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Optimized and validated method for simultaneous extraction, identification and quantification of flavonoids and capsaicin, along with isotopic composition, in hot peppers from different regions

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ABSTRACT

Nineteen hot pepper (*Capsicum annuum* L.) samples from five countries and twenty samples from Romanian producers were analyzed. Concentrations of flavonoids and capsaicin were simultaneously quantified for the first time with the method developed and validated in the present paper. $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ isotopic values were also measured. Maximum concentrations of studied compounds were detected in methanol extracts, after 12 h incubation of the samples assisted by ultrasound, at the 1:8 ratio of sample to solvent. The extraction recovery ranged from 90.60% to 115.05%. Capsaicin and four flavonoids were quantified in studied samples at different concentration ranges: capsaicin (28.23–2322.35 μg/g), vitexin (2.93–33.46 μg/g), isoquercetin (3.19–155.58 μg/g), kaempferol-3-glucoside (2.31–2462.25 μg/g) and myricetin (1.55–78.79 μg/g). The association between these analytical techniques and chemometric tools proved that kaempferol-3-glucoside is one of the strongest markers for country and maturity stage discrimination.

1. Introduction

Hot pepper (*Capsicum annuum* L.) belongs to the *Solanaceae* family and is a vegetable of great importance in nutrition, source of pigments, source of vitamins C and E, carotenoid compounds and antioxidant properties (Arnnok, Ruangviriyachai, Mahachai, Techawongstien, & Chanthai, 2012; Ornelas-Paz et al., 2010). They are also rich in flavonoids and other phytochemicals, which may contribute to the antiradical activity, have a protective role against coronary heart disease, stroke, diabetes, and some forms of cancer (Menichini et al., 2009; Oboh & Rocha, 2007; Ornelas-Paz et al., 2010).

Phenolic compounds are bioactive organic metabolites that have attracted the interest of scientists in the past two decade because of their powerful antioxidant activities. Their content in plants is affected by different factors that may vary from one region to the other: agricultural practices, climatic stress factors, and postharvest processing conditions (Materska & Perucka, 2005). Within this context, previous studies have aimed to evaluate the levels of these compounds in pepper samples (Alvarez-Parrilla, de la Rosa, Amarowicz, & Shahidi, 2011; Bae et al., 2014; Campos, Gómez, Ordoñez, & Ancona, 2013; Lin & Tang, 2007; Zhuang, Chen, Sun, & Cao, 2012). Capsaicin is also an active component belonging to a distinctive class of compounds named capsaicinoids. They are responsible for spicy flavor in peppers (Barbero, Liazid, Palma, & Barroso, 2008). Several studies have shown that the concentration of capsaicin in hot peppers depends on maturity stages, growing conditions and geographical origin (Barbero et al., 2014; Howard, Talcott, Brenes, & Villalon, 2000; Ruiz-Lau et al., 2011).

There is an increasing pressure from the consumers of European Countries about a number of issues related to high quality food and a well-defined geographical origin. Mislabeling the products is one of the most common forms of falsification found in food industry. In Romania there is a real problem with high amounts of different fruits and vegetables, obtained by intensive farming and coming from other areas at lower prices and labeled like “regional products” or “organic”. This has a negative impact on small producers and regional food quality. Kelly, Heaton, and Hoogewerff (2005) were highlighted an important reason regarding the enthusiasm among consumers for food with a clear identity: specific culinary, organoleptic qualities, health benefits associated with regional products and confidence in the foods produced outside their local area. Therefore, many studies are focused on tracing the geographical origin of food, with different approaches and different markers: stable isotope ratios ($^{1}H/^{2}H$; $^{18}O/^{16}O$; $^{13}C/^{12}C$) and multielemental content for geographical origin of raw milk (Magdas et al., 2016), polyphenolics and carbohydrates as indicators of botanical and geographical origin of Serbian red spice paprika (Mudrić et al., 2017), $\delta^{2}H$ and $\delta^{18}O$ isotopic analysis combined with chemometrics for

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traceability of bell peppers (De Rijke et al., 2016), multi-element composition to discriminate between the different wine regions of Australia (Martin, Watling, & Lee, 2012).

Flavonoids (vitexin, isoquercetin, kaempferol-3-glucoside, and myricetin) and capsaicin are found and quantified in hot pepper samples. Different factors are investigated in order to optimize the simultaneous extraction of phenolic compounds: extraction solvent, extraction time (with and without ultrasound), ratio of sample to solvent and drying process of the samples. Beside flavonoids and capsaicin content, $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ isotopic values are measured as potential markers of geographical origin. Analytical techniques, such IRMS or HPLC, provide large data sets, which become difficult to interpret or to highlight the most meaningful information. It is well known that isotopic measurements might be good indicators for geographical origin of food products: milk (Magdas et al., 2016), wines (Hosu et al., 2016) or for distinguishing organic versus conventional vegetables (Feher, Magdas, Dehelean, Cristea, & Voica, 2017). Moreover, the analysis of bioactive compounds using HPLC provides information regarding the products quality (smell, taste). The association between these two analytical techniques, along with chemometric techniques applied on obtained experimental data, revealed some new insights in the matrices under investigation.

The present study focused on two objectives: (1) to develop and optimize an efficient extraction method for simultaneous determination of flavonoid compounds and capsaicin in hot pepper samples from different regions and (2) to evaluate the possibility of discrimination between hot pepper samples from different countries of origin and between different fruit maturity stages while using a combination of phenolic compounds and stable isotope ratios ($\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$) as markers, using analysis of variance (ANOVA) and principal component analysis (PCA).

2. Materials and methods

2.1. Samples and chemicals

Nineteen fresh hot pepper (*Capsicum annuum* L.) samples were purchased in 2015 and 2016 from local supermarkets (Cluj-Napoca, Romania) originated from five countries (Morocco, Spain, Italy, Hungary and Turkey), according to their label. Additional twenty samples were obtained from Romanian local producers: fourteen from Transylvania area (northern Romania) and six from south Romania. The pepper samples were in different ripening stage: green, orange and red. The whole pepper fruits were carefully selected and finely chopped to achieve homogeneity and kept under refrigeration conditions (−20 °C) before further analysis.

Analytical standards of vitexin, isoquercetin, kaempferol-3-glucoside, myricetin, luteolin, kaempferol, chrysin and capsaicin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). HPLC grade acetonitrile, ethanol, ultrapure water, acetone and methanol were purchased from LGC Standards GmbH (Wesel, Germany). Hexane (HPLC grade) was supplied from Sigma-Aldrich (Saint Louis, MO, USA) and acetic acid (Suprapure) from Merck (Darmstadt, Germany).

2.2. Instrumentation

The compounds of interest were separated and quantified using an Accela UHPLC (Ultra-high Performance Liquid Chromatography) system from Thermo Scientific (Bremen, Germany) equipped with a photodiode array detector (PDA), an autosampler with a column oven and a tray compartment and a quaternary pump with a built-in solvent degasser, all controlled by Xcalibur software. The separation was achieved using reversed-phase chromatography with gradient elution. The analytical column used for separation of vitexin, isoquercetin, kaempferol-3-glucoside, myricetin, luteolin, kaempferol, chrysin and capsaicin was a Hypersil Gold 50 × 2.1 mm packed with 1.9 µm particles (Thermo Scientific, Bremen, Germany). The mobile phase was acetonitrile (solvent A) and HPLC water containing 0.1% acetic acid (glacial) (solvent B). For confirmation, the compounds detected in samples were compared with a known amount of injected standards. Besides this, the flavonoids and capsaicin peaks were confirmed by mass spectrometric analysis in both negative and positive ion mode. ESI-MS analysis was performed on a LCQ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, Bremen, Germany).

The isotopic measurements of $\delta^{18}O$ and $\delta^{2}H$ were made using a liquid-water isotope analyser (DLT-100, Los Gatos Research, USA) and determination of $\delta^{13}C$ were carried out on an Elemental Analyser (Flash EA1112 HT, Thermo Scientific, USA), coupled with an isotope ratio mass-spectrometer IRMS (Delta V Advantage, Thermo Scientific, USA).

2.3. Optimization of extraction conditions

2.3.1. Drying the samples

In the sample preparation step, drying procedures before further analysis have been shown to have an influence on the different groups of phenolic compounds. Two drying technologies were evaluated in parallel in order to study the effect on the content of flavonoids and capsaicin in hot pepper: freeze-drying and thermal drying. For thermal drying, the samples were kept in a laboratory dryer at 55 °C for 48 h. Freeze-drying process was performed at −75 °C and was conducted for 48 h. A quantity of 0.5 g of lyophilized pepper sample and 0.5 g of thermal dried sample were mixed separately with 4 ml methanol and extracted for 12 h in an ultrasound bath. The samples were than centrifuged and the supernatant of each sample was collected and injected.

2.3.2. Extraction method optimization

Freeze-dried samples of hot peppers (0.5 g) were mixed with 4 ml of different extraction solvents or mixture of solvents in order to compare the extraction efficiency: ethanol (EtOH) 100%, methanol (MeOH) 100%, EtOH:MeOH 50:50%, hexane (Hex) 100%, Hex:EtOH 50:50%, acetone (Ac) 100%, and Hex:Ac 50:50%. All the samples were extracted in duplicate. The homogenized mixtures were kept on a horizontal shaker for 4 h at room temperature. After extractions, the samples were centrifuged; the supernatant of the mixture was collected and then injected in UHPLC system for analysis.

The experiment for extraction time optimization of flavonoids and capsaicin was performed in two ways. In the first approach 0.5 g of lyophilized pepper samples with 4 ml of MeOH was extracted for 2, 4, 6, 12, and 24 h. In the second approach, 0.5 g of lyophilized pepper samples with 4 ml of MeOH was extracted for 2, 4, 6, 12, and 24 h assisted by ultrasound. All the samples were extracted in duplicate. The homogenized mixtures from the first approach were placed on a horizontal shaker, at room temperature, for the different incubation times. The samples from the second approach were placed in an ultrasonic bath at room temperature. After the defined exposure time, the samples were centrifuged at 4000*g* for 15 min, the supernatant was collected and injected in UHPLC system. Both approaches were evaluated in order to find the best extraction conditions.

For optimization of the ratio of sample to solvent, different proportions were used: 1:4, 1:6 and 1:8. Freeze-dried samples of hot peppers (0.5 g) were mixed with 2, 3 and 4 ml of methanol and extracted for 12 h in an ultrasound bath. The samples were than centrifuged and injected in the UHPLC system.

2.3.3. Chromatographic conditions

Before selecting the appropriate UHPLC program conditions for phenolic compounds separation, a number of preliminary trials were conducted with different mobile phase composition (water, methanol and acetonitrile), flow rate, gradient, working temperatures and injection volume. Water containing 0.1% acetic acid and acetonitrile were selected for the method development, coupled with 30 °C working temperature, and flow rate of 500 µl/min and sample injection volume

of 2 µl. The optimum gradient program was as follows: 0–0.3 min from 100% to 76% B, 0.3–0.5 min from 76% to 100% B, 0.5–14 min from 100% to 0% B, 14–15 min from 0% to 100% B, then 100% B for 2 min.

2.4. Method validation

2.4.1. Calibration curves

Stock solution (1000 µg/ml) of a mixture of seven phenolic standards and capsaicin were prepared in ethanol. To make the working solutions for calibration curves, the mixture was successively diluted to obtain the appropriate concentrations of 0.5, 1, 5, 10, 20, and 40 µg/ml. Calibration curves for all the compounds were obtained by plotting peak area versus analyte concentration. The linear regression equations were calculated with $y = ax \pm b$, where x is concentration and y is the peak area of each analyzed compound. The acceptance of linearity was established by the correlation coefficient (R^2), which should not be less than 0.990.

2.4.2. Specificity

The specificity was tested by measuring the analyte response in the presence of other components of the sample. For this purpose, the blank sample and the spiked sample were injected in the UHPLC system. No interfering peaks were observed.

2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ tests were performed on samples containing very low concentrations of analytes. LOD represents the lowest amount of analyte that can be detected, typically, three times of the signal-to-noise ratio. LOQ was determined by injecting the diluted standard solutions until signal-to-noise ratios were 10:1.

2.4.4. Precision

For the retention time and peak area instrumental precision, repeated injections were made to evaluate the intra-day and inter-day accuracy. Intra-day precision was calculated at two concentrations levels (5 and 40 µg/ml) by six replicate injections each in the same day under the same experimental conditions. The inter-day variation was made by repeated injections for four different days.

2.4.5. Recovery

The extraction recovery was performed for every combination of solvents: EtOH 100%, MeOH 100%, EtOH:MeOH 50:50%, Hex 100%, Hex:EtOH 50:50%, Ac 100%, and Hex:Ac 50:50%, by adding the standard solution, obtaining a concentration of 5 µg/ml of each studied compound. All the solutions were also prepared without adding the standards (blank), and were extracted in parallel. All the samples were extracted in duplicate.

2.5. Isotopic measurements

The isotopic values were expressed in δ‰ which is according to equation (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014) as follows:

$$\delta^i X = \frac{R_{sample}}{R_{standard}} - 1$$

where i represents the mass number of the heavier isotope of the element X (e.g. ^{13}C, ^{2}H, ^{18}O), R_{sample} is the isotope ratio of the sample (for example, $^{13}C/^{12}C$) and $R_{standard}$ is that of an international standard. The delta values are multiplied by 1000 and are expressed in units “per mil” (‰). The isotopic values are expressed against international standards Vienna – Standard Mean Ocean Water for $\delta^{18}O$ and $\delta^{2}H$ and Vienna Pee Dee Belemnite for $\delta^{13}C$.

For $\delta^{18}O$ and $\delta^{2}H$ analyses, a cryogenic distillation system under static vacuum was used to extract the water contained in hot pepper samples, always with quantitative recovery of water, as was previously described (Magdas & Puscas, 2011). The $\delta^{18}O$ and $\delta^{2}H$ measurements were made using a Liquid-Water Isotope Analyzer (DLT-100, Los Gatos Research, USA), calibrated against five laboratory-used standards (Std): working standard 1 ($\delta^{18}O = -9.57‰$, $\delta^{2}H = -154.1‰$), working standard 2 ($\delta^{18}O = -15.55‰$, $\delta^{2}H = -117.0‰$), working standard 3 ($\delta^{18}O = -11.54‰$, $\delta^{2}H = -79.0‰$), working standard 4 ($\delta^{18}O = -7.14‰$, $\delta^{2}H = -43.6‰$), and working standard 5 ($\delta^{18}O = -2.96‰$, and $\delta^{2}H = -9.8‰$), respectively. The water working standards are certificated materials and were produced by Los Gatos Research, USA and purchased from the same provider. The standardization of $\delta^{18}O$ and $\delta^{2}H$ values against international V-SMOW scale was made by calibration of the working standards with IAEA V-SMOW-2, Standard Light Antarctic Precipitation (SLAP-2, $\delta^{18}O = -55.5‰$, $\delta^{2}H = -427.5‰$ versus V-SMOW-2), and Greenland Ice Sheet Precipitation (GISP, $\delta^{18}O = -24.76‰$, $\delta^{2}H = -189.5‰$ versus V-SMOW-2) standards. The limit of uncertainty of the isotopic analysis was ± 0.2‰ for $\delta^{18}O$ and ± 0.6‰ for $\delta^{2}H$. Each sample was analyzed six times, the last three injections being taken into account for the results calculations.

The determination of $\delta^{13}C$ from hot pepper samples (freeze-dried before analysis) was carried out on an Elemental Analyser (Flash EA1112 HT, Thermo Scientific, USA), coupled with an isotope ratio mass-spectrometer IRMS (Delta V Advantage, Thermo Scientific, USA). For the quality control of $\delta^{13}C$ analysis, three replicates of NBS-22 oil standard, having a certified value of −30.031‰ versus V-PDB (V-Pee Dee Belemnite, IAEA, Vienna, Austria), were analyzed at the beginning of each sequence and then three replicates from each sample were measured. The uncertainty for the carbon isotope ratio measurements was ± 0.3 for $\delta^{13}C$.

2.6. Statistical analysis

All experimental data was analyzed using SPSS program (IBM, USA). Two statistical tests were applied, namely analysis of variance (ANOVA) and principal component analysis (PCA). In this particular case, ANOVA was used for comparing the organic compounds and isotopic content, which are able to differentiate the hot pepper according to the color (green, red, orange) and to the geographical area of growing (Morocco, Spain, Italy, Turkey, Romania). Last comparison was made in order to evidence some characteristics parameters that differ in Romanian samples compared to the foreign ones.

3. Results and discussion

3.1. Optimization of extraction parameters

3.1.1. Drying the samples

The drying process of the samples is an important step in sample preparation prior to extraction. This approach was chosen in order to evaluate and compare the effect of drying methods on the studied bioactive compounds. Analysis of data showed that there was a significant decrease in flavonoid concentrations when the samples were treated by thermal drying. Capsaicin concentration was not affected by drying processes. The freeze-dried process demonstrated a higher level of flavonoids (Fig. 1).

3.1.2. Comparison of extraction solvent

For extraction optimization of flavonoids and capsaicin, various solvents or mixtures of solvents were used: EtOH 100%, MeOH 100%, EtOH:MeOH 50:50%, Hex 100%, Hex:EtOH 50:50%, Ac 100%, and Hex:Ac 50:50%. High concentrations of compounds were obtained in the MeOH extracts for vitexin, isoquercetin, kaempferol-3-glucoside and myricetin. For luteolin, kaempferol and chrysin the highest concentrations were observed in Hex/Ac extracts. The extraction optimization for all compounds using different solvents is shown in Fig. S1 (Supplementary Data). Because some compounds like luteolin, kaempferol and chrysin were not detected in real hot pepper samples,

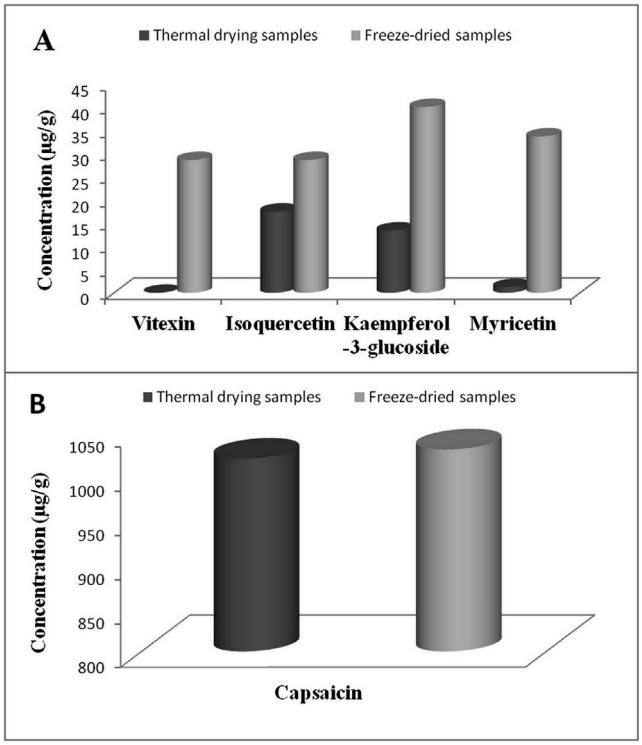

Fig. 1. Effect of drying methods on (A) flavonoid compounds and (B) capsaicin.

methanol was found to be the most suitable solvent for the efficient extraction of vitexin, isoquercetin, kaempferol-3-glucoside, myricetin, and capsaicin in further experiments. As far as we know, this is the first work that shows simultaneous solvent extraction optimization of flavonoids together with capsaicin in hot peppers. A method was reported in literature regarding simultaneous separation and quantification of capsaicinoids and ascorbic acid from pungent peppers (Bae, Jayapraskasha, Crosby, Jifon, & Patil, 2013).

3.1.3. Extraction time

Previously published papers reported different approaches for extraction of phenolic compounds: 3 h extraction with EtOH from fresh pepper (Bae, Jayapraskasha, Jifon, & Patil 2012), 30 min with ultrasound in 80:20 (v/v) methanol/water from three different freeze-dried non-pungent peppers (Morales-Soto, Gómez-Caravaca, García-Salas, Segura-Carretero, & Fernández-Gutiérrez, 2013), three times extraction with 80% methanol in ultrasonic bath for 20 min at room temperature from spice pepper (Mudrić et al., 2017), extraction of capsaicinoids from fresh pepper with methanol at 50 °C, assisted by ultrasound for 15 min (Barbero et al., 2014).

The experiment for extraction time optimization was performed in two ways: with and without ultrasound at different incubation times (2, 4, 6, 12, and 24 h). The concentrations of flavonoids and capsaicin obtained with different extraction conditions are presented in Table 1. Maximum extraction was observed in methanol after 12 h incubation of the samples assisted by ultrasound. For this reason, the conditions selected for an efficient further extraction were 12 h assisted by ultrasound.

3.1.4. Ratio of sample to solvent

For optimization of ratio sample to solvent, different proportions were compared (1:4, 1:6 and 1:8). No significant differences were observed in flavonoid concentrations at various ratios. The concentration of capsaicin is higher at ratio 1:8. For this reason, the ratio selected for an efficient extraction was 1:8 (Fig. S2) (Supplementary Data).

Table 1
Concentrations of flavonoids and capsaicin at different extraction times (with and without ultrasound).

Compound	Extraction time on horizontal shaker	Concentration (µg/g)*	Extraction time with ultrasound	Concentration (µg/g)*
Vitexin	2 h	12.71 ± 1.61	2 h	17.58 ± 2.79
	4 h	20.93 ± 0.49	4 h	18.19 ± 2.56
	6 h	19.71 ± 1.01	6 h	22.13 ± 3.64
	12 h	14.63 ± 2.23	12 h	24.38 ± 0.82
	24 h	17.55 ± 2.05	24 h	17.98 ± 0.47
Isoquercetin	2 h	5.36 ± 1.14	2 h	5.63 ± 0.21
	4 h	8.22 ± 0.44	4 h	11.34 ± 0.09
	6 h	7.48 ± 4.16	6 h	13.38 ± 1.71
	12 h	3.26 ± 0.86	12 h	32.28 ± 3.05
	24 h	5.41 ± 0.38	24 h	17.42 ± 0.55
Kaempferol-3-		Glucoside	2 h	7.39 ± 3.23
	2 h	23.41 ± 4.24		
	4 h	16.19 ± 0.28	4 h	29.08 ± 0.42
	6 h	22.52 ± 2.39	6 h	29.25 ± 5.70
	12 h	16.10 ± 0.62	12 h	32.55 ± 2.83
24 h	16.94 ± 1-.33	24 h	34.79 ± 0-.20	
Myricetin	2 h	8.41 ± 1.58	2 h	18.27 ± 0.31
	4 h	12.75 ± 2.36	4 h	20.76 ± 1.18
	6 h	18.10 ± 1.60	6 h	22.87 ± 0.45
	12 h	16.01 ± 1.96	12 h	25.09 ± 0.76
	24 h	16.34 ± 0.72	24 h	23.88 ± 0.60
Capsaicin	2 h	662.87 ± 106	2 h	627.59 ± 28.95
	4 h	705.50 ± 32	4 h	637.02 ± 21.90
	6 h	716.49 ± 16.85	6 h	826.01 ± 23.78
	12 h	608.38 ± 13.11	12 h	846.97 ± 19.50
	24 h	749.52 ± 16.45	24 h	762.75 ± 103

* Values are means ± standard deviation of duplicate samples.

3.2. Mass spectrometric analysis

Full scan MS spectra in the positive and negative ion mode from *m/z* 110–2000 were recorded. In ESI ionization, all studied flavonoids showed the deprotonated $[M-H]^-$ ion as the base peak of MS spectra, and no fragmentation ions were observed at significant intensities. For capsaicin, the peak confirmation was obtained in positive mode ionization $[M+H]^+$. In detail, the products ions for peaks confirmation are at *m/z* 431.15 $[M-H]^-$ for vitexin, *m/z* 463.11 $[M-H]^-$ for isoquercetin, *m/z* 447.21 $[M-H]^-$ for kaempferol-3-glucoside, *m/z* 317.18 $[M-H]^-$ for myricetin and *m/z* 306.2 $[M+H]^+$ for capsaicin.

3.3. Method validation

In present study, method validation was carried out in order to evaluate the linearity, specificity, instrumental precision, limits of detection (LODs), limit of quantification (LOQs) and recovery for seven flavonoids and capsaicin. For the linearity, linear graph for concentration of each flavonoid (vitexin, isoquercetin, kaempferol-3-glucoside, myricetin, luteolin, kaempferol, chrysin) and capsaicin (0.5, 1, 5, 10, 20, 40 µg/ml) was plotted against peak area. The correlation coefficient (R^2) obtained for each compound (vitexin 0.9994; isoquercetin 0.9993; kaempferol-3-glucoside 0.9990; myricetin 0.9987; luteolin 0.9998; kaempferol 0.9996; chrysin 0.9992; capsaicin 0.9997) demonstrates excellent relationship between peak areas and concentrations.

Instrumental precision of the proposed method was determined on the same day with two different concentrations and on the four different days. The relative standard deviation RSD (%) for the retention times was less than 0.57% and for the peak areas less than 5.2%, and

illustrated the good precision of the analytical method. All the results (RSD %) obtained for both concentrations are presented in Table 2.

Limits of detection (LODs) and limits of quantification (LOQs) were obtained by repeated injections of diluted standard solutions in order to obtain a ratio of signal to noise 3:1 for LOD and 10:1 for LOQ. The LOD values for flavonoids ranged from 0.1 to 0.2 μg/ml and for capsaicin was 0.05 μg/ml, while the LOQ ranged from 0.3 to 0.4 μg/ml for flavonoids and was 0.1 μg/ml for capsaicin.

The recovery was calculated for every combination of solvents after 4 h incubation. The highest extraction yields for vitexin (95.81–115.05%), isoquercetin (102.51–105.5%), kaempferol-3-glucoside (99.64–109.64%), and myricetin (90.6–110.54%) were obtained in the MeOH extracts. For luteolin (84.1–87.79%), kaempferol (93.47–95.94%), and chrysin (95.9–96.76%), the highest recovery percentages were observed in Hex/Ac extracts. Good yields were also obtained in MeOH (85.53% for luteolin; 83.11% for kaempferol; 87.78% for chrysin) and EtOH:MeOH (84.72% for luteolin; 83.37% for kaempferol; 86.95% for chrysin) extracts. For capsaicin, best values were observed in EtOH (105.64–108.73%), EtOH:MeOH (103.9%) and MeOH (101.27%). Percentages higher than 100% could be explained by the sample matrix interference. Lower levels of compounds were detected in hexane extracts (3.96% for vitexin, 0.44% for isoquercetin, 0.24% for kaempferol 3-glucoside, 0.63% for myricetin, 2.96% for luteolin, 1.86% for kaempferol, 16.23% for chrysin, and 79.37% for capsaicin).

The recovery (%) levels of all studied compounds were obtained for every combination of solvents used (Fig. S1).

The developed and validated UHPLC method was employed to simultaneous quantification of flavonoids and capsaicin concentrations in hot pepper samples.

3.4. Identification and quantification of flavonoids and capsaicin

Phenolic compounds (vitexin, isoquercetin, kaempferol-3-glucoside, and myricetin) and capsaicin content were extracted and quantified in thirty-nine freeze-dried commercial samples of hot pepper (*Capsicum annuum* L.) using the method developed and validated in the present work. The concentrations obtained revealed a great variability in the flavonoids and capsaicin content among different country of origin, different stage of maturity and different growing conditions (organic or conventional). Regarding the variability from stage of maturity point of view, Estrada, Bernal, Díaz, Pomar, and Merino (2000) studied the changes in content of capsaicinoids, lignin and free phenolics during the maturation process in pepper fruits. They observed that capsaicinoids increase with development, while free phenolics have maximum levels in early stages. Barbero et al. (2014) found that capsaicin content increased until day 40 of fruit ripening and started to decrease progressively until day 80 of ripening. In another study Ruiz-Lau et al. (2011) suggested that capsaicin accumulation in pepper fruits was higher in plants exposed to water deficit. This could be an explanation for the great variability of compound concentrations in studied hot peppers.

The concentrations of analyzed compounds for thirty-nine samples (with sample code) found in hot peppers are listed in Table 3 and are expressed in μg/g of lyophilized sample. The phenolic compounds luteolin, kaempferol and chrysin were not detected in the examined hot peppers or were below limit of detection (LOD).

Regarding the quantified flavonoids, only kaempferol-3-glucoside was found in all analyzed samples. Capsaicin was also present in all samples except one (**T-GP2**). The range of kaempferol-3-glucoside concentrations was 2.31–2462.25 μg/g, the highest amount was observed in **H-GP** (2462.25 μg/g), **T-GP1** (1724.74 μg/g), **T-GP2** (1496.18 μg/g) and **RN-GP3** (1057.53 μg/g). Low concentrations of kaempferol-3-glucoside were measured in **RN-RP3** (2.31 μg/g), **S-OP** (10.53 μg/g), **RN-RP2** (19.15 μg/g), **I-OP** (21.52 μg/g) and **M-OP** (23.81 μg/g). It can be observed that the highest content of kaempferol-3-glucoside was measured in green hot peppers (early stage maturity), while the lowest in orange and red hot peppers (mature pepper fruits). Vitexin was found in seventeen commercial samples at very low concentrations, from 2.93 μg/g to 33.46 μg/g. The highest quantity was obtained in **T-RP2** (33.46 μg/g), **RN-RP1** (28.26 μg/g) and **M-RP** (23.5 μg/g), while low concentrations of vitexin were detected in **M-GP** (2.93 μg/g), **RS-GP3** (3.55 μg/g), **RS-GP2** (4.49 μg/g) and **RS-GP1** (4.55 μg/g). As can be seen, the highest amount of vitexin was found in red peppers and the lowest in green hot peppers. Regarding the country of origin, the samples from Romania have the smallest amount of vitexin (from twenty samples, vitexin was detected only in eight). In literature, Mudrić et al., (2017) reported low concentrations of vitexin in Serbian hot, semi-hot and sweet paprika in the range of 0.15–3.50 μg/g. Isoquercetin was detected in all peppers except the samples from Morocco (**M-RP**, **M-OP**, **M-GP**) and the red pepper samples from Spain and Turkey (**S-RP**, **T-RP1**, **T-RP2**). Isoquercetin concentrations range between 3.19 μg/g and 155.58 μg/g with the highest content in green hot peppers from Italy (**I-GP**). The content of isoquercetin in small amount was observed in **S-OP** (3.19 μg/g), **I-RP2** (4.09 μg/g) and **I-RP1** (5.62 μg/g). In a previous paper, Morales-Soto et al. (2013) reported the identification of isoquercetin (quercetin 3-O-β-d-glucopyranoside) in three different pepper varieties. Myricetin was found at low concentrations almost in all the samples, excepting one orange pepper sample from Morocco (**M-OP**) and four green pepper samples, one from Hungary and three from Turkey (**H-GP**, **T-GP4**, **T-GP5** and **T-GP6**). The concentrations range between 1.53 μg/g in **S-OP** and 78.79 μg/g in **I-GP**. Similar concentrations of myricetin were reported by Miean and Mohamed (2001) in green chili (11.5 μg/g of dry weight) and red chili (29.5 μg/g of dry weight).

Capsaicin content in studied samples shows a great variability and seems to be influenced by numerous factors such as: climatic conditions, areas of production and especially the stage of maturity of the pepper fruits. The highest concentration of capsaicin in studied peppers was observed in **T-GP5** (2322 μg/g), **T-GP6** (2230 μg/g), **RN-OP1** (2009.44 μg/g) and the lowest in **S-OP** (28.23 μg/g), **H-GP** (110.85 μg/g), **RS-RP1** (118.16 μg/g) and RS-GP1 (197.15 μg/g). Except for several pepper samples where a big amount of capsaicin was found in green peppers (early stage maturity), the highest values in the rest of the samples tends to be in mature hot peppers (red and orange). Similar results were reported by Menichini et al. (2009) for habanero hot peppers from Italy: 2498 μg/g for mature stage and 357 μg/g for immature stage.

3.5. Determination of the isotopes

Beside flavonoids and capsaicin content, $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ isotopic values were measured for establishing a correlation between these characteristics and geographical origin of hot peppers as potential markers. The isotopic content ($\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$) of thirty-nine hot pepper samples is presented in Table 3 and is expressed in δ‰.

3.6. Chemometric results

Principal component analysis (PCA) is a technique applied for pattern recognition, which aims to explain the variance of a large data set, by grouping all variables into new ones, called principal components, with a minimum loss of information (Karadaş & Kara, 2012). The suitability of this technique for an experimental dataset is usually tested by applying two criteria: Kaiser-Meyer-Olkin (KMO) and Bartlett's test of sphericity. KMO values vary between 0 and 1. Values greater than 0.5 are considered appropriate for this analysis to continue and mean that pattern of correlation between variables are compact, and PCA is able to yield reliable factors. Bartlett's test measures the null hypothesis, that the correlation matrix is an identity matrix and becomes significant for $p < .05$.

The aim of applying chemometric methods to isotopic values and

Table 2
Intra-day and inter-day instrumental precision for flavonoids and capsaicin at two concentration levels (5 and 40 μg/g).

Intra-day precision (RSD %) (n = 6)																	Inter-day precision (RSD %) (n = 4)			
Compound	Day 1				Day 2				Day 3				Day 4							
	5 μg/ml		40 μg/ml		5 μg/ml		40 μg/ml		5 μg/ml		40 μg/ml		5 μg/ml		40 μg/ml		5 μg/ml		40 μg/ml	
	RT	A	RT	A	RT	A	RT	A	RT	A	RT	A	RT	A	RT	A	RT	A	RT	A
Vitexin	0.56	3.33	0.57	0.97	0.38	1.29	0.50	1.30	0.46	1.81	0.45	2.35	0.19	3.21	0.27	1.78	0.28	1.45	0.21	2.09
Isoquercetin	0.25	4.06	0.19	3.21	0.17	2.80	0.16	1.82	0.23	3.14	0.17	2.69	0.12	2.98	0.16	5.20	0.11	1.09	0.14	1.05
Kaempferol-3-glucoside	0.21	1.75	0.19	2.74	0.19	1.80	0.13	2.23	0.25	2.04	0.25	1.76	0.33	2.30	0.27	1.56	0.23	4.04	0.36	3.28
Myricetin	0.1	2.99	0.12	2.17	0.12	3.67	0.13	3.02	0.18	2.16	0.12	2.37	0.13	3.32	0.13	3.28	0.32	3.94	0.25	1.85
Luteolin	0.11	1.05	0.10	3.39	0.15	1.94	0.16	3.61	0.18	3.34	0.23	2.62	0.15	2.80	0.19	2.77	0.28	2.37	0.25	1.42
Kaempferol	0.13	1.77	0.15	2.48	0.18	1.96	0.21	3.83	0.17	3.40	0.24	3.83	0.15	2.91	0.20	3.09	0.36	1.58	0.25	3.65
Chrysin	0.19	3.2	0.21	2.8	0.21	2.02	0.26	2.40	0.24	2.48	0.30	1.94	0.22	3.14	0.24	2.23	0.33	3.34	0.28	2.69
Capsaicin	0.12	4.02	0.13	3.5	0.10	2.12	0.14	2.68	0.15	1.75	0.15	1.76	0.16	1.29	0.17	1.75	0.16	2.41	0.21	2.30

RT: RSD (%) for retention time; A: RSD (%) for peak area.

Table 3
The concentrations of studied compounds for thirty-nine samples (with sample code) found in freeze dried hot peppers.

Country of origin	Sample code	Phenolic compound concentrations[a,b]					Isotopic content		
		Vitexin [μg/g ± SD]	Isoquercetin [μg/g ± SD]	Kaempferol 3-glucoside [μg/g ± SD]	Myricetin [μg/g ± SD]	Capsaicin [μg/g ± SD]	$^{13}C/^{12}C$ [‰]	$^{18}O/^{16}O$ [‰]	$^{2}H/^{1}H$ [‰]
Morocco	M-RP	23.5 ± 4.38	n.d.	108.825 ± 11.91	3.625 ± 0.65	1290.775 ± 13.06	−29.2	0.4	−2.6
	M-OP	n.d.	n.d.	23.815 ± 2.05	n.d.	808.455 ± 40.3	−27.9	−1.7	−21.1
	M-GP	2.935 ± 0.28	n.d.	37.615 ± 2.94	2.885 ± 0.12	293.475 ± 33.82	−28.4	−1.8	−19.8
Spain	S-RP	5.125 ± 1.11	n.d.	78.625 ± 5.56	2.375 ± 0.14	242.385 ± 39.23	−28.2	−0.7	−14.1
	S-OP	n.d.	3.195 ± 0.24	10.535 ± 1.40	1.535 ± 0.04	28.235 ± 0.72	−28.0	−0.3	−17.8
	S-GP	n.d.	12.295 ± 1.35	38.625 ± 2.05	1.745 ± 0.48	376.275 ± 16.27	−29.4	−1.8	−26.7
Italy	I-RP1	12.695 ± 0.29	5.625 ± 0.88	70.165 ± 7.82	10.775 ± 0.07	320.255 ± 87.15	−29.0	0.2	−13.9
	I-RP2	16.65 ± 0.6	4.095 ± 0.8	64.715 ± 2.16	1.635 ± 0.42	1413.985 ± 173.43	−31.0	0.6	−3.1
	I-OP	n.d.	25.335 ± 0.5	21.525 ± 0.82	13.075 ± 0.70	1331.685 ± 0.84	−28.3	3.7	5.6
	I-GP	n.d.	155.585 ± 1.11	143.385 ± 13.90	78.795 ± 3.54	448.165 ± 78.51	−26.9	0.6	−15.7
Hungary	H-GP	n.d.	14.75 ± 3.09	2462.255 ± 134.54	n.d.	110.85 ± 43.30	−28.5	−1.2	−37.2
Turkey	T-RP1	13.765 ± 0.66	n.d.	169.315 ± 10.55	15.15 ± 1.98	281.745 ± 6.85	−28.2	0.7	3.0
	T-RP2	33.465 ± 0.50	n.d.	122.585 ± 5.74	4.125 ± 0.50	1244.875 ± 200.42	−28.2	0.5	−2.4
	T-GP1	12.995 ± 0.44	14.985 ± 0.50	1724.745 ± 29.23	8.645 ± 1.65	420.325 ± 19.43	−29.1	−0.6	3.5
	T-GP2	17.065 ± 1.71	38.165 ± 0.04	1496.185 ± 25.88	8.995 ± 1.61	n.d.	−28.8	2.2	−9.2
	T-GP3	n.d.	13.645 ± 4.38	114.225 ± 4.04	3.755 ± 1.09	545.95 ± 30.49	−28.8	1.6	−2.5
	T-GP4	n.d.	61.575 ± 2.99	425.275 ± 54.39	n.d.	316.875 ± 72.93	−28.2	−4.1	−37.4
	T-GP5	n.d.	16.025 ± 0.55	751.135 ± 57.96	n.d.	2322.355 ± 71.75	−29.6	1.0	−7.2
	T-GP6	n.d.	47.635 ± 4.42	257.655 ± 5.98	n.d.	2230.935 ± 56.87	−27.1	−0.3	−17.2
Romania North	RN-RP1	28.265 ± 4.27	10.25 ± 0.22	94.475 ± 3.28	1.555 ± 0.61	1476.485 ± 37.17	−30.7	2.4	−5.1
	RN-RP2	n.d.	12.655 ± 1.50	19.155 ± 3.92	4.255 ± 0.12	1080.455 ± 39.74	−21.1	−2.8	−33.7
	RN-RP3	n.d.	12.535 ± 0.41	2.315 ± 0.10	4.675 ± 0.18	1595.785 ± 26.40	−28.1	−1.7	−34.4
	RN-RP4	n.d.	12.825 ± 0.66	68.725 ± 1.45	6.745 ± 0.98	1209.445 ± 12.81	−27.7	1.8	−24.2
	RN-RP5	n.d.	10.535 ± 0.57	239.75 ± 19.73	6.995 ± 1.02	445.775 ± 6.24	−29.4	−3.3	−36.2
	RN-RP6	n.d.	17.495 ± 3.28	208.745 ± 23.2	7.355 ± 1.74	1103.565 ± 41.66	−29.4	−3.1	−37.0
	RN-OP1	n.d.	12.325 ± 2.34	432.075 ± 9.74	12.865 ± 0.37	2009.445 ± 58.11	−29.4	3.0	−0.8
	RN-OP2	16.545 ± 0.93	48.255 ± 3.54	487.235 ± 30.87	12.085 ± 1.01	286.425 ± 46.66	−28.2	−0.3	−16.4
	RN-GP1	10.585 ± 2.76	33.35 ± 3.58	892.145 ± 47.09	3.075 ± 0.24	1020.145 ± 109.9	−29.4	4.0	1.1
	RN-GP2	n.d.	24.335 ± 1.15	67.815 ± 1.05	3.755 ± 00.46	444.025 ± 80.2	−27.7	0.0	−18.8
	RN-GP3	5.725 ± 0.07	77.665 ± 4.51	1057.535 ± 65.77	3.795 ± 0.2	650.545 ± 41.90	−31.0	3.5	−0.6
	RN-GP4	n.d.	23.945 ± 1.76	60.595 ± 13.07	1.715 ± 0.05	751.825 ± 98.4	−28.4	−1.9	−31.3
	RN-GP5	n.d.	28.665 ± 1.28	32.095 ± 0.85	2.975 ± 0.2	1320.115 ± 66.37	−28.1	−0.7	−21.1
	RN-GP6	n.d.	26.45 ± 3.16	328.85 ± 29.75	4.45 ± 0.6	1463.985 ± 114.32	−29.0	−2.4	−34.8
Romania South	RS-RP1	n.d.	65.15 ± 3.65	101.675 ± 12.74	3.375 ± 0.63	118.165 ± 14.99	−27.4	−0.5	−29.5
	RS-RP2	n.d.	79.75 ± 18.76	84.35 ± 1.98	8.475 ± 0.82	751.295 ± 81.64	−27.5	−1.2	−25.1
	RS-GP1	4.55 ± 0.21	35.585 ± 7.06	290.195 ± 32.89	16.965 ± 2.93	197.15 ± 30.77	−25.5	0.0	−16.9
	RS- GP2	4.495 ± 0.04	35.275 ± 0.30	144.665 ± 3.88	6.815 ± 0.81	744.155 ± 75.78	−30.1	3.1	−14.3
	RS-GP3	3.55 ± 0.36	31.625 ± 1.69	192.355 ± 12.79	3.345 ± 0.19	580.545 ± 55.40	−29.6	−1.5	−37.5
	RS-GP4	5.995 ± 0.06	22.045 ± 0.12	115.855 ± 8.40	6.475 ± 0.47	647.455 ± 70.18	−25.6	−2.2	−32.6

n.d.– not detected; RP – red pepper, OP – orange pepper, GP – green pepper; M – Morocco, S – Spain, I – Italy; H – Hungary; *T* – Turkey; RN – Romanian North; RS – Romanian South.

[a] The concentration values are expressed in μg/g ± standard deviation (SD) of lyophilized pepper samples;

[b] luteolin, kaempferol, and chrysin were not detected in the real pepper samples.

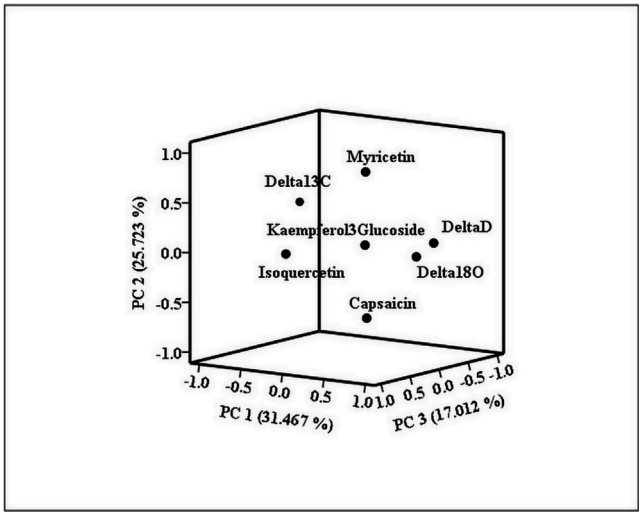

Fig. 2. Loading plot obtained after applying PCA using isotopic values along with phenolic compounds.

phenolic compounds determination was to establish and to evidence the parameters that characterize country of origin for real hot peppers. Besides this, establishing a differentiation between colors of hot pepper was investigated.

The matrix used for chemometric interpretation was formed of 38 hot pepper samples with 7 variables (organic compounds along with isotopic content), as description parameters. Regarding the geographical origin of hot pepper samples, five countries were compared (Morocco, Spain, Italy, Turkey, and Romania). The sample from Hungary was omitted from this interpretation. In this case, kaempferol-3-glucoside ($p = .042$) had the highest values in samples from Turkey, myricetin ($p = .024$) presented a higher average value in samples from Italy, compared with samples produced in Turkey or Romania. When ANOVA was applied in order to evidence some characteristic compounds for Romanian samples, only δ^2H content ($p = .003$) was obtained as being statistically significant. There were no differences regarding organic content.

Furthermore, an ANOVA test was applied to compare the hot pepper samples according to their color. Only one compound, kaempferol-3-glucoside ($p = .049$) is able to realize a clear separation between the samples. After applying Tukey post hoc test, it was observed that content of kaempferol-3-glucoside was much higher in green peppers than in red peppers.

In this study PCA was applied on the entire matrix in order to obtain the main components which are loaded by measured parameters. The value of the initial KMO test was 0.510 and Barttlet's test of sphericity was significant ($p = .001$). The main parameters employed when PCA was compiled were extraction method selected which was principal components (PC), matrix rotation was made using Varimax method with Kaiser Normalization and only components with eigenvalues higher than 1 were retained for further interpretation. Thus, PC 1 had 31.467% from total variance and had high loading of all three measured isotopic ratios, PC 2 had 25.723% and had loadings of isoquercitin and myricetin, and PC 3 had 17.012% and was formed of capsaicin and kaempferol-3-glucoside compounds. The loading plot obtained for each measured parameters after applying PCA is presented in Fig. 2. It can be concluded that PC 1 represents the geographical origin of hot pepper samples. This because evapo-transpiration processes enriches both ^2H and ^{18}O isotopes in plant water. The enrichment degree depends on factors like microclimate conditions (i.e. relative humidity and isotopic signature of available water for the plant development) but also on other factors like soil type or sun exposure (Christoph, Hermann, & Wachter, 2015). Although, $\delta^{13}C$ is not a specific marker for geographical origin as δ^2H and $\delta^{18}O$ are, it could give information regarding the climatic and indirect geographical origin in which plant had grown. Beside this, PC2 and PC3 comprise elements that are characteristic for phenolic profile.

4. Conclusions

Current work proposed a new optimized and validated UHPLC method for simultaneous extraction and quantification of flavonoids and capsaicin in hot peppers. The optimal working conditions for phenolic compounds quantification in peppers were experimentally proven to be methanol, as extraction solvent, at 12 h extraction time assisted by ultrasound, at the 1:8 ratio of sample to solvent. Compared with thermal drying, the freeze-dried process, used in sample preparation, demonstrates a higher level in detected flavonoid concentrations. The extraction recovery ranged from 90.60% to 115.05%. The correlation coefficient obtained for each compound demonstrates excellent relationship between peak areas and concentrations ($R^2 > 0.9987$). The developed method was applied in order to analyze flavonoids (vitexin, isoquercetin, kaempferol-3-glucoside, and myricetin) and capsaicin content in thirty-nine freeze-dried commercial samples from different country of origin. The studied compounds were quantified in hot pepper samples at different concentration ranges: capsaicin (28.23–2322.35 μg/g), vitexin (2.93–33.46 μg/g), isoquercetin (3.19–155.58 μg/g), kaempferol-3-glucoside (2.31–2462.25 μg/g) and myricetin (1.55–78.79 μg/g). To the best of our knowledge, this is the first work that proposes an optimized and validated method for simultaneous extraction of flavonoids together with capsaicin in hot peppers.

Besides phenolic content determination in hot pepper samples, isotopic content was also measured, in order to improve the establishment of geographical origin. The association between these analytical techniques and chemometric tools proved that kaempferol-3-glucoside is one of the strongest markers both for country and maturity stage discrimination. This is the first approach that attempt to discriminate the country of origin of hot pepper fruits and maturity stages using a combination of markers like flavonoids, capsaicin and stable isotopic measurements correlated with chemometric tools (ANOVA and PCA).

Conflict of interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.10.031.

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