



Analytical Methods

Classification and fingerprinting of kiwi and pomelo fruits by multivariate analysis of chromatographic and spectroscopic data

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ABSTRACT

The fingerprinting capacity of two analytical techniques, HPLC and UV–Vis spectroscopy in the case of fruits samples (kiwi and pomelo) has been investigated. The multivariate exploratory techniques were used for sample discrimination according to the fruit species and subspecies. The combination of principal component analysis with linear discriminant analysis is opening new directions in the fingerprinting analysis. The classifications obtained were independent by the analytical techniques, which signalise that both of them may be successfully employed in the fingerprinting methodologies if they are combined with appropriate chemometric methods. One of the biggest advantages of the proposed chemometric method is the ability to discriminate different types and species and subspecies of fruits in just one analysis, which indicates that it is one of the simplest and less time consuming methods. It is strongly sustained by the good results obtained in the case of kiwi and pomelo fruit samples that were simultaneously analysed.

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1. Introduction

Nowadays, the main world wide tendency is the consumption of natural occurring products, such as vegetables and fruits. Some of the most consumed fruits are kiwifruit and pomelo, known also as Chinese grapefruit. None of these fruits have been intensively analysed to elucidate their chemical composition. The most known kiwifruits belong to *Actinidia deliciosa* species, from the *Actinidia* genus, *Actinidiaceae* family. The fruit flash colours are usually green, but there are known some red, purple, yellow and orange species (Ferguson, 1990). It has been shown that kiwifruit is not only tasty but it is also rich in vitamin C, catechins and polyphenolic acids (Imeh & Khokhar, 2002; Mattila, Hellström, & Törrönen, 2006). Recently studies have reported the identification and characterisation of a new tocopherol analogue (tocomonol) in the kiwifruit. Data about this compound antioxidant capacity allows to evaluate its involvement in the total antioxidant activity generally attributed to kiwifruits (Fiorentino, Mastellone et al., 2009). On the other side pomelo (*Citrus maxima* of *Citrus grandis*; *Citrus* genus, *Rutaceae* family) is a Southeast Asia fruit with white, pink or red flash. Pomelo fruit is characterised by significant amount of vitamin C and flavonoids, especially naringine and neohesperidin

(Franke, Custer, Arakaki, & Murphy, 2004). The polyphenols and vitamin C, presented in both fruits, are well known for their beneficial biological activity, mainly in the decrease risk of cancer and cardiovascular diseases (Fiorentino, D'Abrosca et al., 2009; Granado-Serrano et al., 2007). The level of chemicals and indirectly the resulted properties depends and varies in function of many important factors. The main differences appear between the different fruit species. However, the concentration of the chemical constituents can vary significantly depending on the growing conditions of the plant (region, soil, temperature, humidity, meteorological conditions). Other significant influences are the geographical origins, cultivation and harvesting methods, post harvesting processing and fruits storage (Dumarey, van Nederkassel, Deconinck, & Vander Heyden, 2008). The wide complexity composition of plant materials makes the analysis of individual compound to not be useful or feasible, because it is very laborious and often unjustified expensive. The development of a suitable analytical procedure to separate and evaluate all constituents is practically impossible to realise. The natural synergic activity of compounds is well known and the fruits properties are a consequence of the entire chemical composition complexity. Considering this aspects, the global assessment of the fruits is recommended, instead of focusing on specific individual compounds. However, knowing the major constituents may sustain and in the same time may explain a specific classification or different fruit properties. Such a possibility is

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offered by the fingerprinting methods, which are comprehensive characterising the analysed sample (Ni, Lai, Brandes, & Kokot, 2009). This method has as purpose the quality control of the vegetal material. It was firstly introduced for the characterisation of the herbal medicines and extended to other types of vegetal materials. Even more, the fingerprint methodology is widely involved in the authenticity and origin control of fruits, herbs or derived products (Costas-Rodríguez, Lavilla, & Bendicho, 2010). The US Food and Drug Administration (2000) and the European Medicines Agency (2001) recommend that the appropriate fingerprinting procedure involves chromatographic techniques. However, other techniques, such as the spectral ones may lead to interesting and useful results.

The chromatographic methods of fingerprinting are preferred for the sample characterisation since even at the first view it may offer some screening information about the chemical composition of the samples (Gong, Liang, Fung, & Chau, 2004). The accepted chromatographic techniques are including thin layer chromatography (Ciésła, Bogucka-Kocka, Hajnos, Petruczynik, & Waksmundzka-Hajnos, 2008), high performance liquid chromatography (Zhai, Hu, Huang, & Chen, 2010), gas chromatography (Cardeal, de Souza, Gomes da Silva, & Marriott, 2008), highly speed counter current chromatography (Gu, Zhang, Su, Chen, & Ouyang, 2004) and so on. The chromatographic fingerprinting has also been intensively employed in the species authenticity and origins investigations. However, nearby chromatography other analytical techniques were indicated as being representative in the fingerprinting procedures. The spectroscopic techniques may offer important information that may be used to identify the cultivation area and contaminated products (Ni, Zhang, Hou, Shi, & Guo, 2009). One of the most remarkable spectroscopic techniques is the nuclear magnetic resonance (NMR) spectroscopy, which is considered to be a powerful tool to determine metabolite fingerprinting and/or metabolic profiling of plant or animal extracts (Pereira et al., 2006; Taglienti, Massantini, Botondi, Mencarelli, & Valentini, 2009). Moreover, the FT-IR spectroscopy has been successfully used to classify the plant samples of different geographical areas (Wu et al., 2010; Yu, Sun, Fan, Zhou, & Noda, 2005), while the UV-Vis spectroscopy has not been intensively used because the obtained spectra are difficult to discriminate the samples, without the involvement of an adequate chemometric methodology (Casale, Oliveri, Armanino, Lanteri, & Forina, 2010), or the use of derivative spectroscopic analyses.

A significant disadvantage of routine fingerprinting methods is the fact that they usually require a large number of experiments and interpretation procedure, which are often tedious and time consuming. An alternative may be offered by an efficient simultaneous investigation of different types of samples. Focused on these considerations, the main goal of this study has been the development of a new simple and fast method for the simultaneous discrimination and authentication of various samples of fruits based on chemometric analysis of HPLC and UV-Vis spectroscopy data. The proposed method has been tested on four subspecies of kiwifruit and three subspecies of pomelo fruit. It has been evidenced that the UV-Vis spectrometry may be also considered a competitive technique in the fingerprinting approaches. In addition, the methodology developed in this paper might be also extended in the authenticity and origin control of fruits, herbs or derived products.

2. Experimental

2.1. Fruits samples and extraction procedure

All compounds and solvents were obtained from Merck (Darmstadt, Germany) in analytical degree purity. Water was purified by use of a Millipore Waters (Milford, MA, USA) Milli-Q system. All fruit cultivars that reached commercial maturity stage were

harvested in an orchard located in Heanam County, Jeonnam Province, Korea in 2009. The fruits were stored in a cool and dry atmosphere till the sample preparation has been performed. Kiwifruit belong to some subspecies of *Actinidia chinensis*, namely: Hayward (HW), Haenam (HN), Bidan (BD) and Daeheung (DH), while pomelo fruit belong to some subspecies of *C. maxima*, named as follows: Kao Paen (KP), Thong Dee (TD) and Tha Knoi (TK).

The fruits extraction pre-treatment consists in peeling and chopping of the fresh fruit flash, followed by their freezing at $-80\text{ }^{\circ}\text{C}$ for 2 h. The frozen piece of fruits was lyophilised for 72 h at 0.2 Pa and stored at $4\text{ }^{\circ}\text{C}$. Before extraction the lyophilised samples were powdered by means of a mortar and pestle. Recently, the ultrasound assisted extraction technique is one of the most used phytochemical compounds extraction because of its significant advantages expressed in terms of simplicity and extraction capacity; thereby it was used to extract the chemical compounds from the fruit matrices. The extraction conditions were typical for a large spectrum of polyphenolic compounds extraction, such as flavonoids and polyphenolic acids. The extractions were performed in an Elmasonic S15H ultrasonic bath (Singen, Germany), with a frequency of 37 kHz and a power of 95 W. Each sample (2.5 g) was extracted for 30 min with 30 mL extraction solvent (ethanol: water, 70:30, v/v). The hydroalcoholic mixture will be able to extract mainly the polyphenols. Three parallel extractions were performed for each fruit subspecies. The obtained extracts were filtered through quantitative paper filters with grade of $40:8\text{ }\mu\text{m}$ (Whatman, GE Health, Germany) and rinsed trice with 5 mL of the extraction solvent. The filtrate was evaporated to dryness by using a rotary evaporator (Heidolph Laborota 4000 eco, Suarlee, Belgium) and the residuum has been diluted quantitatively with 10 mL of extraction solvent. The evaporation had as purpose the concentration of the fruit extract. Previously of the chromatography the extracts were filtered through nylon microfilters of $0.22\text{ }\mu\text{m}$ (Whatman, GE Health, Germany), and directly injected in the HPLC system.

2.2. Chromatography

The chromatography was performed on an Agilent 1100 Series LC (Agilent Technologies, Santa Clara, CA, USA) system consisting of a vacuum degassing unit, a binary pump, an autosampler injector, a column thermostat, and a diode array detector (DAD). The system has been directly connected to 1100 MSD mass spectrometer, equipped with an electrospray ion source and ion trap analyzer (Agilent Technologies, Santa Clara, CA, USA). The injection volume of the fruit extracts was $20\text{ }\mu\text{L}$ per sample. The chromatographic analyses were carried out on a LiChrosphere RP-18e column ($250 \times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$ particle size). A binary gradient elution system composed of 0.1% solution of formic acid in water as solvent A and 0.1% solution of formic acid in acetonitrile: methanol (1:1; v/v) as solvent B was applied for the fingerprint analysis with gradient elution as follows: 0.00 min, 15% B; 0.00–10.00 min, 15–22% B; 10.00–17.00 min, 22–27% B; 17.00–17.01 min, 27–40% B; 17.01–25.00 min, 40–50% B; 25.00–30.00 min, 50% B. The separations were performed with a flow rate of 1 mL min^{-1} , which was directly injected in the ESI source, without any splitting. The column temperature has been maintained at $30\text{ }^{\circ}\text{C}$. The analysis time was of 30 min. The ESI mass spectrometer was set to run in negative SCAN mode with a capillary voltage of 4000 V and sample cone voltage of 70 V. The MS pseudoions range has been chosen between 50 and 2500 m/z . The HPLC-MS method was tested on 16 phytophenolic compounds (catechin, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, sinapic acid, rutin, naringin, quercetin, naringenin, kaempferol, apigenin, phloretin and luteolin), in order to evaluate its separation capacity. All the standards were Sigma-Aldrich products quality. The polyphenols standard solutions ($10\text{ }\mu\text{g mL}^{-1}$) were prepared in methanol.

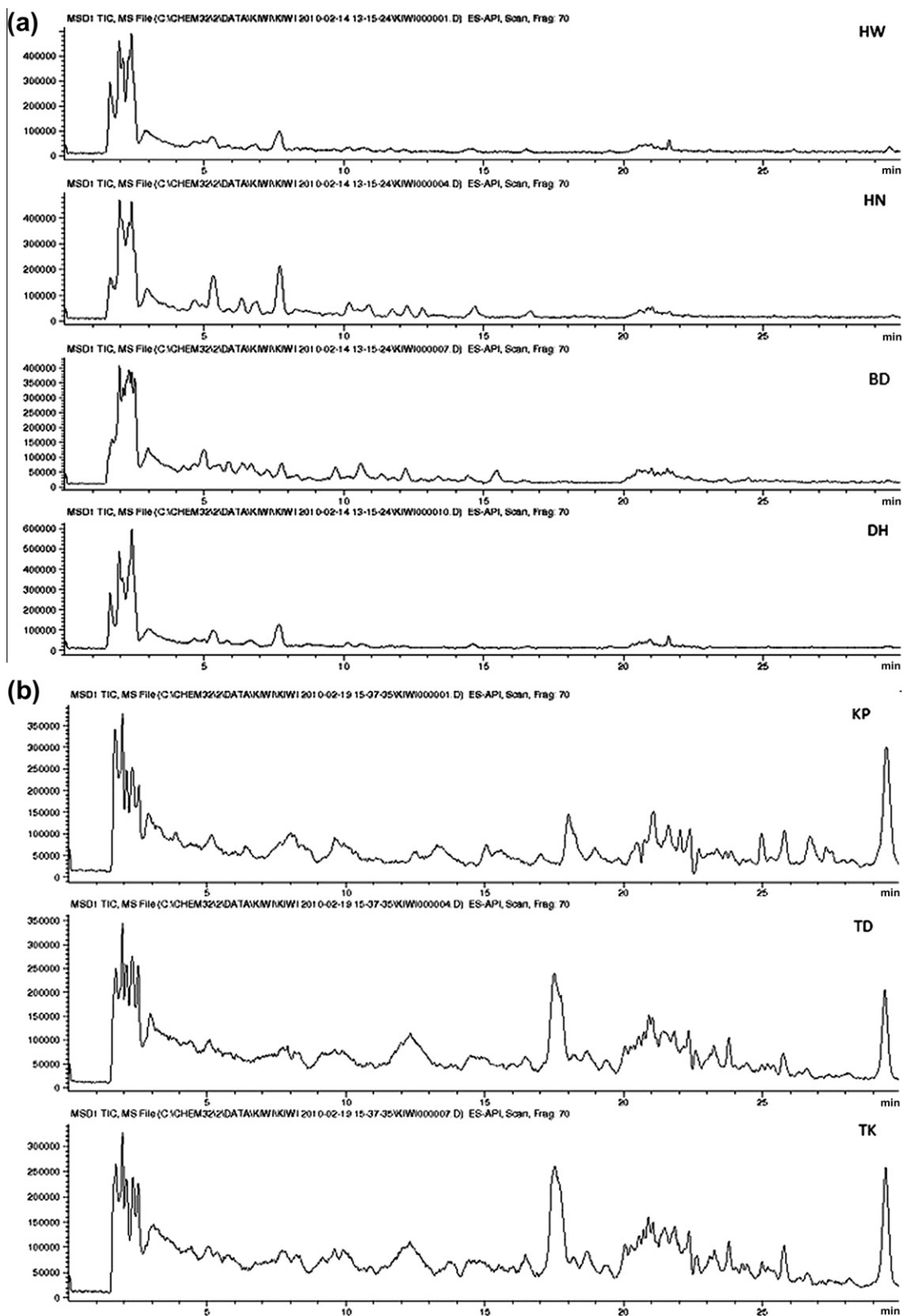


Fig. 1. The HPLC–MS chromatograms obtained for kiwifruit (a) and pomelo fruit (b).

2.3. UV–Vis Spectroscopy

The UV–Vis absorbance spectra were recorded using a V-550 Jasco spectrophotometer (ABL&E Jasco, Cluj-Napoca, Romania) on a wavelength range starting from 200 to 500 nm. Previously of

spectroscopic investigation, all fruits extracts were 10 times diluted as follows: 1 mL of each fruit extract was introduced in a 10 mL volumetric flask, which was then filled with methanol. The obtained mixture was homogenised under ultrasonic conditions for 10 min. All spectra were recorded with a resolution of 0.5 nm.

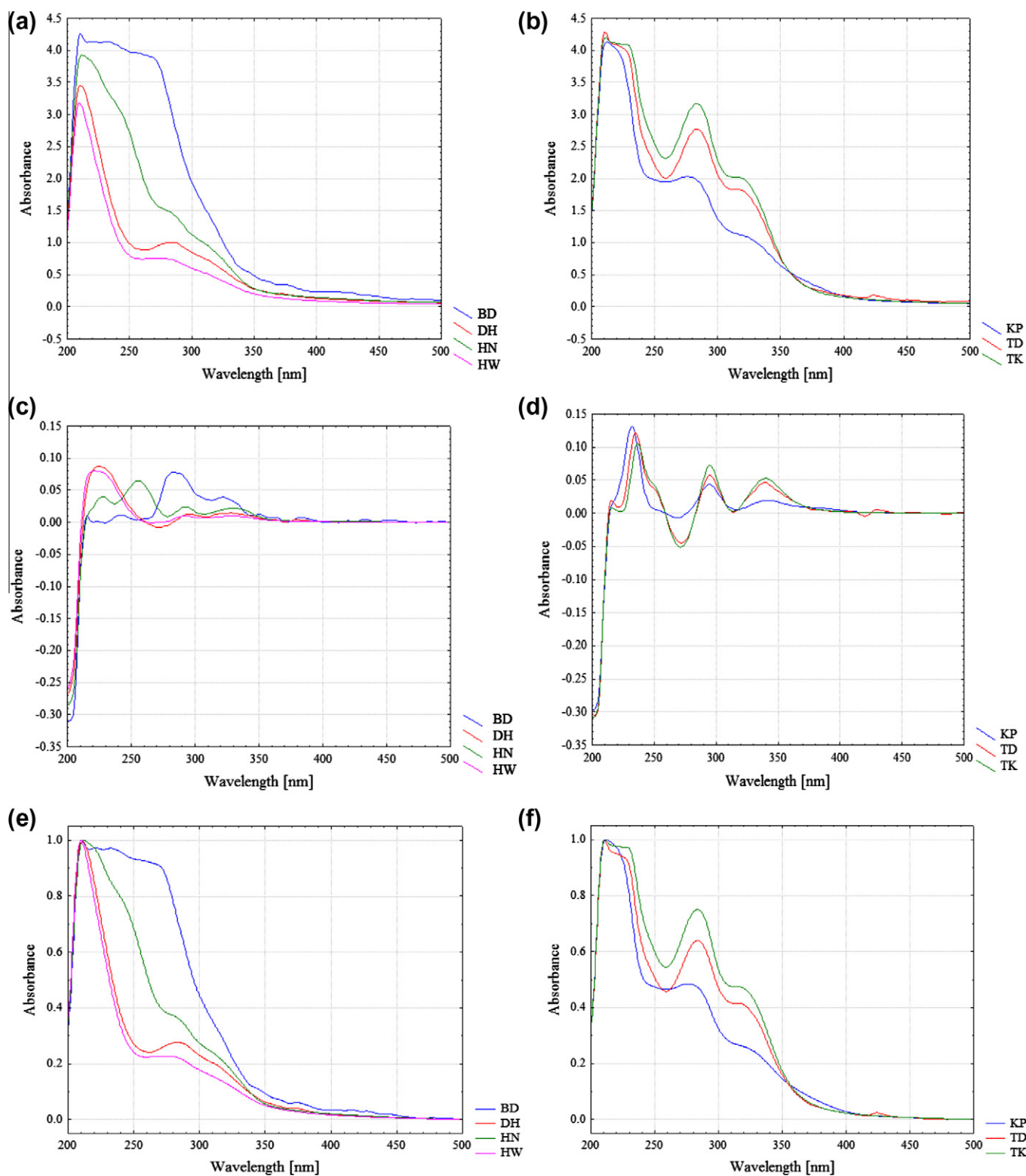


Fig. 2. The UV-Vis zero order (a, b), first order (c, d) and normalised (e, f) spectra of kiwifruit and pomelo extracts.

The obtained zero order spectra were normalised by using the following Eq.:

$$y_i = \frac{x_i - x_{\min}}{x_{\max} - x_{\min}} \quad (1)$$

where y_i represents the normalised value, x_i is the initial absorbance value at given wavelength and x_{\max} and x_{\min} are coinciding to the maximum and minimum absorbance values for the zero order UV-Vis spectra. Moreover, the first order spectra have been computed according to the Savitzky-Golay algorithm (Savitzky & Golay,

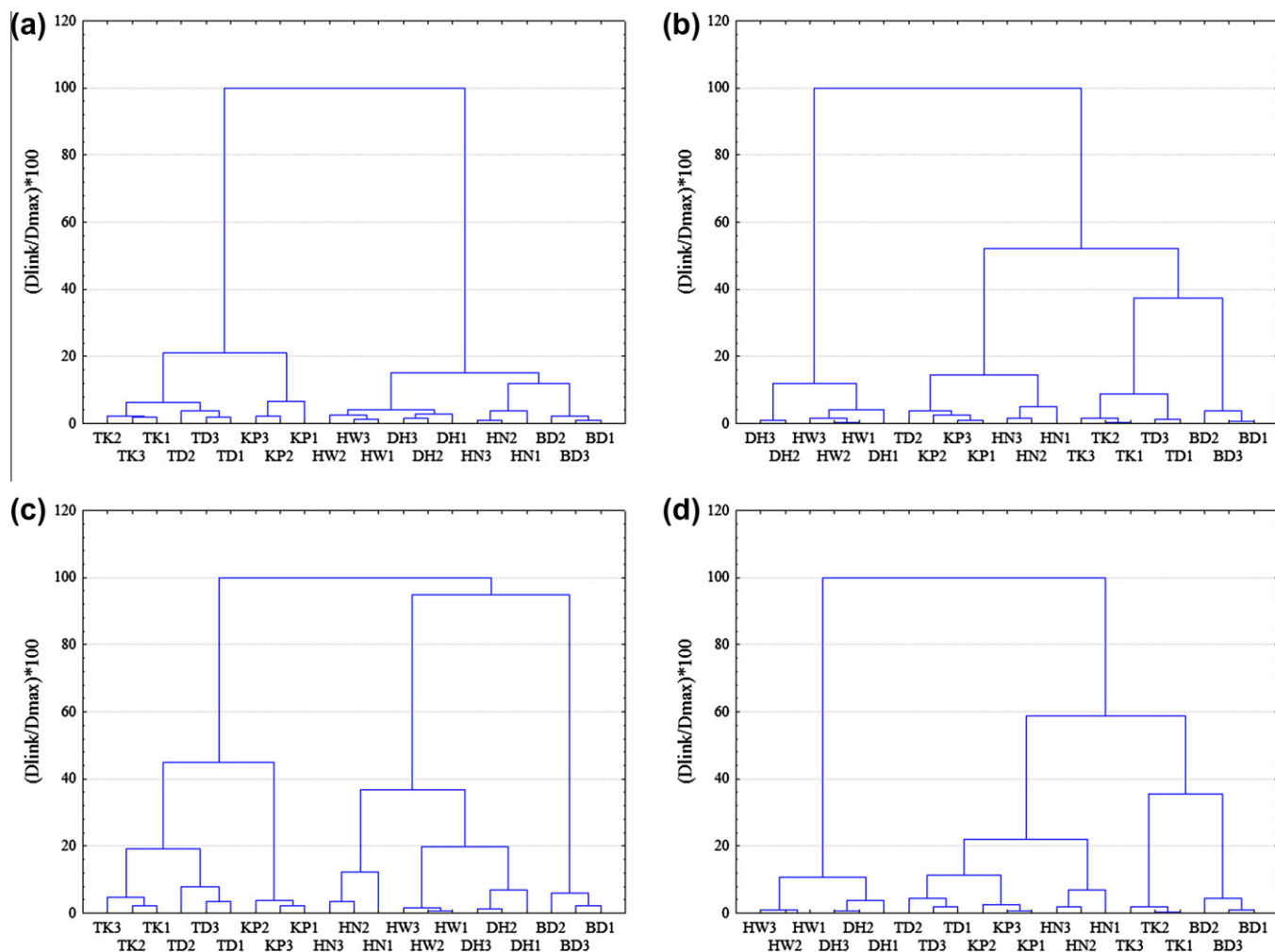


Fig. 3. Hierarchical clustering of kiwi and pomelo fruits using HPLC digitized chromatogram (a) and UV-Vis spectra data: (b) zero order spectra; (c) first order spectra; and (d) normalised zero order spectra.

1964) included in the Spectra Analysis option of Jasco spectrophotometer software.

2.4. Chemometric methods

Nowadays, many chemical problems are solved by the computational methods, especially those classified as multivariate exploratory techniques. While the computational methods were introduced in the fingerprinting methodology a new analytical level has been reached. In this order, the most used techniques are the principal component analysis (PCA), partial least squares (PLS), cluster analysis (CA), linear discriminant analysis (LDA), and so on (Chen et al. 2008; Xu et al. 2009). CA is a well-known and widely used unsupervised clustering procedure with its hierarchical and non-hierarchical approaches. CA is a multivariate analysis technique used to sort samples (in our case fruits extracts) into groups. The Ward's method as the amalgamation rule and the squared Euclidean distance as metric were used to establish clusters. PCA is the most preferred multivariate technique because it is considered a sophisticated technique that is reducing the dimensionality of the original dataset by explaining the correlation amongst a large number of variables (digitized chromatograms and spectra) in terms of a smaller number of underlying factors

(principal components or PCs) without losing much information (Kong, Zhao, Xiao, Jin, & Li, 2009). The PCs are a very useful tool for examining the relationships between objects, looking for groups and trends, sorting out outliers. The PCA and CA are unsupervised techniques which offer useful information about samples by graphical representation as 2D and 3D patterns or as a dendrogram, but sometimes when the similarities are very prominent they are not enough for a highly sustained conclusion. On the other side, the LDA is a supervised classification technique based on the linear discriminant functions, which maximises the ratio of between-class variance and minimises the ratio of within-class variance. LDA selects directions, which accomplish maximum separation among the given classes. The Euclidean distance is used in the LDA algorithms in order to classify unknown samples and the stepwise algorithm to extract the most important variables (Moţ, Soponar, & Sârbu, 2010; Ni, Peng, & Kokot, 2008; Xiang et al., 2010). It is also possible to visualise how the functions discriminate between groups by plotting the individual scores for the discriminant functions. In addition, the combination of the PCA and LDA may offer some remarkable information for classification and discrimination of the considered samples. The chemometric analysis has been performed by using Statistica 7.1 software (StatSoft, Inc., Tulsa, USA).

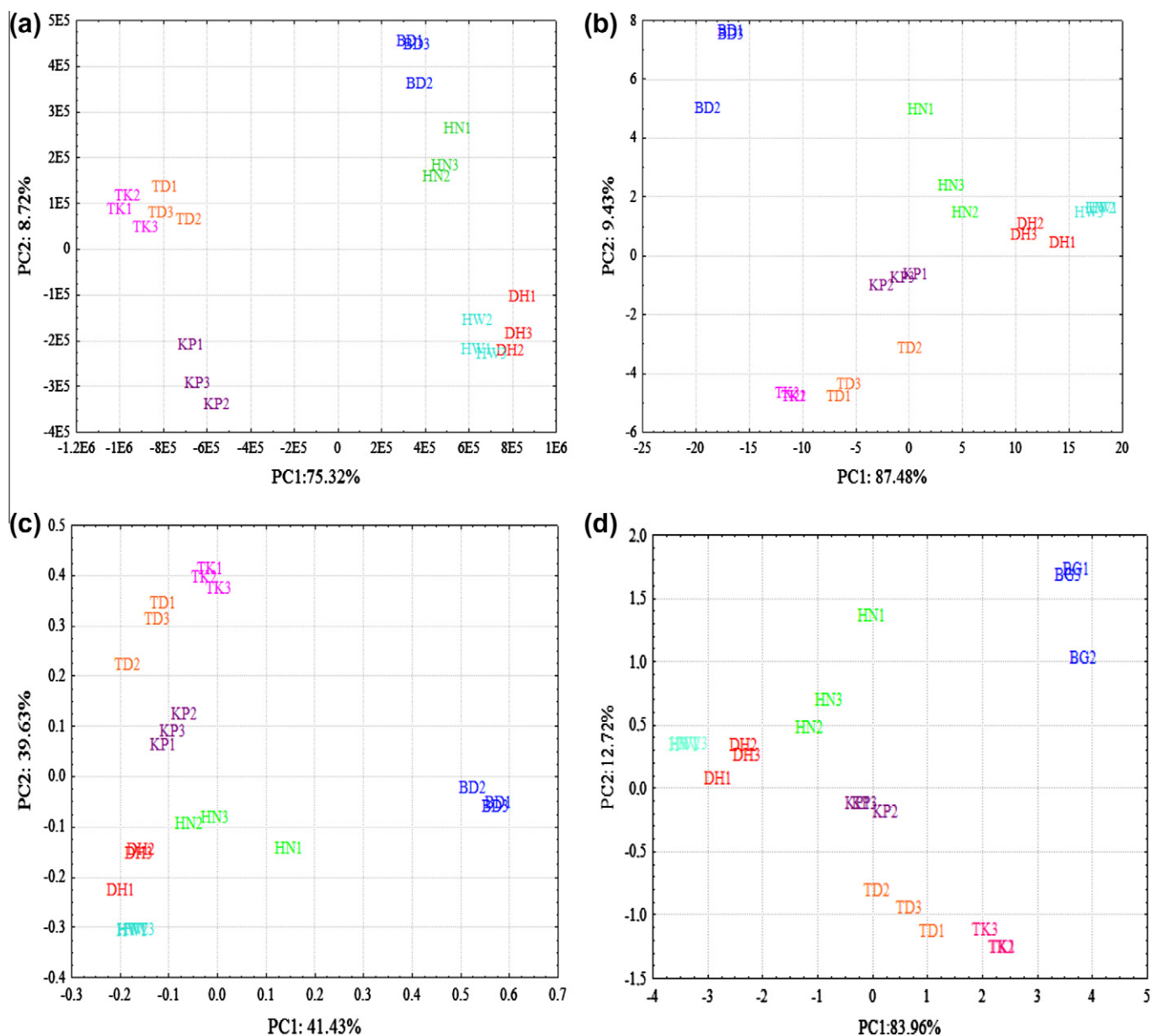


Fig. 4. PC1–PC2 score plot: (a) HPLC–MS chromatogram; (b) zero order spectra; (c) first order spectra; and (d) normalised zero order spectra.

3. Results and discussion

The HPLC fingerprints are very difficult to be efficiently analysed by a simple visual screening. By carefully examination of the HPLC–MS chromatograms (Fig. 1) and the UV–Vis spectra (Fig. 2) corresponding to kiwi and pomelo subspecies, there may be appreciated that they presents some significant differences. Three of the kiwifruits are recommended by the chromatograms (HW, HN and DH) to be highly similar, while the kiwifruit extracts UV–Vis spectra indicated that the HN species has a chemical composition a bit different by the HW and DH. The BD kiwifruit has been remarked as being different by the rest of the kiwifruit subspecies. On the other side, for pomelo fruits both the chromatograms and the spectra are suggesting that the TD and TK are presenting high similarity, while the KP is a bit different. The kiwifruit chromatograms are indicating that the first 10 min are differentiating the samples, while those corresponding to pomelo fruits are leading to more significant differences between 10 and 20 min. According to the phytochemical standards separation there may be

appreciated that in the first 10 min the most polar compounds are eluted, such as catechins and small molecule phenolic acids (4-hydroxybenzoic acid, chlorogenic acid and caffeic acid), while between 10 and 20 min the intermediary polarity compounds should be observed, like phenolic acids (*o*-, *m*-, *p*-coumaric acids and sinapic acid) and glycosylated flavonoids (rutin and naringin). However, after 20 min the flavonoids aglicons could be detected (quercetin, kaempferol, luteolin, naringenin and phloretin). All these observations are leading to the conclusion that the kiwifruits are discriminated by very polar phenolic compounds, while the glycosylated flavonoids are influencing the pomelo fruit characteristics. The use of parallel sample analysis is strongly sustaining the above presented considerations.

The UV–Vis spectra were showing that the most specific UV range is mainly between 200 and 350 nm. The kiwifruit extracts exhibit the maximum absorption between 210 and 220 nm, but the spectra were quite simple, while the pomelo spectra were more specific, presenting two absorption maxima around 280 and 320 nm. However, the (dis-)similarity level of the analysed

Table 1
Classification matrix of kiwi and pomelo fruits (21 samples) using scores corresponding to the first 14 principal components.

Data	Group	Correct classification (%)	Observed classification							
			G1	G2	G3	G4	G5	G6	G7	
Chromatograms	BG	100	3	0	0	0	0	0	0	0
	DH	100	0	3	0	0	0	0	0	0
	HN	100	0	0	3	0	0	0	0	0
	HW	100	0	0	0	3	0	0	0	0
	KP	100	0	0	0	0	3	0	0	0
	TD	100	0	0	0	0	0	3	0	0
	TK	100	0	0	0	0	0	0	3	0
	Total	100	3	3	3	3	3	3	3	3
Zero order spectra	BG	100	3	0	0	0	0	0	0	0
	DH	100	0	3	0	0	0	0	0	0
	HN	100	0	0	3	0	0	0	0	0
	HW	100	0	0	0	3	0	0	0	0
	KP	100	0	0	0	0	3	0	0	0
	TD	100	0	0	0	0	0	3	0	0
	TK	100	0	0	0	0	0	0	3	0
	Total	100	3	3	3	3	3	3	3	3
First order spectra	BG	100	3	0	0	0	0	0	0	0
	DH	100	0	3	0	0	0	0	0	0
	HN	100	0	0	3	0	0	0	0	0
	HW	100	0	0	0	3	0	0	0	0
	KP	100	0	0	0	0	3	0	0	0
	TD	100	0	0	0	0	0	3	0	0
	TK	100	0	0	0	0	0	0	3	0
	Total	100	3	3	3	3	3	3	3	3
Normalised zero order spectra	BG	100	3	0	0	0	0	0	0	0
	DH	100	0	3	0	0	0	0	0	0
	HN	100	0	0	3	0	0	0	0	0
	HW	100	0	0	0	3	0	0	0	0
	KP	100	0	0	0	0	3	0	0	0
	TD	100	0	0	0	0	0	3	0	0
	TK	100	0	0	0	0	0	0	3	0
	Total	100	3	3	3	3	3	3	3	3

fruits is impossible to be comprehended by a simple visual screening.

The dendrograms (Fig. 3) obtained by applying the CA on the digitized chromatogram (21 samples \times 1775 variables), and UV-Vis spectra (21 samples \times 601 variables) offer some more information about the (dis)similarities observed between the analysed samples. The Ward's method used for cluster building is regarded as being one of the most efficient rules of amalgamation, because it uses an analysis of variance approach to evaluate the distance between clusters. The procedure is more efficient when the distance between clusters is computed by squared Euclidean method, which is not affected by the addition of new objects to the analysis or by outliers. In our particular case, by studying Fig. 3a and c is easy to observe that, according to CA, the chromatograms and first order spectra offer the most significant classifications since the fruit samples are associated according to the fruit species reality. Once again the HW and DH kiwifruit are highly resembled, while the BD is more different by the rest of kiwis. On the other side, the fruit samples (12 kiwi subspecies and nine pomelo subspecies) are more weakly classified by the CA applied on the zero order spectra (Fig. 3b) and normalised zero order spectra (Fig. 3d), which are mixing the fruit types and the obtained classifications are inconclusive. However the similarities and differences indicated by the CA applied on the chromatograms and first order spectra are a direct consequence of the high level of resemblance existed between HN-DH-HW kiwifruits subspecies and TD-TK pomelo subspecies.

By applying PCA on the digitized chromatograms and spectra some new information about the fruits origin, similarity and differences may be obtained. The projected dots of the chromatograms and spectra were localised in a confined cluster in the 2D-projection plot of PCA (Fig. 4). The observation indicates that both analytical results are correctly associating the samples according to fruits

species. In all cases the first 20 PCs explain the total variance (100%) of the data. The variance corresponding to the PC1 are accounting more than 75% in the case of the HPLC chromatograms and around 87% and 41% for zero and first order UV/Vis spectra. The low value in the case of first order spectra might be explained by two main directions of variation in the spectra data as a result of the first derivative. The PC1 of normalised spectra takes 84% from the variance of raw data. The first two PCs obtained on the chromatographic data accounts more than 84%, while the two PCs corresponding to zero and first order spectra take 98% and 81%, respectively. Finally, the normalised spectra 97% refunded in the first two PCs. The obtained PCA patterns gave an illustrative and elegant suggestion about the CA results. For example, the PC1-PC2 score plot corresponding to the chromatograms (Fig. 4a), zero order spectra and normalised zero order spectra (Fig. 4b and d) illustrate the HW-DH and TK-TD almost as single groups, while the first order spectra (Fig. 4c) are mixing the kiwi-fruit subspecies, but the fruit species may still be distinguish. However, the BD subspecies of kiwi can be remarked as being totally different by the other kiwis.

Even if the involved methods seem to be very efficient in the simultaneous evaluation of different types of fruit, there are some problems when the fruit subspecies are highly similar from chemical composition point of view. The previous obtained results are highly descriptive, but they are not always sufficient of a beyond doubt classification of the kiwis or pomelo subspecies. The combination of PCA with LDA led to the most efficient discrimination of the investigated groups of fruit. The results obtained by applying LDA to the first 14 principal components (Table 1) indicate a total separation of samples (100%) within seven groups, in a good agreement with their nature (species and subspecies) and origin (cultivar), and independently of the analytical technique. These statements are supported and well illustrated in the Root

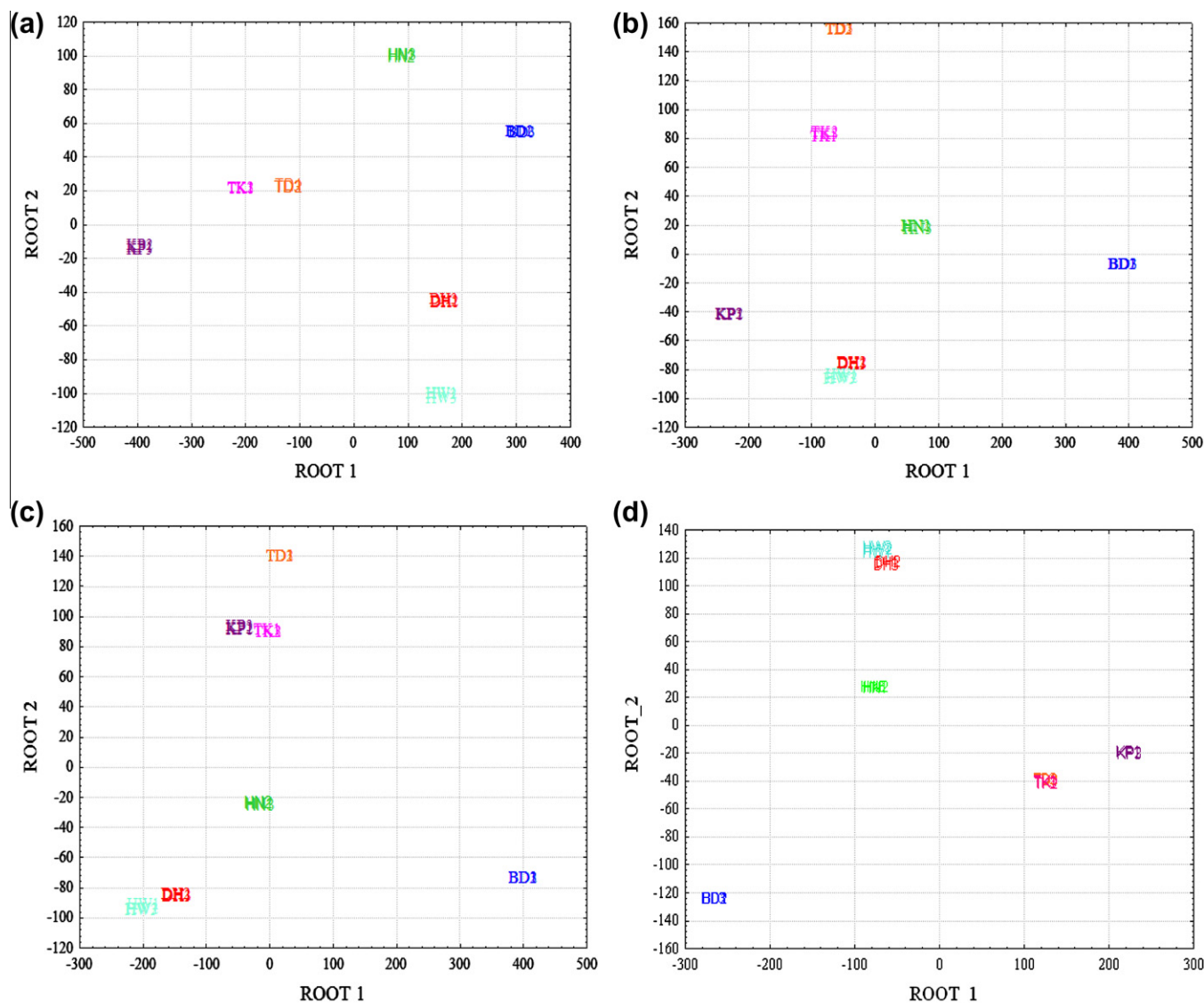


Fig. 5. Root 1–Root 2 canonical score plot: (a) digitized chromatogram; (b) zero order spectra; (c) first order spectra; and (d) normalised zero order spectra.

1–Root 2 score plots (Fig. 5), which clearly indicate that the chromatography (Fig. 5a) is more efficient in the fruits sample classification, since all fruit subspecies and species are very substantially separated, without any overlapping. Moreover, the UV–Vis spectroscopy is also correctly classifying the investigated samples, but in all cases the kiwi DH and HW subspecies are very close. However, comparing all spectra taken into consideration is easy to appreciate that the zero order spectra (Fig. 5b) are the most efficient in the PCA–LDA analysis. Even more, the first order spectra (Fig. 5c) are grouping the pomelo KP and TD groups very close, while the normalised zero order spectra (Fig. 5d) are leading to a total overlapping of TK and TD groups. All the above presented analyses are indicating that the combination of PCA with LDA will lead to more powerful classification and discrimination of vegetal samples, firstly according to their species and secondly taking into consideration the subspecies variance. Furthermore, this type of analyses may open new pathways in the fingerprinting procedures. The UV–Vis spectroscopy led to HPLC comparable results, which is a strong argument for employing it in other investigations. Another aspect that must be considered is the fact that the UV–Vis method is simpler and less expensive, so it may be viewed as a competitive and friendly technique in the fingerprint analyses.

The methodology developed in this paper might be also extended in the authenticity and origin control of fruits, herbs or derived products.

4. Conclusions

A simultaneous fingerprinting analysis of some kiwi and pomelo fruits has been performed by employing the HPLC and UV–Vis spectroscopy. The analysis of the data has been performed by multivariate exploratory techniques, such as cluster analysis, principal component analysis, and linear discriminant analysis applied on the scores corresponding to the first principal components. Four subspecies of kiwi and three of pomelo were analysed in order to investigate the most efficient technique of simultaneous discrimination. The employed chemometric methods offer sustainable results, but the most powerful seems to be the combination of PCA with LDA. The digitized chromatograms led to a better classification and discrimination of the fruits samples than the UV–Vis spectra, but the zero order spectra led to very competitive results. All results have indicated that the BD kiwi subspecies is the most different one, while DH and HW present high similarities. The TK and TD subspecies of pomelo are majorly related.

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