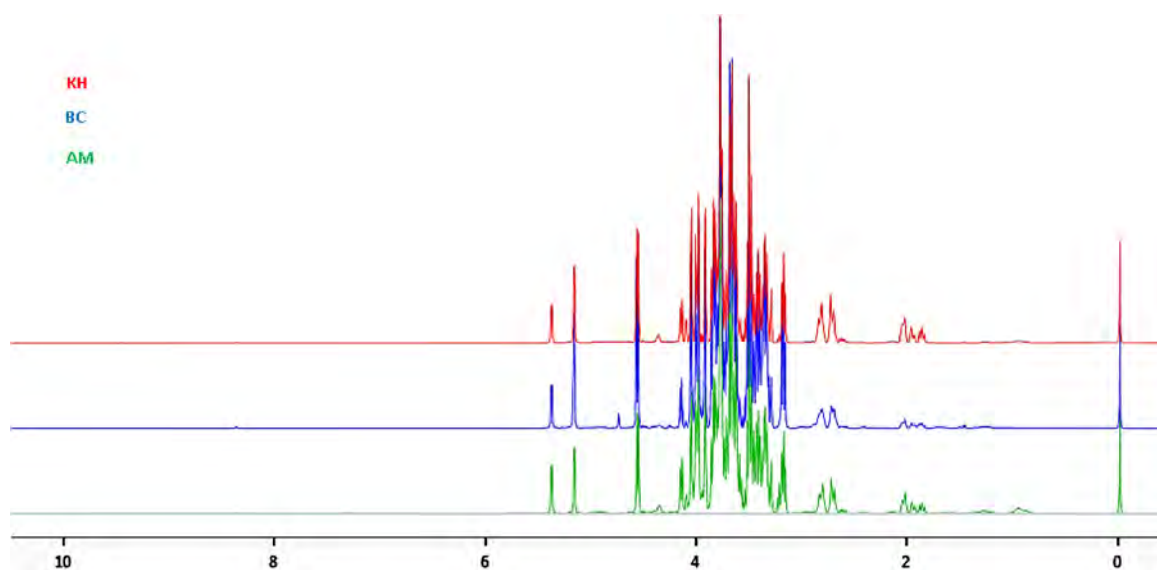


Fig. 1. The representative ^1H NMR spectra of kiwifruits Hayward (KH), Bidan conv (BC), and *A. arguta* (AM).

3. Results and discussion

3.1. ^1H NMR spectra inspection and identification of metabolites

In this study, the discrimination of three different varieties of kiwifruits was achieved based on their ^1H NMR fingerprint; and identification of important metabolites responsible for their separation are described. The overlay spectra of the representative kiwifruit samples are shown in Fig. 1. The apparent visualization to the ^1H NMR spectra of the samples showed the presence of various groups of metabolites in different kiwifruit species. A comprehensive inspection of the ^1H NMR spectra of the different kiwifruit samples based on its characteristic signals allowed the identification of organic acids (citric and aspartic acids), phenolics, flavonoids, sugars, fatty acids and amino acids (phenylalanine, alanine, arginine, glutamate, glutamine, leucine, isoleucine, tryptophan, hydroxy-L-proline, and tyrosine). A total of 41 metabolites were identified by comparing with literature data [2,15,16,21,33], Chemomx database and 2D NMR. The identified metabolites and their NMR characteristics as well as the presence in each sample are listed in Table 1. Based on Fig. 1, the most intense signals are observed in the δ 3.00–5.00 region, representing the carbohydrate constituents. There are differences in both quantitative and qualitative aspects of some metabolites of the different fruit samples, which can be detected from the ^1H NMR spectra. The principal peak areas of the spectra impart an immediate measure of metabolites concentration permitting the whole metabolites to be quantified based on a single internal standard. Different peak intensities can be observed at δ 5.20 (alpha glucose) and δ 5.40 (sucrose). Other identified sugars were UDP-glucose, sucrose, α -glucose, β -glucose β -xylose, α -xylose, and mannose.

Fig. 2 shows 1D NMR spectra of three different kiwifruits focusing on the region of δ 0.02–3.00 and δ 6.00–9.50 in the NMR spectra. The signals of phenolics and flavonoids (quercetin, kaempferol, and their derivatives) at the region δ 6.00–8.50 were identified. Meanwhile, the low field signal at δ 8.36 was assigned to formate. The attributable peaks belong to trigonelline, which was the product from vitamin B3 metabolism, was detected at δ 8.86 and δ 9.12 [10]. Different level of signal intensities can be observed in the aliphatic region. The high field region from δ 0.50 to δ 3.00 displayed signals of aspartate, citric acid and GABA.

On the basis of spectral analysis, BC demonstrated remarkable differences that can be observed in the content of quinic acid and shikimic acid. An earlier study done by Mittelstadt et al. [14] has shown that one of the novel unique features of kiwifruit is the occurrence of higher amount of quinic acid which plays a role in the flavor of the fruit. The higher content of quinic acid in BC might result in a distinctive flavor of BC as compared to the other two varieties. BC is also rich in flavonoids such as quercetin, kaempferol, hesperidin, and rutin. Meanwhile, the apparent variation in the metabolite content of KH from the other two varieties was based on the greater presence of syringic acid and organic acids. The differences in the metabolite content between kiwifruits especially the phenolics and flavonoids content were also reported in the earlier studies [1,2,5,15,33–35]. The obtained ^1H NMR data were further evaluated using multivariate data analysis (MVDA) to determine the discriminatory features between kiwifruit samples.

3.2. Multivariate data analysis

PCA, as an unsupervised classification method, can be performed without a prior knowledge of the data set, simplifying the dimensionality of the numerous variables while virtually sustaining the variances. The outcome of the PCA evaluation comprised of score plot which signifies the variation of the classes based on the metabolome similarities, and loading plots which offer information as to which NMR chemical shifts are contributing in the grouping attained in the score plots.

From Fig. 3(A), three different clusters were formed between studied fruit samples. The first two PCs showed an accumulated variance of 83.9%, where PC1 explained 55.9%, followed by PC2 with 28.0%. The three kiwifruit samples were separately clustered. The BC sample was obviously discriminated from the other kiwifruits by PC1. As shown in Fig. 3(B), the left lower quadrant of the PCA loading score plot which corresponded to the KH sample showed a lower intensity in most of the signals in the aliphatic regions (δ 0.50– δ 3.00), compared to other regions of the plot. This was consistent with the trend demonstrated in the 1D NMR spectra in Fig. 2. Based on the loading plot (Fig. 3), BC sample was separated from the other kiwifruit samples by its higher content of glucose, xylose, alanine, arginine and identified flavonoids as well as actinidic, syringic and protocatechuic acids. The metabolites include GABA, citric acid, glutamate, glutamine, hydroxyl-L-proline, shikimate, quinic acid,

Table 1
The identified metabolites and their characteristics.

No.	δ H(ppm), multiplicity, J value (Hz)	Tentative	Samples		
			AM	BC	KH
1	2.85, d, 15.0	Citric acid	+	+	+++
2	2.74, d, 15.0	Aspartic acid	++	+	++
3	2.63, dd, 16.0, 7.5	UDP-glucose (Uridine diphosphate glucose)	+++	+	+
4	7.91, d, 5.87, d, 5.83, d 5.50, m	Sucrose	+++	+	+
5	5.39, d, 3.7	α -Glucose	+	+++	+
6	4.17, d, 8.6	β -Glucose	+	+++	+
7	5.18, d, 3.5	β -Xylose	+	+++	+
8	4.58, d, 8.0	α -Xylose	+	+++	+
9	4.54, d, 7.2	Mannose	+++	+	+
10	5.20, d, 3.3	Phenylalanine	+	++	+
	5.12, br s				
	7.40, m (2H)				
	7.35, m				
	7.30, d, 7.4 (2H)				
11	1.46 d, 7.0	Alanine	+	+++	++
12	1.65–1.75, m	Arginine	+	+++	++
13	2.24–2.34, m	Glutamate	+	+	+++
14	2.10–2.20, m	Glutamine	+	+	+++
15	0.95, m	Leucine	+++	+	+
16	0.89, t	Isoleucine	+++	+	+
17	4.40, m	Hydroxy-L-Proline	+	++	+++
18	8.01, d, 8.0	Kaempferol	–	++	+
	6.95, d, 8.0				
	6.32, br d (small d)				
	6.10, br d (small d)				
19	7.65, d, 2.0	Rutin	–	++	+
	7.60, dd,				
	6.82, d, 8.5				
	6.38, d,				
	6.19, d,				
	1.05, d, 7.0				
	4.51, br s (small d)				
	5.05, d, 8.0				
20	8.36, s	Formate	+	+	+
21	9.12, s	Trigonelline	–	+	–
	8.86, m				
	8.08, t (not equal)				
22	7.70, d, 8.0	Tryptophan	+	+++	+
	7.54, d, 8.0				
	7.20, t, 7.0				
23	2.04, m	4-Aminobutyrate (GABA)	++	+	+++
	1.87, m				
24	3.94, m	Tyrosine	+	+	++
	7.15, d, 8.0				
	6.82, d, 8.0				
25	7.57, d, 13.0	Caffeic acid derivatives	+	++	+
	7.28, br s (small d)			+	
	7.22, d, 8.0				
	6.95, d, 8.0				
	6.55, d, 13.0				
26	2.06, m	Quinic acid	+	++	–
	1.97, m				
	1.89, dd, 10.0, 12.0				
27	6.70, m	Shikimate	+	++	+
	2.19, dd, 17.0, 7.0				
	4.43, t, 4.0				
	4.01, m				
28	7.39, br s (small d)	Protocatechuic acid (3,4-dihydroxybenzoic acid)	–	++	–
	7.35, br d (dd), 8.0				
	6.92, d, 8.0				
29	6.77–6.84, m	Catechol	+	++	+
	4.52, d, 7.20				
	2.94, dd, 15.7, 6.2				
	2.47, dd, 15.0, 8.0				
30	7.26, s, 2H	Syringic acid	++	+	+
	3.89, s				



Table 1 (Continued)

No.	δ H(ppm), multiplicity, J value (Hz)	Tentative	Samples		
			AM	BC	KH
31	4.65, s 4.62, s 5.32, t, 5.4	Actinidic Acid	+	+++	+
32	2.42, m	Malate	-	+	-
33	2.83; 2.80; 2.79, dd, 15.6, 4.8 2.68, dd 6.85, d, 8.0 (2H) 7.17, d, 8.0 (2H)	Afzelechin	+	++	+
34	3.21, s	Choline	+++		
35	8.96, s 8.54, br s 8.25, br s	Niacin (low concentration)	-	+	-
36	0.95, d, 8.0 1.03, d	Valine	+++	+	+
37	2.41, t, 6.9 0.89, s 0.87, s	Pantothenic acid	+	+	+
38	2.68, s, br t 1.37, s	α -Tocopherol	+	+	+
39	1.29, m	Fatty acids	+++	+	+
40	6.97, d, 2.7 6.46, d, 2.7	Hesperidin	++	++	+
41	7.52, d, 3.5 6.66, d, 3.5	Quercetin derivatives	+	++	+

Table 2

The content of total polyphenols, flavonoids and total antioxidant capacities of methanol extracts of kiwifruit samples (per g DW).

Indices	KH	BC	AM
Polyph, mgGAE	6.43 \pm 0.21 ^c	57.42 \pm 5.12 ^a	12.85 \pm 1.09 ^b
Flavon, mgCE	2.18 \pm 0.24 ^b	4.56 \pm 0.44 ^a	3.43 \pm 0.34 ^{ab}
ABTS, μ MTE	14.17 \pm 1.51 ^c	149.22 \pm 14.12 ^a	28.85 \pm 2.29 ^b
DPPH, μ MTE	7.65 \pm 0.76 ^c	80.33 \pm 8.72 ^a	15.45 \pm 1.65 ^b
FRAP, μ MTE	10.23 \pm 1.76 ^b	97.14 \pm 9.43 ^a	14.59 \pm 1.44 ^{ab}
CUPRAC, μ MTE	24.54 \pm 2.38 ^b	132.87 \pm 12.11 ^a	34.18 \pm 6.32 ^{ab}

Mean \pm SD (standard deviation) of 5 measurements. Average in rows marked with different letters differ significantly ($P < 0.05$). Abbreviations: ABTS, 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; FRAP, Ferric-reducing/antioxidant power; DPPH, 1,1-diphenyl-2-picrylhydrazyl; CUPRAC, Cupric reducing antioxidant capacity; Polyph, polyphenols; Flavon, flavonoids; CE, catechin equivalent; GAE, gallic acid equivalent; DW, dry weight; TE, Trolox equivalent; *Actinidia arguta*, AM (select *arguta*); *Actinidia deliciosa*, KH (kiwifruit 'Hayward'); BC (Bidan conventional), *Actinidia eriantha*.

phenylalanine, and afzelechin, were suggested to be responsible for the separation of KH sample for the rest. In addition, the AM samples dominate the high content of sucrose, choline, valine, leucine, tyrosine, mannose, and pantothenic acid. PCA scores plot illustrated distinct clusters between kiwifruit samples based on variation in metabolite content. However, PCA did not offer information regarding the degree of similarity shared between the studied kiwifruits.

The relationship between the three varieties of kiwifruits was also evaluated in the first level of our hierarchical cluster analysis (HCA). As shown in Fig. 4, the three samples were discernible from the each other based on different metabolite constitution. Two major groupings were formed in HCA dendrogram presented as (i) and (ii). Kiwifruit samples labeled as BC were clearly separated from the other two varieties by dominating cluster (i). This indicated the fewer similarities of BC compared to KH and AM, making this sample more distinctive. Meanwhile, all samples for KH and AM were positioned in the cluster (ii), suggesting a closer relationship between these two kiwifruits. The observations here were consistent with the clustering displayed in PCA score plot (Fig. 3). The previous study done by Park et al. [33] has also shown similar

results of the distant relationship between the new cultivar, BC and traditional one, KH based on HCA dendrogram [33].

The clustering pattern observed here might occur due to the differences in geographical origin and environmental factors. AM was cultivated in Poland while BC and KH were originated from South Korea. The variation in origin might explain the higher content of quinic acid, hence the unique taste of this particular variety. Meanwhile, BC was the newly consumed cultivar with a smaller size and distinct color property; green for the flesh and white for the skin [2,33]. The apparent differences in size and color of this kiwifruit may reflect the distant relationship of BC compared to the other two kiwifruit varieties. The results presented here indicated that the different varieties of kiwifruit affect more to the metabolite constituents of kiwifruit as opposed to the origin of cultivation.

3.3. The bioactivity of investigated samples

Polyphenols, flavonoids and antioxidant activities are shown in Table 2. The obtained results in methanol extract differ from the ones shown in our recent studies [1,2], but the significantly highest value in all antioxidants were in Bidan, following by AM and the lowest was in Hayward ($p < 0.05$).

3.4. Fluorescence results

Fig. 5 presents the polyphenol profiles of the studied three different kiwifruit varieties in comparison with caffeic acid. All the samples have three peaks a, b, c (Fig. 5A, Aa, B, Bb, C, Cc, Table 3A) and can be compared with caffeic acid as a standard (Fig. 5D, Dd, Table 3A). The highest fluorescence intensity from all peaks was in Bidan (Table 3A). Peak d had an average value in comparison with the first three and was found in all studied samples, but does not appear in caffeic acid with the highest fluorescent intensity (FI) in Bidan. Peak e appeared in Hayward ($\lambda_{ex}/\lambda_{em}$ 276/602 and FI = 51.8 \pm 4.8) and AM ($\lambda_{ex}/\lambda_{em}$ 280/610; FI = 124.3 \pm 11.5), and FI in AM was twice higher than in Hayward. Peak e1 ($\lambda_{ex}/\lambda_{em}$ 229/613 and FI = 67.9 \pm 5.8) was found only in AM and peak f ($\lambda_{ex}/\lambda_{em}$ 337/422 and FI = 84.5 \pm 7.8) was estimated in Hayward.

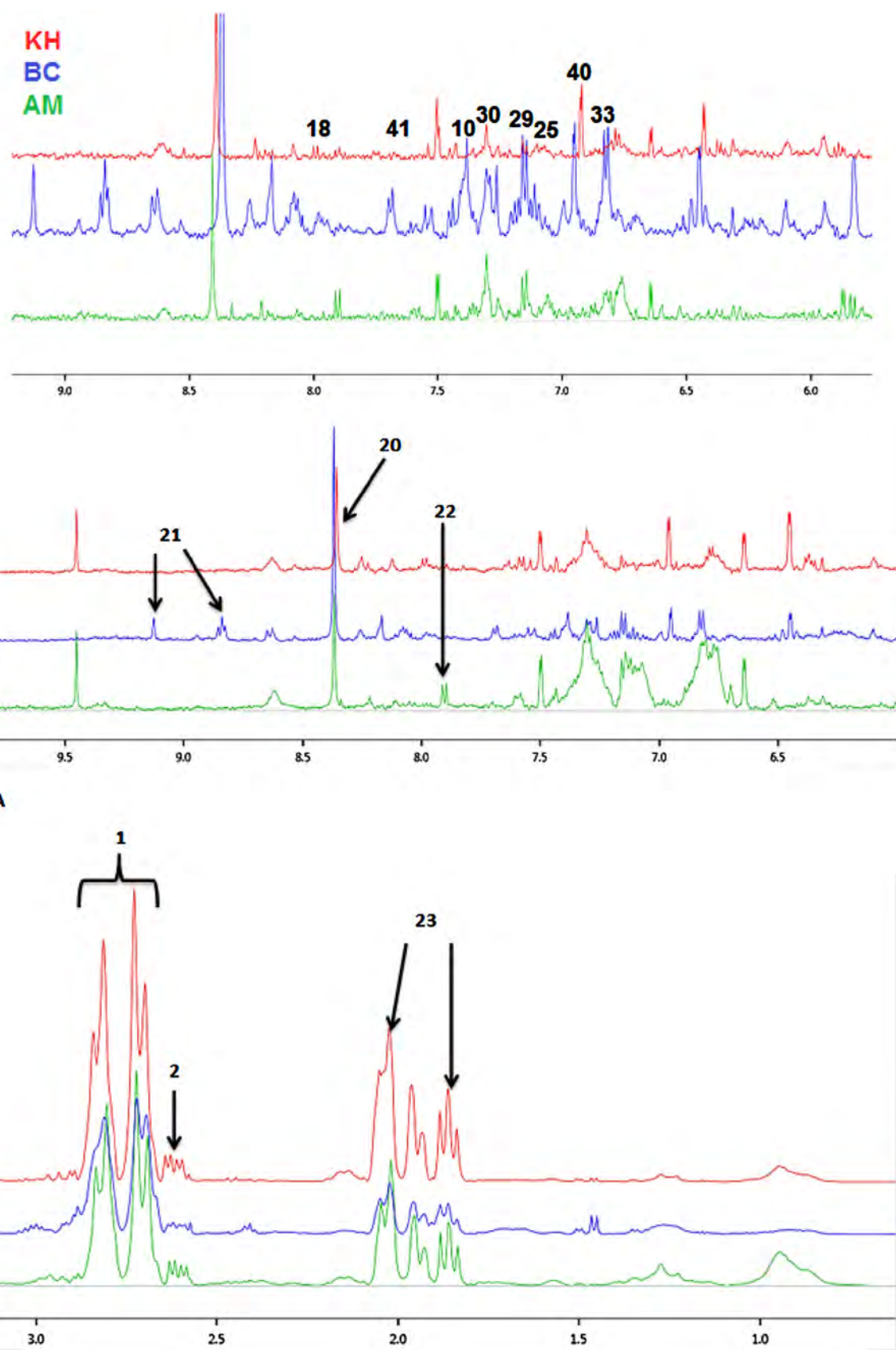


Fig. 2. The representative expanded 1D NMR spectra of the (δ 6.00–9.50, A) (δ 0.02–3.00, B) ranges in kiwifruits Hayward (KH), Bidan conv (BC), and *A. arguta* (AM).

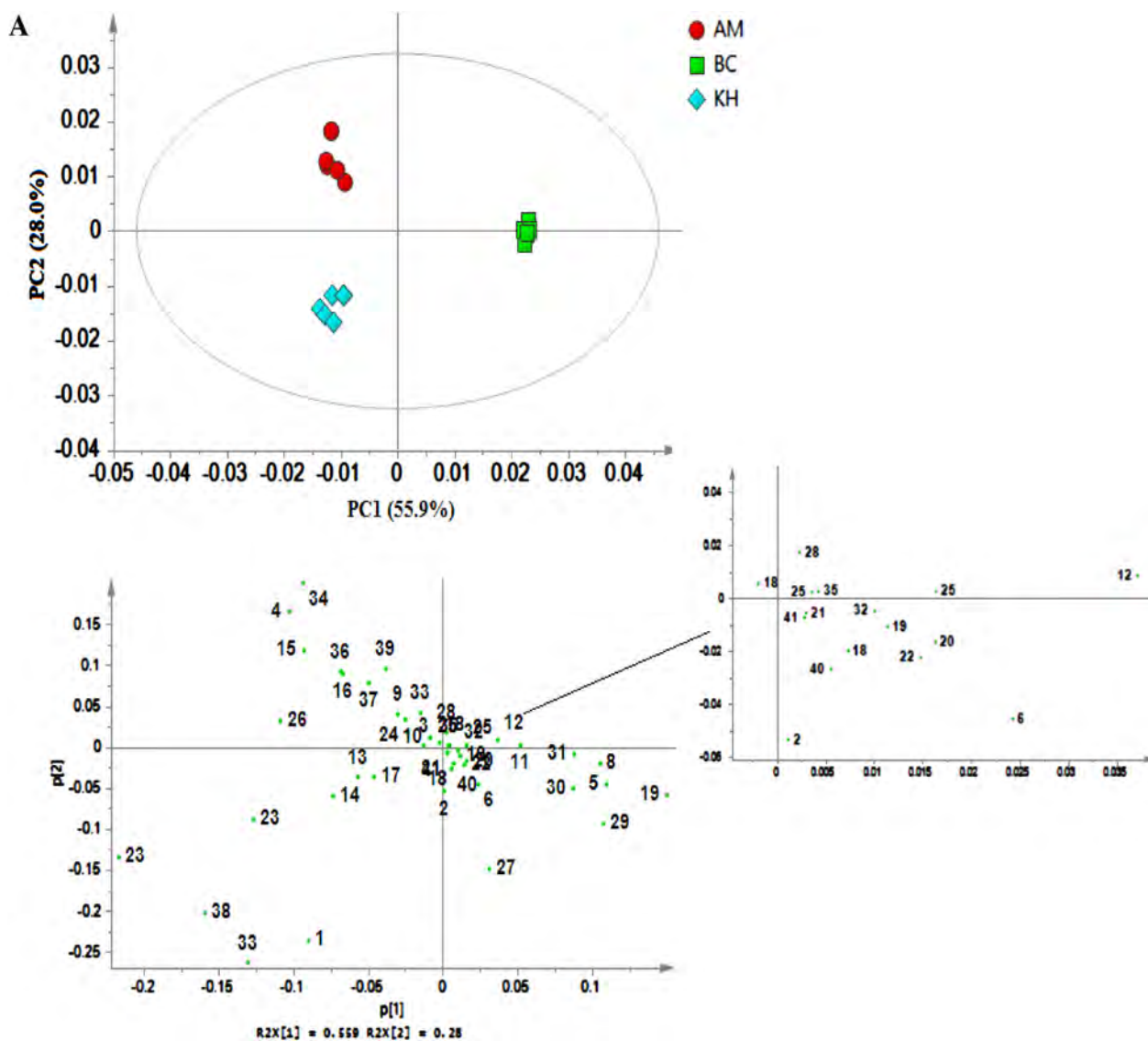


Fig. 3. The PCA score (A) and loading (B) plots of three kiwifruit varieties. Hayward (KH), Bidan conv (BC), and *A. arguta* (AM).

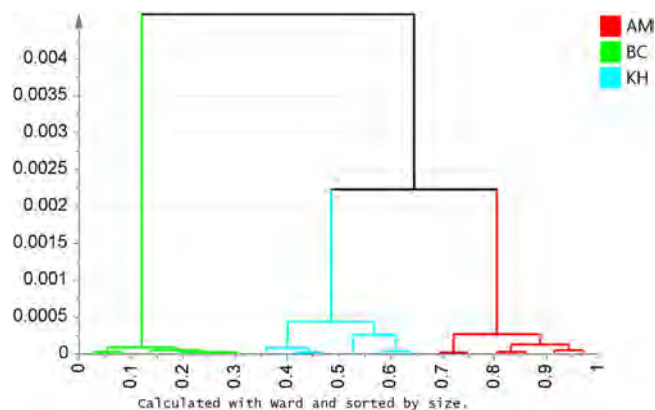


Fig. 4. Hierarchical cluster analysis (HCA) of for three different kiwifruits; Hayward (KH), Bidan conv (BC), and *A. arguta* (AM) based on group average cluster analysis of the different metabolite components.

The obtained spectroscopic data were in correlation with the determined metabolites. Some common fluorescence peaks with their spectral characteristics were found in Hayward and AM, but not in

Bidan. The binding properties of the polyphenols extracts to HSA are shown on Fig. 6 (Table 3B, C). The FI of two peaks **a** and **b** after interaction of HSA with Hayward (Fig. 6A, Aa), Bidan (Fig. 6B, Bb) and AM (Fig. 6C, Cc) can be compared with caffeic acid (Fig. 6D, Dd) and HSA before interaction (Fig. 6E, Ee). Peak **c** was found in all three kiwifruit samples with similar maximum wavelength of $\lambda_{ex}/\lambda_{em}$ 415/674, but the FI was the highest of 579.8 ± 23.6 for AM, following by Bidan of 462.5 ± 17.8 with the lowest of 135.1 ± 7.6 in Hayward. Peak **f** with slightly different wavelength maximum ($\lambda_{ex}/\lambda_{em}$ 360–377/443–468) was found in Hayward and Bidan. FI was lower in Bidan than in Hayward (148.9 ± 8.6 vs. 298.0 ± 12.4). Peaks **c** and **f** were neglected in the calculation of overall binding capacity. The binding properties of the methanolic extracts of the samples are shown in Table 3C. The highest binding properties of polyphenols were in Bidan, followed by AM, CA and Hayward. The calculated binding properties obtained from 2D-FL measurements were lower than by 3D-FL (Table 3C). This can be explained by the time necessary for 3D-FL measurements and occurrence of slight denaturation of proteins. The correlation and the value of the obtained results were similar. Peak **c** was not found (as it was mentioned above) in CA+HSA and HSA+MeOH samples. The fluorescence intensities after the interaction with HSA in the

Table 3

Three-dimensional fluorescence spectral characteristics (FI, fluorescence intensity, A.U., arbitral units) of methanol extracts of kiwifruits (K.): **A**, Hayw, Hayward; Bidan, AM, CA, caffeic acid; **B, C**, methanol extracts of kiwifruits with human serum albumin (HSA).

A									
Peaks	Indices	Samples							
		K.Hayw		K.Bidan		K.AM		CA	
A	$\lambda_{ex}/\lambda_{em}$	231/331		228/335		228/316		249/444	
	FI	196.9 ± 10.1 ^b		289.0 ± 15.4 ^a		253.6 ± 14.6 ^{ab}		232.6 ± 12.8 ^{ab}	
B	$\lambda_{ex}/\lambda_{em}$	278/331		259/337		280/321		295/437	
	FI	220.4 ± 9.6 ^c		599.7 ± 24.8 ^a		458.8 ± 20.6 ^{ab}		341.0 ± 18.7 ^b	
C	$\lambda_{ex}/\lambda_{em}$	410/670		403/669		405/668		337/434	
	FI	270.2 ± 13.5 ^c		987.0 ± 43.2 ^a		670.6 ± 29.6 ^b		341.1 ± 16.5 ^{bc}	
D	$\lambda_{ex}/\lambda_{em}$	278/665		259/669		280/665		–	
	FI	54.6 ± 4.7 ^b		176.9 ± 8.6 ^a		121.7 ± 6.9 ^{ab}		–	

B									
Samples	Peak a			Peak b			Peak c		
	$\lambda_{ex}/\lambda_{em}(nm/nm)$	FI	A.U.	$\lambda_{ex}/\lambda_{em}(nm/nm)$	FI	A.U.	$\lambda_{ex}/\lambda_{em}(nm/nm)$	FI	A.U.
HSA + K.AM	228/350	276.0 ± 20.6 ^c	–	280/354	624.1 ± 23.4 ^c	–	413/672	579.8 ± 22.6 ^a	–
HSA + K.Hayw	228/352	439.5 ± 28.6 ^b	–	280/357	667.3 ± 25.1 ^c	–	415/672	135.1 ± 8.7 ^c	–
HSA + K.Bidan	228/345	177.3 ± 12.4 ^d	–	282/345	517.6 ± 19.6 ^d	–	415/674	462.5 ± 25.1 ^b	–
HSA + CA	228/354	250.0 ± 16.5 ^c	–	278/357	759.1 ± 25.2 ^b	–	–	–	–
HSA	227/349	766.3 ± 27.6 ^a	–	279/352	875.3 ± 27.7 ^a	–	–	–	–
HSA + MeOH	228/349	744.1 ± 22.3 ^a	–	280/352	854.3 ± 24.9 ^a	–	–	–	–

C					
Line numbers	Samples	λ_{em}	FI	Binding(2D/3D)	
1	HSA	355	953.2 ± 23.6 ^a	–	
2	HSA + MeOH	350	902.2 ± 22.5 ^a	5.4/5.5	
3	HSA + K.Hayw	350	788.7 ± 16.5 ^{ab}	12.6/32.6	
4	HSA + CA	354	778.3 ± 14.3 ^{ab}	13.7/38.5	
5	HSA + Quercetin	335	702.6 ± 12.8 ^b	22.1/–	
6	HSA + K.AM	355	641.9 ± 8.4 ^{bc}	28.9/45.5	
7	HSA + K.Bidan	357	594.7 ± 7.9 ^c	34.1/57.7	

investigated samples can be compared with the initial fluorescence intensities (Fig. 5). Only the change in the intensity of the minor peak **c** after interaction with HSA showed the following binding properties (%) as 53.1, 50.0 and 13.5 for Bidan, AM and Hayward. Caffeic acid phenethyl ester as the used standard in the present report exhibited a distinctive binding interaction with HSA comparing with other propolis components [26]. Our results are in line with other recent reports where was shown that the fluorescence emission of HSA was quenched by rosmarinic acid through a combined static and dynamic quenching mechanism, but the static quenching was the major constituent. The rosmarinic acid was bound to HSA with moderately strong binding affinity through hydrophobic interaction [36]. Such interaction was not only with phenolic acids but also with flavonoids. HSA was quenched by honokiol (HK) or magnolol (MG) through a static quenching procedure. Hydrophobic interaction played a major role in the formation of the HK–HSA complex. In the binding interaction between MG and HSA can be involved the hydrophobic interaction strongly and electrostatic interaction [23]. Fluorescence quenching indicated that isovitexin location is within the hydrophobic pocket in subdomain IIA (site 1) of HSA, close to the Trp214 residue [22]. The interaction of kaempferol with HSA, the main *in vivo* transporter of exogenous substances, was investigated by fluorescence [24]. Human serum albumin (HSA) with high affinity binding sites is a major transporter for delivering several endogenous compds and drugs *in vivo* [25]. Curcumin, the yellow pigment from the rhizoma of *Curcuma longa*, and genistein one of the flavonoids found in soybean and chickpeas bind HSA via polypeptide polar group [25].

3.5. Relative quantification of phenolics of kiwifruit varieties and their suggested metabolic pathway

Primary and secondary metabolites of different fruits, including kiwifruits were identified by NMR approach [6,10,11,19,21,35]. The metabolites were relatively quantified using the ID NMR spectral data. The same trend of polyphenol content can be observed here for BC with those reported by Leontowicz et al. [2]. In the current study, BC showed the highest amount of catechol. Meanwhile, in the former study, BC showed the significantly higher amount of polyphenols than the other kiwifruit varieties. Besides that, it has been published that cultivar BC demonstrated significantly higher bioactivity hence higher bioactive compounds than the classic variety, KH. Based on the results obtained here, BC was consistently having the highest amount of phenolics as compared to the other two varieties. It is also worthy to note that hardy kiwifruit, AM contained a higher amount of tyrosine and lower polyphenols. With respect to organic acid, KH displayed the highest amount of syringic acid. The observation here was in agreement with the differences in the amount of organic acid between *A. deliciosa* and *A. arguta* in the study done by Nishiyama et al. [20]. The variation among the levels of metabolites might be due to the genetic diversity between these three kiwifruit varieties. It has been cited before that different variety of kiwifruits has shown variation in gene composition [18]. The variation in genetic make-up might lead to the differences observed in the chemical composition of kiwifruit. Besides that, AM can grow in the cooler regions owing to the ability to frost resistance [2]. The differences in growing regions might be another possible explanation of the different amount of metabolites in these kiwifruits. Other than, AM was cultivated in Poland which was in different origin from KH and BC, which were originated from South

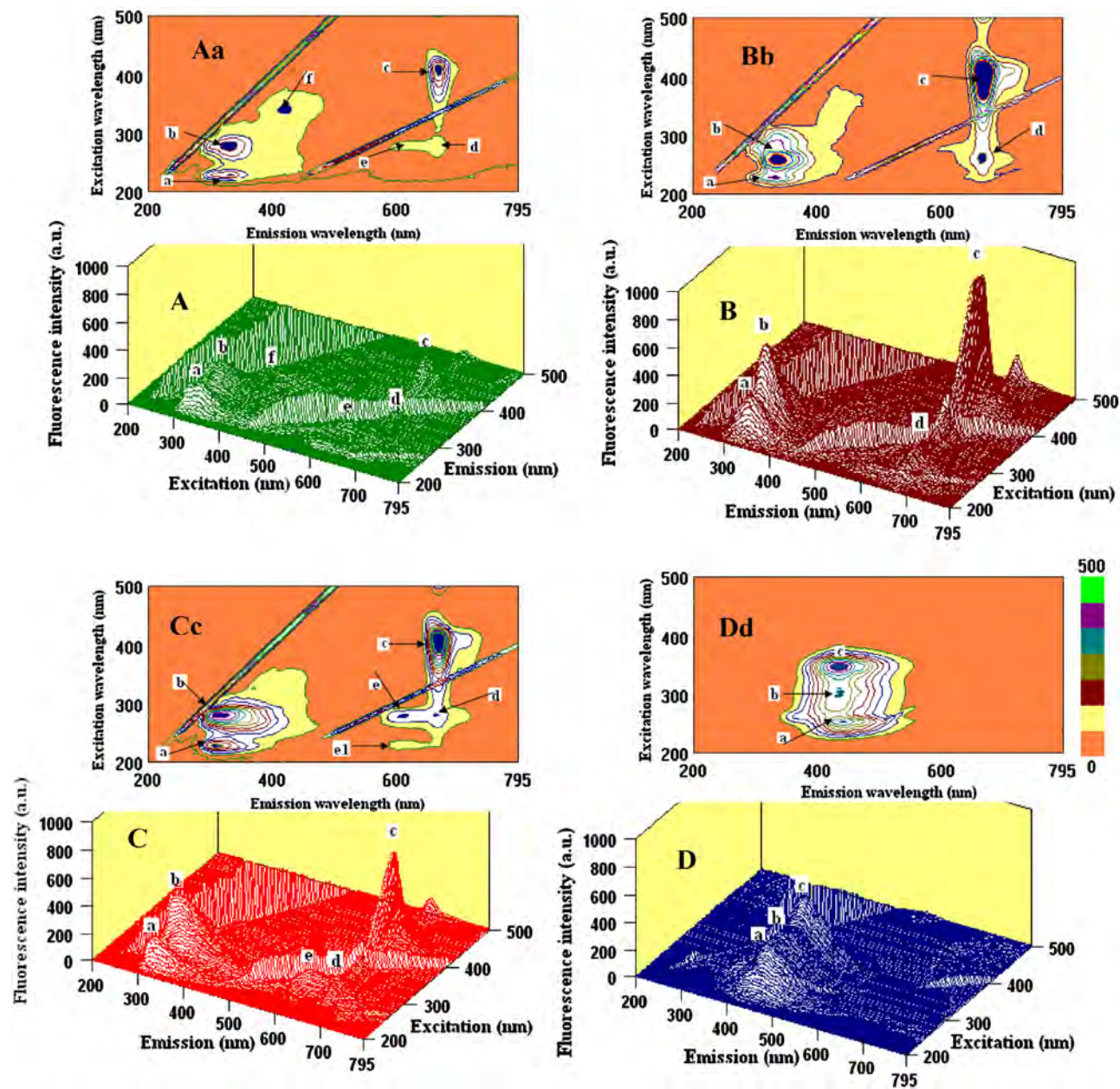


Fig. 5. 3D-fluorescence spectra (A, B, C, D) and corresponding contour maps (Aa, Bb, Cc, Dd) of methanol extracts of kiwifruits Hayward, Bidan, AM; and caffeic acid. Excitation wavelength scan: 200–500 nm. Emission wavelength scan: 200–795 nm. In each sample several peaks are shown (see Table 3A).

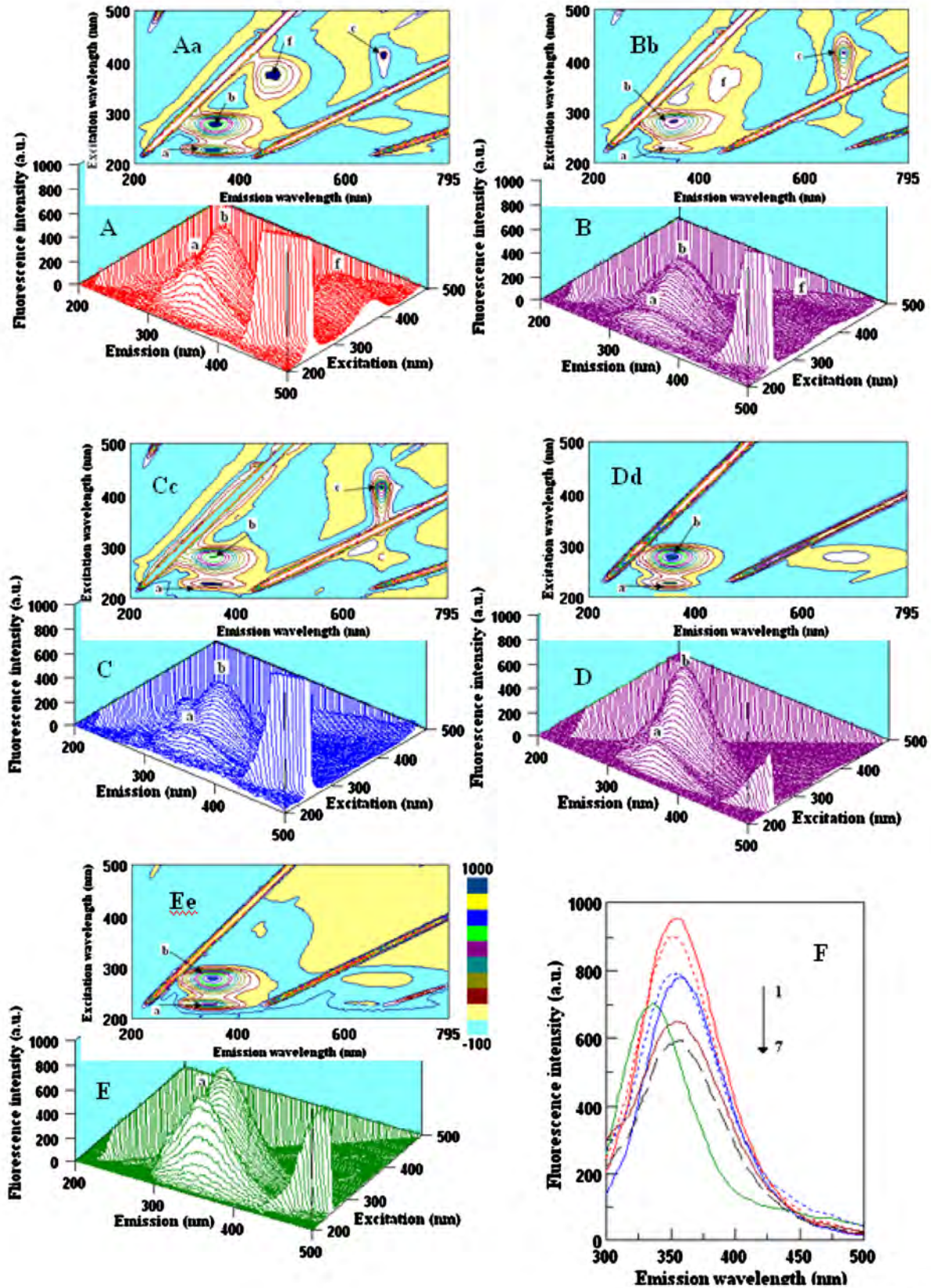


Fig.6

Fig. 6. 3D-FL (A, B, C, D, E) and counter maps (Aa, Bb, Cc, Dd, Ee) of spectral studies of interaction of human serum albumin (HSA) with polyphenols methanol extracts of kiwifruits Hayward, Bidan, *A. arguta* AM, caffeic acid, HSA in methanol. Excitation wavelength scan: 200–500 nm. Emission wavelength scan 200–500 nm. To 20 μ L HSA were added 20 μ L of 0.17 mg/ml of kiwifruit methanol extracts. The reaction was during 1 h at room temperature. CA – Caffeic acid 0.01 mM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

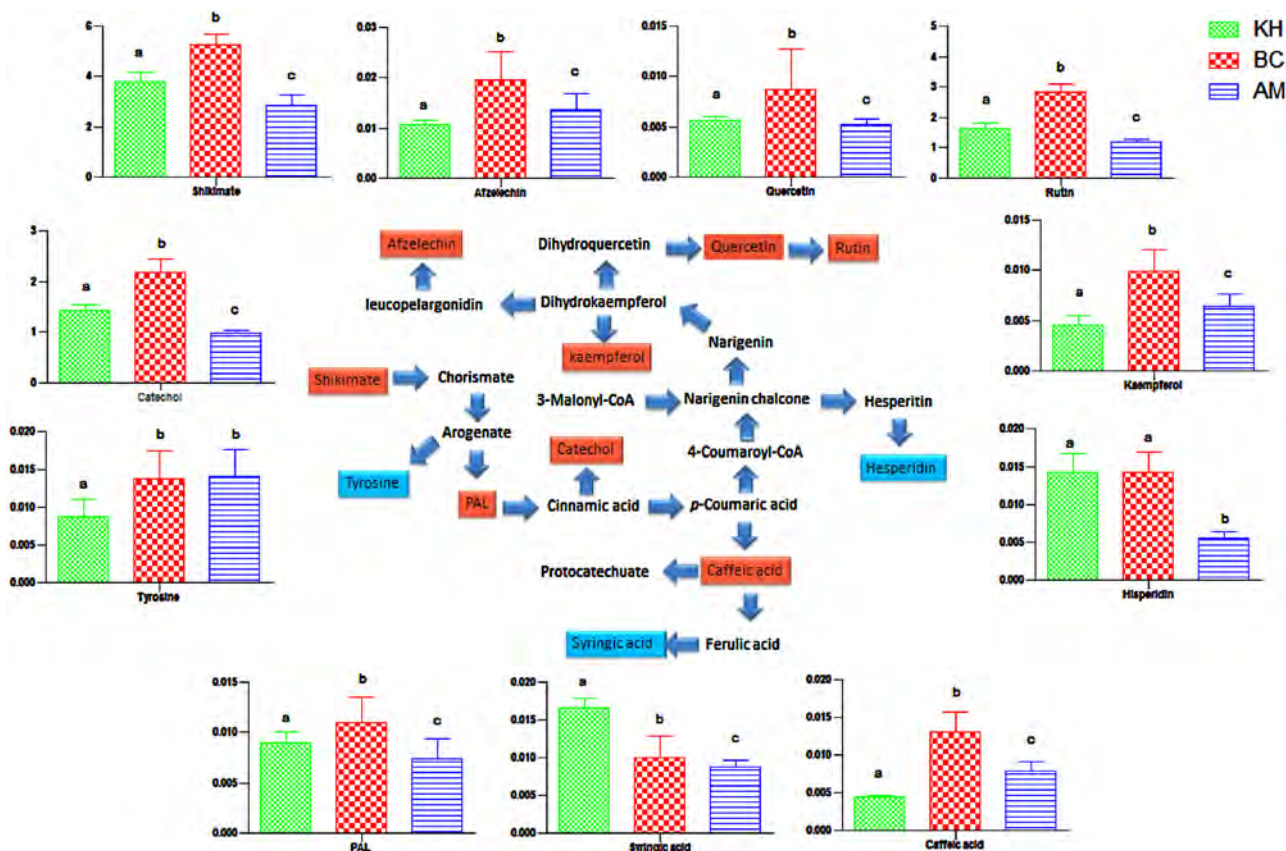


Fig. 7. Metabolic network and the metabolites quantification for kiwifruit varieties. The metabolites highlighted with the red color are higher in Bidan whereas the blue ones are lower compared to other varieties. KH, Hayward; BC, Bidan; AM, *A. arguta*. The letters a, b and c showed significant differences in the amount of metabolites between the varieties (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Korea. Thus, it can be suggested that the geographical origin also plays a role towards the chemical constitution between different kiwifruit varieties. It has been reported before that Korean cabbages showed different amount of phenolic compounds as compared to those cultivated in China [12]. KEGG database was used to design the metabolic pathways, which clarified the phenolics changes described by metabolomics. Fig. 7 shows the proposed pathway for the phenolics identified in kiwifruit varieties by NMR and their variations. This designed pathway links the metabolic pathways as a function to the significantly varied metabolites among the kiwifruit varieties. The phenolic compounds are the secondary metabolites derived from phenylalanine in the phenylpropanoid pathway [10]. Phenylalanine is converted to cinnamic acid, which is the synthesis precursor of vanillic, ferulic, and syringic acids. The high contents of phenylalanine in both *A. arguta* (AM) and *A. deliciosa* (KH) are associated with the potential quantities of most phenolics compared to *A. eriantha* (BC).

4. Conclusions

In the present paper, we showed an NMR investigation of kiwifruit Hayward in comparison with other two different groups. A large number of water-soluble metabolites which were assigned by 1D and 2D NMR experiments was found in three different varieties of kiwifruit. The differences in the metabolic profiles allowed distinguishing different kiwifruit samples. The results of the NMR analysis were corroborated by the amount of total phenolic and flavonoid contents. Antioxidant activities determined by DPPH, ABTS, FRAP and CUPRAC and the binding properties of phenols to BSA showed that kiwifruits exhibit high antioxidant activities and can be used as additives in food and pharmaceutical applications.

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