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Anthocyanin composition of cauliflower (*Brassica oleracea* L. var. botrytis) and cabbage (*B. oleracea* L. var. capitata) and its stability in relation to thermal treatments

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Abstract

Violet cauliflower and red cabbage were analysed for their anthocyanin profiles before and after thermal treatments. Anthocyanins are well-noted as healthy compounds due to their antioxidant properties. Samples were analysed for total anthocyanin content by using a spectrophotometric differential pH method. An MS-based method, combining high-performance liquid chromatography (HPLC) with quadrupole tandem mass spectrometry (HPLC–MS/MS) was developed, aimed to separate, identify and quantify the main anthocyanin forms. The procedure involves a rapid and efficient pre-treatment of the samples by solid-phase extraction, followed by selective determination of all compounds in a single run analysis using HPLC–MS/MS. Structural information for the identification of compounds was obtained from their fragmentation patterns (MS/MS spectra). The compounds were separated by HPLC and detected in the multiple reaction monitoring mode (MRM), which provides a high level of selectivity for targeting the analytes in vegetables. Cauliflower and red cabbage showed differences in their anthocyanin profiles: cyanidin-3,5-diglucoside was absent in cauliflower, while it was well represented in red cabbage, together with the characteristic anthocyanin of *Brassica* genus, cyanidin-3-sophoroside-5-glucoside. The *p*-coumaryl and feruloyl esterified forms of cyanidin-3-sophoroside-5-glucoside were predominant in cauliflower, while the sinapyl one was mostly present in red cabbage. Besides, the stability of cauliflower's anthocyanin profile was evaluated in relation to thermal pre-treatments. All thermal treatments, except microwave heating, drastically reduced total cauliflower anthocyanin content. The amount of individual anthocyanins was expressed as the percentage with respect to total anthocyanin amount, spectrophotometrically measured. Significant individual changes were observed after different thermal treatment with an isomer formation.

Keywords: Violet pigment; Cyanidin glucosides; Brassicaceae; Cauliflower; HPLC–MS/MS

1. Introduction

Among vegetables and fruits, the red–violet colouration is derived mainly from a class of flavonoids called anthocyanins. This quality characteristic largely determines the consumer appeal and impacts significantly on the market value of the produce. There is also an increasing interest in anthocyanins because of their potential health-promoting properties and, above all, for their protection against

Abbreviations: LM02, sample from local market purchased in 2002; LM03, sample from local market purchased in 2003; cv 98, sample of landrace named Sammartinaro harvested in 2003; cv 141, sample of landrace named Natalino harvested in 2003.

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free radicals (Rossetto et al., 2002; Saint-Cricq de Gaulejac, Glories, & Vivas, 1999).

Cauliflowers (*Brassica oleracea* L. var. botrytis) are a source of phytonutrients, acting as antioxidants, such as glucosinolates, ascorbic acid and polyphenols. Some varieties of cauliflowers are violet pigmented as consequence of the presence of anthocyanins. The violet curded landraces of cauliflower, commonly named "Violetto di Catania", can be found in agricultural agri-systems of east Sicily (Branca, Li, Goyal, & Quiros, 2002), and the most traditional are popularly named in relation to the harvesting time. Violet cauliflower extracts show significant antioxidant properties, among which are the scavenging activity of the very reactive hydroxyl radical (Pizzocaro et al., 2000). Anthocyanin pigments were studied by other authors in *B. oleracea* crops, and all the literature reported is related to the anthocyanin composition of red cabbage (*B. oleracea* L. var. capitata) (Hradzina, Iredale, & Mattick, 1977; Idaka, 1987; Idaka et al., 1987).

Cauliflowers are normally submitted to food processing, such as brief thermal treatments before technological use, and are cooked before human consumption. Brief treatments on *Chicorium* samples preserve the vegetable quality by partial or total suppression of enzymatic activities and often result in enhanced antioxidant activity (Papetti, Daglia, & Gazzani, 2002); cauliflower cooking induces a strong decrease of anthocyanin content, except in the case of microwave heat treatment (Di Cesare, Viscardi, Genna, & Ferrari, 2005).

The aim of this work is to propose a method to determine the individual anthocyanin composition of violet cauliflower and the stability of the different forms after heat treatments. We report an advanced protocol, combining a sample pre-treatment by solid-phase extraction, followed by selective determination of all compounds in a single run analysis using HPLC–MS/MS. To our knowledge the analytical methodology is new because, to date, no literature has been specifically found on cauliflower pigment, probably due to the difficult HPLC separation of individual anthocyanins. The incomplete resolution of some anthocyanin forms in HPLC was overcome in our work employing the MS/MS identification.

In this study we have used raw, blanched, microwave-heated and cooked violet cauliflower. The reference used for anthocyanin composition was a sample of red cabbage, for which the anthocyanin composition is already known. Total anthocyanin analysis of the samples was first performed using a spectrophotometric differential pH method. The composition of the vegetable extracts has been investigated by employing HPLC with tandem quadrupole mass spectrometry (MS/MS) for both separation and identification of anthocyanin compounds. Furthermore, the extracts have been analysed using the highly-selective multiple reaction monitoring mode (MRM) and the percentages of individual anthocyanins obtained for the different raw and processed vegetable extracts have been calculated for comparative purposes.

2. Material and methods

2.1. Materials

2.1.1. Reagents and standards

Ultra pure water was obtained from a Millipore Mill-RO 10 Plus deionisation system, followed by a Milli-Quarter system (18 MΩ cm resistivity) and a sub-boiling quartz distillation unit (Quartex s.a., Paris, France). HPLC grade acetonitrile, ethanol and methanol, 96% acetic acid and formic acid were from Merck (Darmstadt, Germany). Nitrogen (99.999%) (desolvation and nebuliser gas) and argon N50 (MS/MS collision gas) were from Air Liquide (Liège, Belgium).

2.1.2. Samples

The reference used for our studies was a sample of red cabbage (*B. oleracea* L. group capitata). It was purchased, in 2002, from a market in Milano (Italy) and was treated in the same way as violet cauliflower samples.

Cauliflower (*B. oleracea* L. var. botrytis) samples (about five curds for each sample) were purchased, harvested and selected by the University of Catania (DOFATA), which is involved in the genetic improvement of the Sicilian landraces of *B. oleracea* crops. After purchasing or harvesting, done in the years 2002 and 2003, the curds were immediately sent from Catania to CRA-IVTPA (Milan). The samples were refrigerated, once delivered to IVTPA, and reduced to small florets. Two aliquots of about 100 g were randomly selected from each sample and immediately frozen at −80 °C before freeze-drying. The samples were reduced to powder in a Waring-blender at −4–0 °C and stored at −20 °C for further analysis.

Fresh floret samples were subjected to thermal treatments. An aliquot of 100 g from a sample harvested during 2002 was blanched in a microwave system (MW) by steam vapour for 2 min and another aliquot was cooked for 12 min in 0.5% NaCl (ratio 1:10 w/w). Samples harvested during 2003 were blanched in boiling tap water for 2 min (ratio 1:10 w/w). After thermal treatments, all samples were refrigerated by dipping in a semi-frozen water solution for 10 min and subsequently frozen at −80 °C, freeze-dried and extracted as the raw samples.

In summary, nine curd samples of landraces of violet cauliflower were analysed:

- three bought from a local market (2002), produce "Violetto di Catania" raw, microwave and cooked (LM02);
- two from a local market (2003), produce "Violetto di Catania" 2003, raw and blanched (LM03);
- two from the DOFATA landrace selection (2003) called "Natalino", raw and blanched (cv 141);

– two from the DOFATA landrace selection (2003) called "Sammartinaro", raw and blanched (cv 98).

2.2. Methods

2.2.1. Total anthocyanin analysis

Total anthocyanin analysis was performed using a spectrophotometric differential pH method, according to Rapisarda, Fanella, and Maccarone (2000), with a few modifications. Two lyophilised samples of 500 mg were treated with 10 ml of buffer solution, pH 1.0 (125 ml of 0.2 M KCl and 375 ml of 0.2 M HCl), and 10 ml of buffer solution, pH 4.5 (400 ml of 1 M sodium acetate, 240 ml of 1 M HCl and 360 ml of water), respectively. The mixture was homogenised and centrifuged twice at 4 °C at 5000*g* for 15 min. The supernatant was collected and its absorbance was read at 510 nm. Total anthocyanin amount was determined by the following equation:

$$C(\mathrm{mg/kgf.w.}) = (\mathrm{ABS\ pH\ 1.0} - \mathrm{ABS\ pH\ 4.5}) \times 484.8/24825 \times F$$

where the terms in parentheses indicate the difference between the absorbance value at 510 nm at pH 1.0 and 4.5 solutions, respectively. 484.8 is the molecular mass of cyanidin-3-glucoside chloride, 24,825 is its molar absorptivity (ε) at 510 nm, and F is the dilution/concentration factor related to the sample weight, the extract volume and the yield of the lyophilisation process. Each sample was analysed in triplicate and the results were expressed as the averages of the three measurements.

2.2.2. Solid phase extraction

Solid phase extraction was carried out on a Chromabond vacuum manifold for 24 columns (Macherey-Nagel, Duren, Germany) connected to a membrane pump (Barnant, Barrington, IL, USA), while tissue disruption was realised using a T25 Ultra-Turrax from IKA Labortechnik (Darmstadt, Germany).

The violet cauliflower and red cabbage extracts to be analysed by LC–MS/MS were prepared as follows: 200 mg of lyophilised cauliflower or 50 mg red cabbage sample were treated with 5 ml of 0.1 N HCl. The mixture was homogenised and centrifuged twice at 5000*g* for 15 min at 4 °C. The supernatant was loaded onto a SPE C_{18} column (50 × 1.5 mm) previously conditioned three times with 2 ml of 0.1 N HCl. The column was rinsed three times with 2 ml of double-distilled water and subsequently, was eluted by adding (three times) 1 ml of EtOH (50% solution) acidified with 0.1% HCOOH (pH 2.2). The eluate was evaporated to dryness and the residue was re-dissolved in the same volume as the collected eluate (red cabbage extract was 5-fold diluted) in a 97:3 solution of water/CH_3CN, acidified with 0.1% HCOOH for further analysis by HPLC–MS/MS.

2.2.3. HPLC conditions

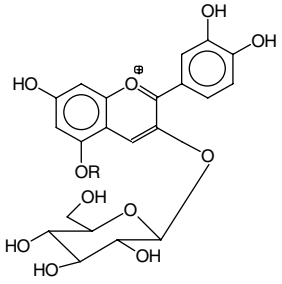
The HPLC equipment used in this work was a Waters Alliance 2690 quarternary solvent delivery system (Waters, Milford, MA, USA). Anthocyanins from red cabbage and cauliflower extracts were separated by reversed-phase HPLC (flow rate of 0.7 ml/min) on a 5 mm Inertsil ODS-3 column (i.d. 4.6 mm, length 50 mm) at 40 °C. The HPLC system includes a DAD detector, set at 520 nm for anthocyanin evaluation, coupled to the electrospray interface (Z-spray source) of a tandem quadrupole mass spectrometer (LC–MS/MS system). A Valco zero dead volume T-piece splitter (7/1) from Waters Micromass was used between the HPLC column and the electrospray interface in order to reduce the flow-rate of the chromatographic effluent to 100 μl/min. Injection volumes of 50 μl were used in HPLC.

The binary mobile phase consisted of solvent A, composed of formic acid 0.1% in water, and solvent B, composed of acetonitrile/formic acid 0.1%. Separation were performed by linear gradients of B in A as follows: time 0–5 min at 3% B in A; time 5–20 min, 3–20% B in A; time 20–30 min at 20% B in A; time 30–35 min, 20–50% B in A; time 35–40 min, 50–3% B in A; time 40–50 min, 3% B in A.

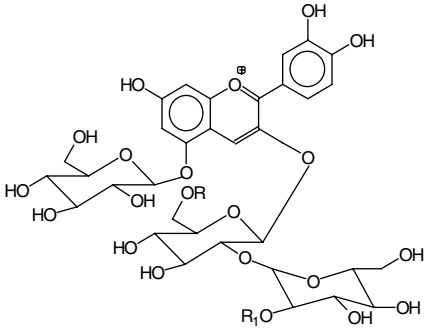
2.2.4. ESI-MS/MS conditions

MS/MS detection of anthocyanins was achieved using a Quattro LC triple stage quadrupole instrument from Waters Micromass (Manchester, UK). The positive ionisation mode was used and the ions were monitored in the MS and MS/MS modes, with a capillary voltage of 3.5 kV and a cone voltage of 65 V. The source block and desolvation temperatures were set at 100 and 300 °C, respectively, while the desolvation and nebuliser gas (N_2) flow-rates were set at 650 and 75 l/h, respectively. Argon was used as a collision gas at 2.5×10^{-3} mbar. Cone voltage was kept constant and collision energy was optimised for each compound separately. MS/MS data were collected and processed with MassLynx software version 3.4 from Waters Micromass (Manchester, UK).

The instrument response was first optimised for the anthocyanins by infusing a constant flow (5 μl/min) of solution of red cabbage extract (used as a reference) in the mobile phase. The instrument was operated in the full scan mode, choosing m/z values from 100 to 1200 for MS1. A TIC (total ion chromatogram) was obtained for reference and the masses of the known pigments were extracted from the TIC chromatogram. The m/z values were obtained from the literature regarding the red cabbage composition on the structure of the major pigments (Fig. 1). The instrument was then operated in the single ion mode (SIM) in order to improve the signal to noise ratio and generate some fragmentation spectra in MS/MS. Selected ions for MS1 were m/z 449, 611, 773, 919, 949, 979, 1081, 1111, 1125, 1141, 1155, 1185.



1- cyanidin-3-glucoside R = H *m/z* 449
2- cyanidin-3-glucoside-5-glucoside R = glucose *m/z* 611



3- Cyanidin-3-sophoroside-5-glucoside R = H R1 = H *m/z* 773
4- Cyanidin-3(6-*p*-coumaryl)-sophoroside-5-glucoside R = *p*-coumaric R1 = H *m/z* 919
5- Cyanidin-3(6-feruloyl)-sophoroside-5-glucoside R = ferulic R1 = H *m/z* 949
6- Cyanidin-3(6-sinapyl)-sophoroside-5-glucoside R = sinapyc R1 = H *m/z* 979
7- Cyanidin-3(6-*p*-coumaryl)-sophoroside-5(6-sinapyl)-glucoside R = *p*-coumaric R1 = sinapic *m/z*1125
8- Cyanidin-3(6-feruloyl)-sophoroside-5(6-sinapyl)-glucoside R = ferulic R1 = sinapic *m/z*1155
9- Cyanidin-3(6-sinapyl)-sophoroside-5(6-sinapyl)-glucoside R = sinapic R1 = synapic *m/z* 1185

Fig. 1. Chemical structure of the main anthocyanins from cauliflower and cabbage.

Then, an elucidation of the structure of pigments was done by fragmentation of the molecular ions in the collision cell of the mass spectrometer (using an Argon flow and optimising the acceleration energy of the ions) and analysis of the generated fragment ions using MS2. The *m/z* values were from 100 to 1200 for MS2. For quantification purposes, multiple reaction monitoring (MRM) mode was used, in order to enhance sensitivity and selectivity (parent ion > fragment ion).

Subsequently, 19 reaction channels were set up, representing the reactions of original anthocyanins, yielding daughter ions by loss of *m/z* 162, from the hydrolysis of a glucose moiety without a water molecule ($180 - 18 = 162$). Each MRM channel was monitored and was set up with the following reaction conditions:

	Chain reaction (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
1	611.22 > 449.09	0.10	65.0	25.0
2	611.22 > 287.19	0.30	65.0	35.0
3	773.36 > 611.22	0.10	55.0	30.0
4	773.36 > 287.19	0.30	65.0	35.0
5	919.22 > 757.21	0.10	60.0	35.0
6	919.22 > 287.19	0.30	65.0	35.0
7	949.32 > 787.33	0.10	60.0	40.0
8	949.32 > 287.19	0.30	65.0	35.0
9	979.28 > 287.19	0.30	65.0	35.0
10	979.28 > 817.33	0.10	60.0	35.0
11	1081.22 > 287.19	0.30	65.0	25.0
12	1111.37 > 287.19	0.30	65.0	25.0
13	1125.05 > 963.43	0.10	60.0	35.0
14	1125.22 > 287.19	0.30	65.0	35.0
15	1155.61 > 993.37	0.10	60.0	40.0
16	1155.61 > 287.19	0.30	65.0	25.0
17	1185.38 > 287.19	0.30	65.0	25.0
18	1185.38 > 1023.24	0.10	60.0	35.0
19	1185.38 > 287.19	0.30	65.0	35.0

2.2.5. Quantification method

Anthocyanins were detected in the visible range by monitoring the DAD detector at 520 nm, and MS/MS data acquisition from each sample was done using the MRM mode (19 MRM channels). The areas from the peaks corresponding to fragment ions obtained by loss of *m/z* 162 from the parent ions, were used for the quantification of single anthocyanins (Channels 1, 3, 5, 7, 10, 13, 15, 18). Other channels were set up to obtain a better response and represented the complete loss of all glycoside units, giving the base structure of the anthocyanidin unit at *m/z* 287 (cyanidin). These channels were not used for quantitative analysis, but only for qualitative. Single anthocyanin content was expressed as a percentage with respect to total anthocyanin content. All analyses were performed in duplicate, and the reported quantitative data presented here are the averages of two measurements.

3. Results and discussion

3.1. Total anthocyanin content

Total anthocyanin analysis of raw samples (Table 1) revealed a content of 756 mg/kg f.w. for red cabbage and an average content of 42.1 mg/kg in samples of violet cauliflower. Among the cauliflower samples, the highest content was found in the curds of raw LM03 (77.2 mg/kg) while the lowest was found in the accession cv 141 (18.1 mg/kg).

3.2. Direct MS(/MS) analysis of the extracts by infusion

The study of anthocyanin composition in cauliflower was initiated by using the anthocyanic profile of red cabbage as the reference, taking into account previous literature data on anthocyanin structures (Fig. 1) (Hradzina et al., 1977; Idaka, 1987; Idaka et al., 1987). The first step was the establishment of the MS and MS/MS profiles of anthocyanins from direct infusion of the red cabbage extract at a concentration of ca. 20 μg/ml (Fig. 2), in order to monitor the existing *m/z* values and to match the experimental data with those existing in the literature.

The typical component of red cabbage pigment is the cyanidin unit glycosylated by three glucose units, giving cyanidin-3-sophoroside-5-glucoside (**3**) at *m/z* 773, and the simultaneous presence of cyanidin 3-glucoside (**1**) at *m/z*

Table 1
Total anthocyanin content, percent recovery after thermal treatments and percent composition of the main anthocyanin in red cabbage and violet cauliflower, not treated (RAW), microwave (MW), blanched (BLANCH), during 2002 (a) and 2003 (b)

(a)	Red cabbage		LM02		
			RAW	MW	COOKED
Ret. Time (min)	Total anthoc. (mg/kg f.w.)	756	32.8	31.3	6.5
	% Recovery			95.4	19.8
	Compound				
16.4	**3**	6.4	4.0	1.5	2.6
19.9	**3 bis**	nd	nd	1.3	2.5
17.1	**2**	1.2	nd	nd	nd
25.3	**4**	6.3	29.9	21.7	28.0
25.8	**5**	2.5	15.1	12.4	11.3
25.9	**6**	24.1	1.6	0.4	4.5
19.6	**6 bis**	1.9	1.0	1.1	1.8
26.3	**7**	14.4	35.7	53.0	42.6
26.7	**8**	3.6	11.5	7.6	3.9
27.1	**9**	39.5	1.1	1.0	2.8

(b)		LM03		cv 98		cv 141	
		RAW	BLANCH	RAW	BLANCH	RAW	BLANCH
Ret. time (min)	Total anthoc. (mg/kg f.w.)	77.2	7.7	40.1	14.2	18.1	4.3
	% Recovery		10.0		35.4		23.8
	Compound						
16.4	**3**	1.4	1.1	0.5	nd	3.0	nd
19.9	**3 bis**	2.1	3.7	3.1	4.2	7.4	13.2
17.1	**2**	nd	nd	nd	nd	nd	nd
25.3	**4**	26.7	26.8	9.0	8.5	19.9	22.4
25.8	**5**	10.7	9.1	8.9	10.2	17.6	15.9
25.9	**6**	4.8	19.0	2.4	2.5	2.0	6.2
19.6	**6 bis**	0.7	3.7	1.0	0.7	1.2	3.0
26.3	**7**	36.2	16.7	27.8	40.0	37.8	36.8
26.7	**8**	5.3	6.1	42.4	29.3	10.0	1.5
27.1	**9**	12.1	14.0	4.8	4.6	1.0	0.9

nd = not detectable.

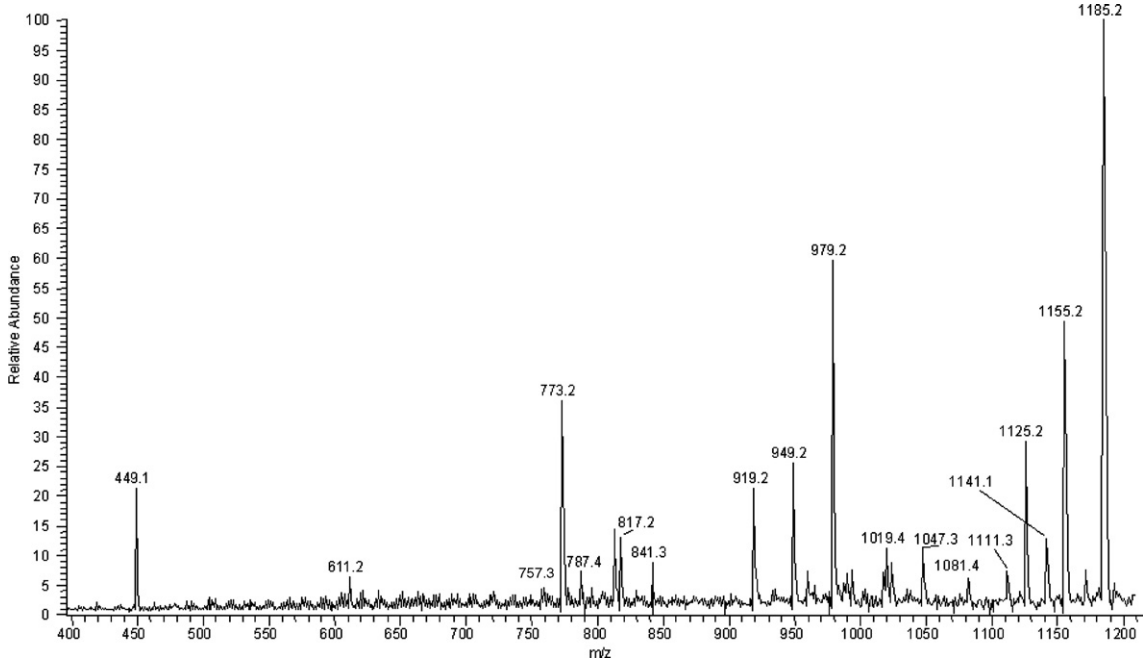


Fig. 2. Direct infusion MS scanning in red cabbage.

449 and cyanidin-3,5-diglucoside (**2**) at *m*/*z* 611 (Fig. 1), which is in agreement with previous authors' findings (Stroh & Seidel, 1965). The sophoroside moiety could be esterified by *p*-coumaric, ferulic or synapic acids, leading to the structures (**4**), (**5**) and (**6**), at *m*/*z* 919, 949 and 979, respectively (Fig. 1). The sophorose moiety could be further esterified by a synapic acid unit, giving the structures (**7**), (**8**) and (**9**), at *m*/*z* 1125, 1555 and 1185, respectively (Fig. 1). The signals at *m*/*z* 1185, followed by 979 were the most abundant in the red cabbage extract. Minor anthocyanin compounds were detected at *m*/*z* 757, 787, 817, 1081, 1111, 1141, already described in previous studies (Idaka et al., 1987). It could be assumed that the red cabbage pigment is mainly characterised by the presence of cyanidin glycosides esterified by synapic acid moieties, as reported in previous studies (Chmielewska, Smardzewska, & Kulesza, 1938).

MS and MS/MS profiles of anthocyanin compounds were also established for violet cauliflower, using the extract from the sample cv 98. The concentration of anthocyanins was about the same as that used for analysis of red cabbage extract (ca. 20 μg/ml). As shown in Fig. 3, the signal-to-noise ratio was lower respect to that observed for the infusion of the red cabbage extract. An explanation of the differences observed could be that the cauliflower sample was less diluted and a higher amount of lyophilised cauliflower sample was extracted in order to obtain the same pigment concentration. Fewer MS compounds were found. Well detected signals corresponded to compound (**1**), *m*/*z* 449; compound (**4**), *m*/*z* 919; compound (**5**) *m*/*z* 949; compound (**7**), *m*/*z* 1125 and compound (**8**), *m*/*z* 1155. The most represented signals of violet cauliflower were at *m*/*z* 949 and 1155, corresponding to the compound esterified by a feruloyl moiety and further by synapic acid. Our results show a significant difference between pigment composition of red cabbage and violet cauliflower, together with the absence of compound (**2**) in violet cauliflower extract. Other low-intense signals, not identified up to now, were detected in violet cauliflower and not in red cabbage, i.e. *m*/*z* 410, 556, 613, 687 and 1029.

3.3. HPLC separation and MS/MS detection of anthocyanins

Raw extracts from red cabbage and violet cauliflower were subjected to HPLC separation, monitoring the signals at 520 nm and simultaneously acquiring MS/MS data. The composition of all monitored LC–MS/MS signals (MRM reactions) of fragment ions generated from red cabbage and cauliflower extracts was summarised above in Section 2.2.4. The chromatographic separation of anthocyanins was not complete, because of the low acidity of the mobile phase and the absence of an ion-pairing reagent in it. Even if a better separation were obtained when using a higher acid medium, low acidic conditions were preferred to reach the highest performance with the pneumatically-assisted electrospray ion source. Fig. 4 shows an example of chromatograms obtained for red cabbage and a cauliflower extract (raw LM03).

Data were also acquired in the MRM mode, the peak attributions and the corresponding MS/MS transitions being shown in Table 2. The presence of all the expected anthocyanins was confirmed for red cabbage, the most intense signals being observed for compounds (**6**) and (**9**).

In cauliflower extracts, the biggest signal found was related to a peak at 25.3 min (identified as compound **4**) (Fig. 4). No peak with the retention time corresponding

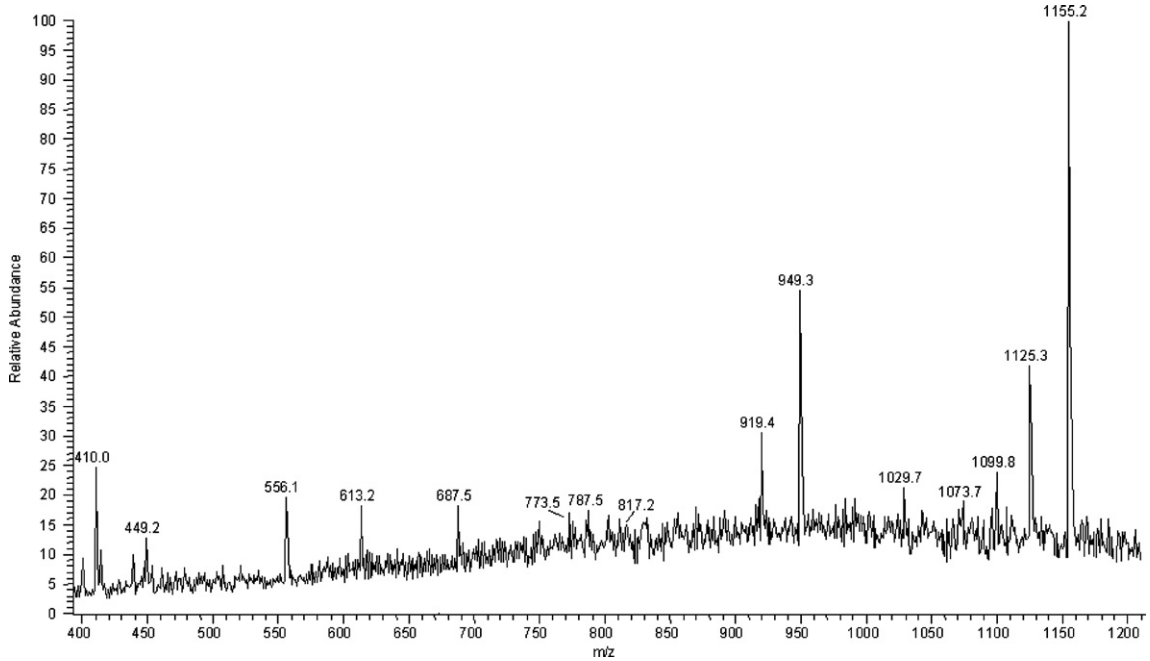


Fig. 3. Direct infusion MS scanning in violet cauliflower cv 98 (landrace "Sammartinaro").

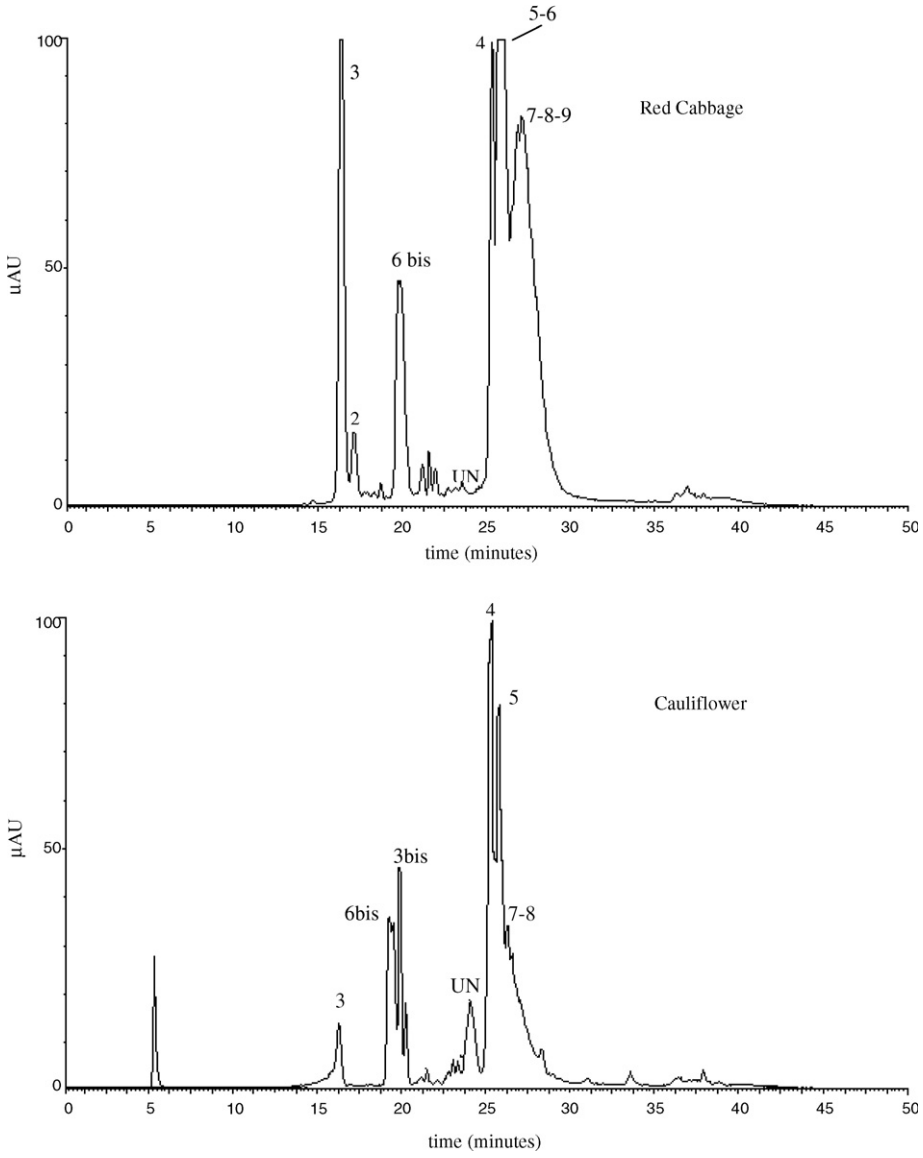


Fig. 4. Example of total ion chromatograms (TIC) by LC–MS/MS (acquisition in the MRM mode) chromatograms of cabbage and cauliflower extracts. Compounds are numbered as in Table 2, UN = unidentified.

to compound (**2**) was observed in cauliflower extract. On the other hand, a closed peak at retention time of ca. 24 min remained unidentified (UN). The presence of two isomers of compounds (**3**) and (**6**), named (**3 bis**) and (**6 bis**), respectively, was highlighted. Compound (**3 bis**) was found in cauliflower and was absent in red cabbage extract.

Table 2
Composition of main anthocyanins from red cabbage and violet cauliflower obtained by LC–MS/MS analysis (signal acquisition in the MRM mode)

Compound nr.	Retention time (min)	Attribution	MRM reaction	*m/z*
1	Not found	Cyanidin-3-glucoside	–	449
2	17.1	Cyanidin-3-glucoside-5-glucoside	611 > 449	611
3	16.4	Cyanidin-3-sophoroside-5-glucoside	773 > 611	773
4	25.3	Cyanidin-3-(6-*p*-coumaryl)-sophoroside-5-glucoside	919 > 757	919
5	25.8	Cyanidin-3-(6-feruloyl)-sophoroside-5-glucoside	949 > 787	949
6	25.9	Cyanidin-3-(6-sinapyl)-sophoroside-5-glucoside	979 > 817	979
7	26.3	Cyanidin-3-(6-*p*-coumaryl)-sophoroside-5-(6-sinapyl)-glucoside	1125 > 963	1125
8	26.9	Cyanidin-3-(6-feruloyl)-sophoroside-5-(6-sinapyl)-glucoside	1155 > 993	1155
9	27.4	Cyanidin-3-(6-sinapyl)-sophoroside-5-(6-sinapyl)-glucoside	1185 > 1023	1185
3 bis	19.9	Isomer of cyanidin-3-sophoroside-5-glucoside	773 > 611	773
6 bis	19.6	Isomer of cyanidin-3-(6-sinapyl)-sophoroside-5-glucoside	979 > 817	979

Data acquisition in the MRM mode was used for anthocyanin quantification in the cauliflower extracts. The main compounds found in MS/MS analysis were confirmed by their MS/MS transitions (m/z values for parent and daughter ions) and their respective retention times (as specified in Section 2.2.5). In raw samples of LM02 and LM03 (Table 1), the two main compounds found were (**4**) and (**7**). They represent more than 50% of total antocyanin composition of violet cauliflower. In contrast to this, landrace cv 98 showed the highest abundance for compound (**8**), 42.4%, followed by compound (**7**), 27.8%. Compounds (**5**) and (**4**) were found to be 9.0% and 8.9% of that of the total anthocyanin content, respectively. For the landrace cv141, the anthocyanin profile was similar to that observed for the LM sample: the main compounds were (**7**), 37.8%, and (**4**), 19.9%, and to a less extent compound (**5**), 17.6%, and (**8**), 10.0%. Compounds (**6**) and (**9**), characteristic of red cabbage, remained with a low abundance in all cauliflower samples.

Main differences observed between samples could be attributed both to different post-harvest evolution (well-known for experimental landraces but not well-known for LM samples) and to intrinsic genetic diversity in the landrace ecotypes with respect to LM ones. It was noticed that the three couples of compounds (**6**)–(**9**), mostly present in red cabbage and characterised by the sinapyl residue, (**4**)–(**7**), characterised by the presence of a *p*-coumaryl residue, and (**5**)–(**8**), characterised by the presence of a feruloyl residue, were always both present in the same sample with a high abundance, suggesting a possible reciprocal metabolic relationship between these compounds.

3.4. Influence of thermal treatments

The different violet cauliflower samples were further used to study the effect of thermal treatment on the determination of total and individual anthocyanin content. In addition to the values for the raw samples, Table 1 also lists the results of the processed samples.

For LM02 the total anthocyanin content, after microwave treatment (MW), remains almost unchanged (95.4% of total content in the raw sample). In contrast to this, the total content after the cooking process (COOKED) was found to be only 19.8% of that of raw sample (Table 1a).

The recovery values obtained after blanching of the cauliflower landrace, were found to be different for the three processed samples (Table 1b). Even if a lower anthocyanin content was found in cv 98 and cv 141 than in sample LM03, a higher resistance to heat treatment was observed for the two first samples. After blanching, total anthocyanin was found to be 35.4% and 23.8% of that of the raw extract for cv98 and cv141, respectively. The value of LM03 was found to be lower than that of the other samples (10%). This observation could probably be linked to the short shelf life of DOFATA selected landrace genotypes, immediately harvested and delivered for analysis, while LM03 samples were purchased in local markets. The shelf life of the LM03 samples was surely unknown and longer than selected genotypes, resulting in lower anthocyanin stability after thermal treatments, as already shown in previous work (Genna, Lo Scalzo, Branca, Argento, & Maestrelli, 2006).

The effect of various processings on the individual anthocyanin compounds of cauliflower was studied in detail. Interestingly, in MW and COOKED cauliflower, the percent values for the individual anthocyanin compounds remained practically the same as that found in the raw sample, with the exception of compound (**7**) (Table 1a). The values for compound (**7**) were found to be 53% (MW) and 42% (COOKED) with respect to 35.7% (RAW), suggesting a better thermal stability of this compound. Another significant change was related to the presence of compound (**3 bis**), with a simultaneous decrease of compound (**3**), leading to the hypothesis of a possible interconversion of (**3**) into (**3 bis**) after both thermal treatments.

For the blanched samples, one of the most abundant compounds found in violet cauliflower (**7**), showed a decrease in LM03, an increase in cv 98 and no variation in cv 141 (Table 1b). Compound (**8**), that was found to be characteristic of cv 98, showed the most important decrease in abundance in both landrace accessions, whereas it remained stable in LM03. The blanching process did not induce much significant change in either compound (**4**) or (**5**) in all assayed varieties. These compounds could result from the hydrolysis of compounds (**7**) and (**8**). The increase of abundance of (**6**) or (**6 bis**) and decrease of (**7**) or (**8**) suggest a possible hydrolysis of the *p*-coumaryl residue in (**7**), and of the feruloyl residue in (**8**), yielding compounds (**6**) and (**6 bis**) in both cases. In cv 98 a different situation was observed: it showed a different individual composition of the raw sample, with the prevalence of compound (**8**). After blanching, an increase of *p*-coumaryl (**7**) and a decrease of feruloyl derivative (**8**) were observed, suggesting a demethoxylation of compound (**8**).

The effect of processing on the individual anthocyanin composition of cauliflower samples is complex as it involves the presence of both anthocyanin isomers and a possible reciprocal interconversion of two compounds. As an example, similar trends were shown for isomer (**3 bis**) after MW and cooking treatments. Its percentage increased in blanched samples compared to raw ones, the highest increase being observed in cv 141. This fact further reinforces the hypothesis on the possible interconversion of (**3**) into (**3 bis**) after blanching. Compound (**2**), which was absent in raw cauliflower, remained undetected after sample treatment. This result suggests that thermal treatments did not induce the hydrolysis of three-glycosylated anthocyanins, yielding compound (**2**), which was a two-glycosylated derivative.

4. Conclusions

An advanced, sensitive, and simultaneous detection method, which affords discrimination of anthocyanins of interest in violet cauliflower before and after heat treatment by HPLC–MS/MS, is reported. Ten main anthocyanin forms were selected and screened simultaneously in the MRM mode to obtain the overall profile and relative abundance of individual anthocyanins in raw and processed violet cauliflowers.

The present study suggests that violet cauliflower anthocyanin composition is different from that of red cabbage taken as a reference. Violet cauliflower is characterised by a lesser amount of total anthocyanins, with absence of cyanidin-3,5-diglucoside and presence of an isomer of cyanidin-3-sophoroside-5-glucoside, the characteristic anthocyanin compound of red cabbage. Predominant anthocyanin in red cabbage was the synapyl diester of cyanidin-3-sophoroside-5-glucoside (**9**) while, in violet cauliflower, there were the corresponding *p*-coumaryl (**7**) or feruloyl (**8**) derivatives. Different assayed landraces of violet cauliflower suggested different compositions in single anthocyanin contents.

Results also showed a different stability of the individual forms after thermal treatments (blanching, microwave-heating and cooking). All samples subjected to thermal treatments showed an isomer production from cyanidin-3-sophoroside-5-glucoside. Additional studies will be needed to elucidate the structure isomer (**3 bis**), since (**3 bis**) seems to play an important role in the evolution of the pigment composition of violet cauliflower after thermal treatments. The good thermal stability of this compound could be useful for a possible application to food processing. The colour retention by natural pigments after thermal treatment is an important quality parameter.

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