Profiling and Simultaneous Quantitative Determination of Anthocyanins in Wild Myrtus communis L. Berries from Different Geographical Areas in Sardinia and their Comparative Evaluation

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ABSTRACT:
Introduction – Myrtus communis L. (Myrtaceae) is a self-seeded shrub, widespread in Sardinia, with anti-inflammatory, antiseptic, antimicrobial, hypoglycemic and balsamic properties. Its berries, employed for the production of sweet myrtle liqueur, are characterised by a high content of bioactive polyphenols, mainly anthocyanins. Anthocyanin composition is quite specific for vegetables/fruits and can be used as a fingerprint to determine the authenticity, geographical origin and quality of raw materials, products and extracts.
Objective – To rapidly analyse and determine anthocyanins in 17 samples of Myrtus communis berries by developing a platform based on the integration of UHPLC–MS/MS quantitative data and multivariate analysis with the aim of extracting the most infor-mation possible from the data.
Methodology – UHPLC-ESI-MS/MS methods, working in positive ion mode, were performed for the detection and determination of target compounds in multiple reaction monitoring (MRM) mode. Optimal chromatographic conditions were achieved using an XSelect HSS T3 column and a gradient elution with 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Principal com-
ponent analysis (PCA) was applied to the quantitative data to correlate and discriminate 17 geographical collections of Myrtus communis.
Results – The developed quantitative method was reliable, sensitive and specific and was successfully applied to the quantifica-tion of 17 anthocyanins. Peonidin-3-O-glucoside was the most abundant compound in all the extracts investigated. Conclusion – The developed methodology allows the identification of quali-quantitative differences among M. communis samples
and thus defines the quality and value of this raw material for marketed products. Moreover, the reported data have an imme-diate commercial value due to the current interest in developing antioxidant nutraceuticals from Mediterranean plants, including Sardinian Myrtus communis.

Keywords: Myrtus communis; anthocyanins; UHPLC-QTRAP-MS/MS; PCA

Introduction

Myrtus communis L. (Myrtaceae), the common myrtle, is an ever-green shrub that is widely distributed in the Mediterranean area, where it self-propagates. In Italy, myrtle is typically found in coastal regions and is one of the most characteristic species of Sardinia (Kirtikar and Basu, 1988; Nassar et al., 2010; Asif et al., 2011; Melito et al., 2014).

Previous authors have described numerous varieties and forms for the species Myrtus communis (Picci and Atzei, 1996) and, in fact, two subspecies are reported: M. communis ssp. Communis and M. communis ssp. Tarentina (L.) Nyman (Fiori, 1925). Our sampling took into account only the typical Sardinian subspecies M. communis ssp. Communis.

Different parts of the plant are used for medicinal, food, spice and cosmetic purposes. Myrtle extracts obtained from leaves and berries are known to possess anti-inflammatory, antiseptic, antimicrobial, antihaemorrhagic, hypoglycemic, disinfectant and balsamic properties (Alipour et al., 2014). The leaves contain flavo-noids (i.e. quercetin, catechin and myricetin derivatives), and

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essential oils (Yoshimura et al., 2008; Aidi Wannes et al., 2010; Ghasemi et al., 2011; Petretto et al., 2016). In Sardinia, the leaves and berries of this plant are employed for the production of a sweet myrtle liqueur, which is also reported to exhibit strong antioxidant activity (Tuberoso et al., 2010). The berries are characterised by a high content of polyphenols, primarily antho-cyanins, which are strongly associated with the red-purple colour of the liqueur (Aidi Wannes et al., 2010; Barboni et al., 2010; Sumbul et al., 2012).

The production process of the "Myrtle Liqueur" consists of an hydroalcoholic infusion (> 40°) of Myrtus communis berries for not less than 15 days (AA.VV., 1998). The production of myrtle li-queur is currently more than three million bottles per year, and this liqueur is becoming one of the most typical Sardinian products exported abroad. Thus, because of increasing consumer demand, coupled with the expectation of quality products, a rigorous set of "Product Specifications of Production" were established by the Producers' Association of Traditional Myrtle from Sardinia. These product specifications allow the certification of the quality, authen-ticity and geographical origin of the myrtle liqueur (Franco et al., 2002; Montoro et al., 2006a, 2006b; Barboni et al., 2010).

The anthocyanin composition of different vegetables and fruits is quite specific and can be used as a fingerprint to determine the authenticity, origin, quality and safety of raw materials, extracts, and products. In particular, increasing interest has been focused on establishing a clear geographical origin for raw materials and food products (Montoro et al., 2006a; Stoj et al., 2006; Krüger et al., 2013).

The currently employed methods of establishing composition are based on spectroscopic, biological, separation and electro-chemical procedures, but recently, much interest has been focused on the application of sophisticated analytical techniques, such as liquid chromatography mass spectrometry (LC–MS) analy-ses, in conjunction with statistical data analysis methods (López-Díez et al., 2003; Pereira et al., 2005; Luykx and van Ruth, 2008; Piras et al., 2009).

High-performance liquid chromatography coupled to mass spectrometry (HPLC–MS) is the most frequently used technique for analysing anthocyanins. HPLC allows for the powerful and rapid separation of anthocyanins, and MS provides immediate and highly sensitive detection (Fulcrand et al., 1994; Revilla et al., 1999; da Costa et al., 2000; Flamini, 2003). HPLC–MS has become an important tool for anthocyanin characterisation, as this tech-nique provides both a mass spectrum of intact molecular and frag-ment ions and retention times to elucidate structural information without requiring the isolation of compounds (Lopes-da-Silva et al., 2002).

In this context, the aim of the present work was to develop an integrated reliable method that employs ultra-high performance liquid chromatography-triple quadrupole linear ion trap tandem mass spectrometry (UHPLC-QTRAP-MS/MS) techniques and princi-pal component analysis (PCA) for a comprehensive study on an-thocyanins occurring in a large number of Myrtus communis berries collected from different areas of Sardinia.

UHPLC coupled with hybrid linear ion trap/triple quadrupole detection provides efficient separation and high selectivity for both qualitative and quantitative analyses. The method was also validated according to the guidelines of the European Medicines Agency (EMEA) relating to the validation of analytical methods (EMEA,). Furthermore, the data were analysed using PCA to explore and visualise the correlations and discriminations be-tween 17 collections of Myrtus communis representing different geographical areas of Sardinia and to identify individual markers contributing to the classification. Chemometric analysis has been shown to be able to classify samples based on different tech-niques (NIR, NMR, GC, etc.), and to be an effective method for de-termining food authenticity and the geographical origin of botanical matrices (Bondia-Pons et al., 2014; Petretto et al. 2015; Tomita et al., 2015; Wolfender et al., 2015). Much less literature data are available on the use of LC–MS/MS targeted analysis data in combination with PCA to give a rapid screening system.

Experimental

Reagents and chemicals

Solvents used for extraction, LC–MS grade methanol, acetonitrile and formic acid were from Sigma-Aldrich Chemical Company (St Louis, MO, USA). HPLC grade water (18 mΩ) was prepared by using a Millipore (Bed-ford, MA, USA) Milli-Q purification system. Standards of cyanidin, delphinidin, malvidin, cyanidin-3-O-glucoside, cyanidin-3-O-arabinoside, cyanidin-3,5-di-O-glucoside, delphinidin-3-O-glucoside, malvidin-3-O-glu-coside, pelargonidin-3-O-glucoside chloride were purchased from Extrasynthese (Lyon, France).

Sampling sites

Myrtus communis berries analysed in this work were collected at the follow-ing 17 sites in Sardinia: San Gavino Monreale (gm), Montevecchio (mv), Morgongiori (mg), Paulilatino (p), Abbasanta (ab), Mara (m), Alghero (al), Stintino (st), Porto Torres (pt), Sassari (ss), Trinità d'Agultu (t), Santa Teresa di Gallura (tg), La Maddalena (lm), Olbia (o), Sant'Antonio di Gallura (ag), Luras (l), Bortigiadas (b).

The berries were collected in February 2013 from wild shrubs and imme-diately extracted. Plants were identified by Dr. M. Chessa; voucher speci-mens were dried and deposited at the Erbarium SASSA of Sassari University at number 514.

Sampling extraction and preparation

A modified extraction procedure was developed on the basis of that re-ported by Montoro et al. (2006a) based on the traditional recipe for the preparation of the liqueur. In brief, fresh berries were extracted by using ethanol/water (70:30 v/v; sample to solvent ratio 13:25 w/v) under ultra-sound agitation for 1 h and then stored in the dark overnight. Samples were filtered and the solvent was completely removed using a rotary evaporator under vacuum at 30 °C in the dark. The dried samples were dissolved in ul-trapure water with the same volume of extraction and filtered through 0.20-µm syringe PVDF filters (Whatmann International Ltd., Maidstone, UK).

For qualitative analysis, each extract was diluted 1:100 with water and a 20 µL aliquot injected into the analytical system. For quantitative determi-nation, each extract was diluted 1:100 with water and a 5 µL aliquot injected into the analytical system; determinations were replicated three times each.

ESI-MS and ESI-MS/MS analyses

Full scan electrospray ionisation mass spectrometry (ESI-MS), MS/MS and MS^3 analyses of standards and samples were performed on an AB Sciex API4000 Q-Trap (Foster City, CA, USA) spectrometer. The analytical parame-ters were optimised by infusing each standard solution (1 µg/mL in meth-anol 50%) into the source at a flow rate of 10 µL/min. Data were acquired in the positive ion MS, MS/MS and MS^3 modes. Direct infusions of each indi-vidual standard compound were used to optimise the multiple reaction monitoring (MRM) conditions, which was done automatically by the Analyst 1.6.2 software, and then manually checked for selection of the quantifier and qualifier ions.

UHPLC-QTRAP-MS/MS conditions

Qualitative on-line HPLC-ESI-MS/MS analyses of extracts were performed using a UHPLC system interfaced to an AB Sciex (Foster City, CA, USA) API4000 Q-Trap instrument in ion trap mode. LC analyses were conducted using a system equipped with a Flexar UHPLC AS system (Perkin–Elmer, Waltham, MA, USA) consisting of degasser, Flexar FX-10 pump, autosampler and PE 200 column oven. Samples were injected (5 µL) into an XSelect HSS T3 column (Waters, Milford, MA, USA) (100 mm × 2.1 mm i.d., 2.5 µm d). Mobile phase A was water containing 0.1% formic acid while mobile phase B was acetonitrile containing 0.1% formic acid. Elution was carried out at 40 °C according to the following gradient: 0–0.5 min, isocratic 5% B; 0.5– 19 min, linear gradient 5–95% B. The flow rate was 300 µL/min and was injected from the chromatograph directly into the ESI source. Qualitative analysis of the compounds was performed using IDA (information depen-dent acquisition). The IDA method created included an IDA criteria (specify the charge state, mass range), enhanced MS scan, enhanced resolution, en-hanced product ion scan or MS/MS scan. Enhanced mass spectrometry (EMS) was conducted with mass range from m/z 200.0 to 800.0 with a scan rate of 10000 amu/s. With regard to the criteria for triggering product ion scans, the three most intense precursor ion exceeding 5000 cps of each cy-cle were triggered for enhanced product ion (EPI) scan. The source temper-ature was held at 500 °C, and MS parameters were those optimised for the ESI-MS and ESI-MS/MS analyses with ion spray voltage at 5500 V. MS data were acquired using the software provided by the manufacturer (Analyst software 1.6.2), and extracted ion fragmentograms (XIC) were elaborated in order to identify anthocyanins from their protonated molecular ions and retention time. In the product ion spectra obtained, the predominant fragments relative to anthocyanin compounds were chosen to develop the MRM method for quantification.

Quantitative on-line HPLC-ESI-MS/MS analyses were performed using the same LC-ESI-MS/MS equipment but with the mass spectrometer having the triple quadrupole analyser in MRM mode. Elution was carried out at 41 °C according to the following flow and solvent gradient: 0–4 min, isocratic 0% solvent B and the flow changes from 300 µL to 350 µL; 4–6 min, linear gradient 0–12% B and the flow achieves 400 µL/min; 6–12 min, linear gradient 12–20% B and flow constant at 400 µL/min; 16–17 min, linear gradient 20–100% B and flow retrieves to 300 µL/min. The API 4000 ES source was operated in positive ion mode and was tuned by infusing solutions of standards (1 µg/µL in methanol 50%) into the source at a flow rate of 10 µL/min. Retention times and the optimised parameters such as selected transitions, declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP), for each analyte were listed in Table 1. The voltage applied was 5500 V. Data acquisition and processing were performed using Analyst software 1.6.2.

Principal component analysis (PCA)

An m × n matrix (where m is the number of samples, and n is the number of variables) was used in PCA. Thus, quantitative data of each chemical marker were used to define a data set with 17 observation and 51 variables. The resulting matrix data was analysed by PCA, performed on the data scaled by unit variance with the Factor MineR package of R 2.15.2 software. The results of the analysis are presented in term of score and loading plots.

Calibration and quantification of anthocyanins

In order to prepare the calibration plot, a sample (1 mg) of each standard was weighed accurately into a 1 mL volumetric flask, dissolved in ethanol 70% (v/v) and the volume made up to the mark with ethanol. The resulting stock solution was diluted with water in order to obtain reference solutions containing 0.1, 1, 5, 10 and 20 µg/mL of external standards.

The calibration curves, for each compound, were made by linear regression by plotting the peak area of external standard against their known concentrations. The result represents the average of curves performed by three injection of each concentration. All quantitative data were elaborated with the aid of Analyst software (AB Sciex, Framingham, MA, USA).

Method validation

LC–MS/MS method was validated according to the EMEA guidelines relat-ing to the validation of analytical methods (EMEA,).

Calibration curves were obtained by plotting the area of external stan-dards (ES) against the known concentration of each compound, each concentration of standard solutions was analysed in triplicate. A good linearity with correlation coefficients (r) in the range from 0.9927 to 0.9982 was achieved for all analytes.

The limit of detection (LOD) and the limit of quantification (LOQ) for each target standard compound were determined, under the optimised condi-tions, by the serial dilution of a standard solution until the signal-to-noise

Table 1. LC–MS/MS conditions for quantitation of compounds 1–17 by positive ion MRM

Compound	t_R(min)	Parent ion	Fragment ion	DP(V)	EP(eV)	CE(eV)	CXP(eV)
1	9.39	627	303	90	14	46	32
2	9.65	611	287	177	14	55	19
3	10.01	465	303	181	14	30	21
4	10.52	449	287	192	12	27	20
5	10.86	435	303	192	10	25	21
6	10.95	479	317	172	13	27	21
7	11.02	419	287	194	11	24	19
8	11.32	433	271	163	10	28	24
9	11.39	449	317	124	10	25	22
10	11.73	463	301	104	11	29	20
11	11.86	419	287	194	11	24	19
12	12.01	493	331	94	12	29	21
13	12.31	449	317	124	10	25	22
14	12.46	463	331	94	11	24	22
15	13.69	463	331	94	11	24	22
16	15.91	611	303	165	14	45	21
17	17.72	639	331	121	6	29	24

Note: DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, cell exit potential.

(S/N) ratios were 3:1 and 10:1, respectively. The LOD for each analyte varied from 0.001 to 0.02 µg/mL and LOQ from 0.004 to 0.09 µg/mL, indicating that the developed method exhibited good sensitivity.

Precision of the developed method was evaluated by determination of the intraday and interday variations. Three aliquots of each same sample were analysed within the same day, and another three aliquots of the same sample were analysed over three consecutive days, one for each day. Per-centage relative standard deviation (RSD) was used to express precision of the method.

Recovery experiments were performed with the optimised parameters to evaluate the extraction efficiency and the analytical method. Standard solutions at three different concentration levels (high, middle and low) were added in a known amount of sample and analysed by LC-MS/MS and then triplicate experiments were performed at each level. Within the same day, the recovery (%) ranged from 94.6% to 106.7%, demonstrating good recovery and precision.

Results and discussion

LC-MS/MS conditions optimisation

Different chromatographic conditions such as column, mobile phase and gradient program were tested in order to achieve opti-mal LC separation of the analytes in a short analysis time. Among different mobile phases examined, 0.1% formic acid in water and 0.1% formic acid in acetonitrile were found to produce satisfactory separation in a minimum analysis time with excellent resolution, peak shape, and mass spectrometric ionisation intensity of the analytes. Regarding analytical columns, the best resolution of anthocyanins was achieved using a Waters XSelect HSS T3 column XP (2.1 mm × 100 mm, 2.5 µm).

The MS conditions were optimised using reference standards of six anthocyanins to achieve optimal MS sensitivity for detection and to obtain abundant fragment ions for structural elucidation. Due to the presence of a positive charge in the chemical structure of anthocyanins, good signal sensitivity was obtained in positive ion mode.

UHPLC-ESI-ion trap/MS/MS detection

For qualitative analysis, an IDA method with EMS survey scans and enhanced resolution (ER) and EPI scans was developed, allowing us to identify anthocyanins using complementary information from chromatographic behaviour and mass fragmentation. In ad-dition, we compared our MS/MS values and retention times with those observed for the analytical standards in LC-ESI-MS/MS anal-yses, when available, and/or with those reported in the literature.

Seventeen anthocyanins, which belonged to the classes of cyanidins, delphinidins, malvidins, petunidins, pelargonidins and peonidins, were identified in the hydroalcoholic extracts of Myrtus communis berries collected from the different areas of Sardinia. The MS/MS mode is a useful tool that provides information about the aglycone and the corresponding sugar unit, based on the ob-served m/z characteristic fragmentation values (303 for delphinidin; 287 for cyanidin; 317 for petunidin; 301 for peonidin; 331 for malvidin and 271 for pelargonidin) (Acevedo et al., 2012). Table 2 summarises the individual anthocyanin data, including the molecular ions and MS/MS fragments obtained using the IDA method.

Delphinidin-3-O-arabinoside (m/z 435), petunidin-3-O-glucoside (m/z 479), petunidin-3-O-arabinoside (m/z 449), peonidin-3-O-glucoside (m/z 463) and malvidin-3-O-arabinoside (m/z 463) were frequently identified in myrtle berries, and the presence of these compounds was established from the existence of a retention time and fragmentation pattern consistent with those reported in the literature (Table 2). In addition, the XICs of m/z 419 (cyanidin-3-O-arabinoside), 449 (petunidin-3-O-arabinoside) and 463 (malvidin-3-O-arabinoside) exhibited two peaks, suggesting the presence of other pentose derivatives (Su, 2012), which have not been reported previously in myrtle berry extracts.

Cyanidin-3,5-di-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, cyanidin-3-O-arabinoside, pelargonidin-3-O-glucoside and malvidin-3-O-glucoside were unequivocally identified by comparing spectral data with standard reference compounds. All of these anthocyanins were frequently identified in berries of Myrtus

Table 2. Characterisation of anthocyanins constituents in Myrtus communis berries using HPLC-ESI-MS/MS in positive ion mode

Compound	MW	MS (m/z)	MS/MS	Tentative identification
1	626	627 $[M]^+$	465,303,256.9,229.2	Delphinidin diglucoside
2	610	611 $[M]^+$	287	Cyanidin-3,5-di-O-glucoside[a]
3	464	465 $[M]^+$	303,285,257,229,201,187,173,153	Delphinidin-3-O-glucoside[a]
4	448	449 $[M]^+$	287,241,231,213,175,157,149	Cyanidin-3-O-glucoside[a]
5	434	435 $[M]^+$	303,285,257,229,201,173,153	Delphinidin-3-O-arabinoside
6	478	479 $[M]^+$	317,302,274,245,217,203	Petunidin-3-O-glucoside
7	418	419 $[M]^+$	287,241,213,189,157,137	Cyanidin pentose
8	432	433 $[M]^+$	271	Pelargonidin-3-O-glucoside[a]
9	448	449 $[M]^+$	317,302,287,274,245,217,203	Petunidin pentose
10	462	463 $[M]^+$	301,286,268,258,229,213,201,187	Peonidin-3-O-glucoside
11	418	419 $[M]^+$	287,241,213,189,157,137	Cyanidin-3-O-arabinoside[a]
12	492	493 $[M]^+$	331,315,287,270,242,213,201,179,150	Malvidin-3-O-glucoside[a]
13	448	449 $[M]^+$	317,302,287,274,245,217,203	Petunidin-3-O-arabinoside
14	462	463 $[M]^+$	331,315,287,269,242,213,201,179	Malvidin pentose
15	462	463 $[M]^+$	331,315,287,269,242,213,201,179	Malvidin-3-O-arabinoside
16	610	611 $[M]^+$	464.6,319,303,284.5,228.7,153	Delphinidin coumaroyl glucoside or delphinidin rutinoside
17	638	639 $[M]^+$	331,315,298.9,287,270.1,242	Malvidin 3-O-p-coumaroylglucoside

[a]Identified using corresponding authentic standards.

communis (Franco et al., 2002; Montoro et al., 2006a; Tuberoso et al., 2010; Piras et al., 2009), except for pelargonidin-3-O-glucoside, which, to our knowledge, has not been reported previously in M. communis. Of particular interest is the presence of peaks that have mass spectra ascribable to delphinidin diglucoside (m/z 627), malvidin coumaroyl glucoside (m/z 639) and delphinidin coumaroyl glucoside/ delphinidin rutinoside (m/z 611), respectively.

The pseudo-molecular ion at m/z 627 exhibited an MS/MS fragmentation pattern with two major ion peaks that correspond to m/z 465 (loss of 162 Da relative to the hexose residue) and m/z 303 (subsequent loss of a second hexose residue); other minor product ions were 257 and 229, suggesting a delphinidin deriva-tive. Based on this fragmentation pattern, we hypothesised that this compound might correspond to delphinidin diglucoside.

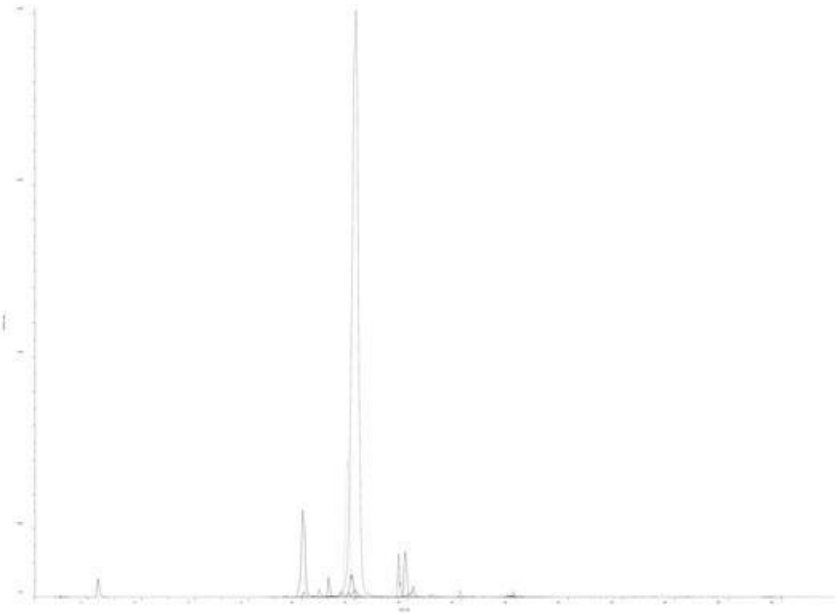

Figure 1. UHPLC chromatographic separation of a myrtle extract.

Table 3. Quantitative results for anthocyanins 1–17 detected in extracts of Myrtus communis berries [µg/mL ± standard deviation (SD) of extract]

	1[a]	2	3	4	5[a]	6[b]	7[c]	8	9[b]
gm	4.02 ± 0.38	0.27 ± 0.02	199.67 ± 11.06	110.37 ± 12.37	0.04 ± 0.01	0.46 ± 0.04	4.25 ± 0.37	0.08 ± 0.01	0.26 ± 0.04
mv	1.34 ± 0.05	0.11 ± 0.06	22.07 ± 1.91	30.13 ± 10.68	ND	ND	0.72 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
mg	0.40 ± 0.18	0.04 ± 0.02	5.59 ± 0.25	12.67 ± 3.15	ND	ND	0.65 ± 0.08	0.01 ± 0.01	0.02 ± 0.01
p	5.24 ± 0.15	0.33 ± 0.05	179.33 ± 6.66	103.60 ± 6.77	ND	0.28 ± 0.03	2.24 ± 0.11	0.06 ± 0.01	0.13 ± 0.01
ab	1.24 ± 0.16	0.25 ± 0.16	107.33 ± 7.57	119.00 ± 15.72	ND	0.14 ± 0.01	3.61 ± 0.29	0.07 ± 0.01	0.15 ± 0.01
m	3.87 ± 0.35	0.21 ± 0.04	174.00 ± 8.19	117.07 ± 27.75	0.02 ± 0.01	0.33 ± 0.03	2.18 ± 0.10	0.04 ± 0.01	0.22 ± 0.02
a	0.79 ± 0.09	0.17 ± 0.02	27.47 ± 2.06	66.77 ± 7.26	ND	0.19 ± 0.03	3.18 ± 0.08	0.05 ± 0.02	0.19 ± 0.02
st	1.17 ± 0.25	0.10 ± 0.01	88.13 ± 6.55	87.73 ± 13.23	0.02 ± 0.01	0.21 ± 0.03	1.56 ± 0.05	0.04 ± 0.01	0.08 ± 0.02
pt	2.36 ± 0.17	ND	103.17 ± 6.93	72.9 ± 8.26	0.02 ± 0.01	0.19 ± 0.03	0.36 ± 0.56	0.05 ± 0.01	0.06 ± 0.01
ss	2.21 ± 0.14	0.14 ± 0.07	146 ± 7.81	80.83 ± 16.50	ND	0.23 ± 0.01	1.8 ± 0.14	0.04 ± 0.01	0.19 ± 0.02
t	0.61 ± 0.05	0.08 ± 0.02	10.6 ± 0.10	16.77 ± 6.01	ND	ND	0.31 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
tg	3.6 ± 0.24	ND	141.33 ± 3.21	118.4 ± 23.49	0.07 ± 0.01	0.49 ± 0.05	0.69 ± 1.15	0.06 ± 0.01	0.14 ± 0.02
lm	1.86 ± 0.09	0.13 ± 0.04	134 ± 11.53	71.17 ± 10.36	0.02 ± 0.01	0.17 ± 0.01	1.48 ± 0.04	0.05 ± 0.01	0.08 ± 0.02
o	3.84 ± 0.42	0.17 ± 0.09	191 ± 3.46	118.37 ± 31.61	0.04 ± 0.02	0.32 ± 0.02	1.98 ± 0.24	0.06 ± 0.01	0.18 ± 0.01
ag	2.21 ± 0.30	0.15 ± 0.01	80.1 ± 7.79	47.4 ± 12.25	ND	0.1 ± 0.02	0.87 ± 0.04	0.02 ± 0.01	0.07 ± 0.02
l	1.53 ± 0.11	0.17 ± 0.03	94 ± 7.55	71.13 ± 3.18	ND	0.16 ± 0.01	0.89 ± 0.06	0.03 ± 0.01	0.09 ± 0.01
b	4.96 ± 0.09	0.24 ± 0.03	175.33 ± 10.69	144.67 ± 30.66	0.04 ± 0.01	0.31 ± 0.02	3.31 ± 0.19	0.06 ± 0.01	0.21 ± 0.02

Note: Each data is the mean of three replicates (mean ± SD). ND, not detected (below LOD).
[a]Quantified as equivalent of delphinidin-3-O-glucoside (3).
[b]Quantified as equivalent of malvidin-3-O-glucoside (12).
[c]Quantified as equivalent of cyanidin-3-O-arabinoside (11).
[d]Quantified as equivalent of cyanidin-3-O-glucoside (4).

In the LC-ESI-MS/MS spectrum, the parent ion at m/z value of 639 exhibited a major fragment ion at m/z 331 $[M-308]^+$, which could correspond to a neutral loss of a rutinoside or coumaroylglucose moiety, and other minor ion peaks at m/z 315, 287, 270.1 and 242, which are characteristic fragmentation values of malvidin derivatives. Due to the long retention time of this compound it is most likely to be coumaroylglucoside. Furthermore, a previous work by Franco et al. (2002), reported the presence of malvidin-3-monoglucoside p-coumarate in commercial red myrtle liqueur. In addition, the fragmentation pattern is consistent with previously reported data (Downey and Rochfort, 2008; Acevedo et al., 2012), suggesting that the identity of this compound is malvidin 3-O-p-coumaroylglucoside.

The pseudo-molecular ion at m/z 611 yielded MS/MS ions at m/z 465 and 303 which have 146 and 162 unit difference, respectively. The loss of 162 amu could correspond to a glucose or caffeoyl unit, while the loss of 146 could correspond to a coumaroyl or rhamnosyl moiety. Other minor peaks were observed at m/z 285, 229 and 153, suggesting a delphinidin derivative. In this case, the long retention time, close to that of yet reported malvidin-3-O-p-coumaroylglucoside, could suggest that identity of this compound is delphinidin coumaroyl glucoside. However, comparison of experimental fragmentation pat-tern with those reported in the literature suggests the identity of this compound could be delphinidin rutinoside (Wu and Prior, 2005; Touriño et al., 2008; Arapitsas et al., 2012).

UHPLC-ESI/triple quadrupole MS/MS with multiple reaction monitoring (MRM)

In the second stage of this study, to obtain accurate data concerning the variations in anthocyanin content among myrtle berry extracts, a quantitative UHPLC-ESI-MS/MS (MRM) analysis was performed. Based on the previously reported fragmentation results, the specific transi-tions from the molecular ions to the fragment ions, declustering po-tential, entrance potential, collision energy and cell exit potential for each monitored compound were selected (Tables 1, 2). The chro-matographic profile contained all of the peaks corresponding to the compounds under investigation, with intensities adequate for quantitative purposes. The method based on the characteristic frag-mentation reactions of anthocyanins was highly specific, with no interfering peaks at the retention times of the marker compounds in the MRM chromatograms (Figure 1).

Quantitative analysis

The UHPLC-ESI-MS/MS (MRM) method developed was applied to the simultaneous determination of 17 anthocyanins in myrtle berries from 17 different sources with high sensitivity and selectiv-ity. The quantitative analysis was performed using an external standards method. Anthocyanins are eluted in accordance with their polarity: the most polar delphinidin derivatives elute first, followed by cyanidin, petunidin, pelargonidin, peonidin and malvidin derivatives.

The mean contents of compounds 1–17 are shown in Table 3. In general, the results demonstrate that glucoside derivatives of anthocyanins are the most abundant compounds in all of the sam-ples. In particular, peonidin-3-O-glucoside is the most important compound in all the extracts, followed by malvidin-3-O-glucoside, delphinidin-3-O-glucoside and cyanidin-3-O-glucoside.

Differences were observed between the samples in both total and individual anthocyanin content. The samples collected from San Gavino Monreale and Alghero are the richest samples in terms of total anthocyanins (2292 µg/mL and 2283 µg/mL, respectively), followed by the samples from Paulilatino (1869 µg/mL), Bortigiadas (1818 µg/mL), Mara (1801 µg/mL) and Abbasanta (1734 µg/mL); in contrast, the extracts from Trinità d'Agultu and Morgongiori exhibited the lowest anthocyanin contents (474 µg/mL and 412 µg/mL, respectively).

Discrimination of Myrtus communis berries from different sources by PCA

Quantitative data were analysed using PCA to explore and visual-ise correlation and discrimination among 17 collections of Myrtus communis representing different geographical areas of Sardinia

Table 3. Quantitative results for anthocyanins 1–17 detected in extracts of Myrtus communis berries [µg/mL ± standard deviation (SD) of extract]

	10[d]	11	12	13[b]	14[b]	15[b]	16[a]	17[d]
gm	1672.89± 30.39	11.73 ± 0.36	278± 6	1.78 ± 0.15	0.75 ± 0.03	7.12 ± 0.25	0.11 ± 0.01	0.07 ± 0.01
mv	427.86± 13.24	1.74 ± 0.06	82.17± 1.62	0.24 ± 0.02	0.22 ± 0.03	2.02 ± 0.05	ND	ND
mg	338.31± 18.69	1.38 ± 0.03	51.13± 3.91	0.11 ± 0.01	0.20 ± 0.01	1.32 ± 0.09	ND	0.02 ± 0.01
p	1297.26± 80.38	10.75 ± 0.45	260.00± 2.65	1.78 ± 0.02	0.39 ± 0.01	8.46 ± 0.34	0.07 ± 0.02	0.06 ± 0.02
ab	1296.02± 82.65	8.85 ± 0.26	191.67± 9.29	1.14 ± 0.04	0.35 ± 0.03	4.34 ± 0.10	ND	0.02 ± 0.01
m	1198.38± 42.72	7.29 ± 0.17	285.33± 9.87	1.86 ± 0.15	0.95 ± 0.02	9.23 ± 0.25	0.03 ± 0.01	0.04 ± 0.01
a	1891.17± 92.39	6.25 ± 0.34	271.67± 7.57	0.92 ± 0.04	1.71 ± 0.06	12.69 ± 0.29	ND	0.03 ± 0.01
st	775.5± 48.09	6.54 ± 0.40	119.67± 8.02	0.81 ± 0.13	0.2 ± 0.03	3.47 ± 0.23	0.01 ± 0.01	0.02 ± 0.01
pt	722.64± 31.07	5.48 ± 0.19	133.67± 6.43	0.8 ± 0.05	0.19 ± 0.01	4.45 ± 0.11	0.15 ± 0.05	0.1 ± 0.01
ss	1027.99± 77.64	7.15 ± 0.28	235± 8.54	1.7 ± 0.15	0.41 ± 0.01	5.99 ± 0.25	ND	0.02 ± 0.01
t	359.45± 7.06	1.16 ± 0.05	82.27± 3.20	0.17 ± 0.01	0.15 ± 0.01	2.27 ± 0.09	ND	0.03 ± 0.01
tg	1024.88± 92.92	7.06 ± 0.67	217± 5.57	1.44 ± 0.08	0.37 ± 0.05	6.04 ± 0.22	0.04 ± 0.02	0.02 ± 0.01
lm	629.98± 10.61	7.13 ± 0.46	140.67± 2.31	1.24 ± 0.07	0.25 ± 0.02	4.63 ± 0.07	0.05 ± 0.01	0.03 ± 0.01
o	968.28± 61.40	8.49 ± 0.32	216.67± 6.66	2.04 ± 0.08	0.45 ± 0.01	6.88 ± 0.17	0.03 ± 0.01	0.02 ± 0.01
ag	595.15± 23.30	4.19 ± 0.25	138.33± 3.06	0.96 ± 0.03	0.32 ± 0.03	4.81 ± 0.08	0.02 ± 0.01	ND
l	667.29± 12.70	5.25 ± 0.16	141.67± 6.11	1.21 ± 0.05	0.2 ± 0.02	4.1 ± 0.30	0.03 ± 0.01	0.03 ± 0.01
b	1249.38± 72.75	9.69 ± 0.57	221± 3.00	1.64 ± 0.01	0.68 ± 0.03	6.65 ± 0.19	0.14 ± 0.01	0.07 ± 0.01

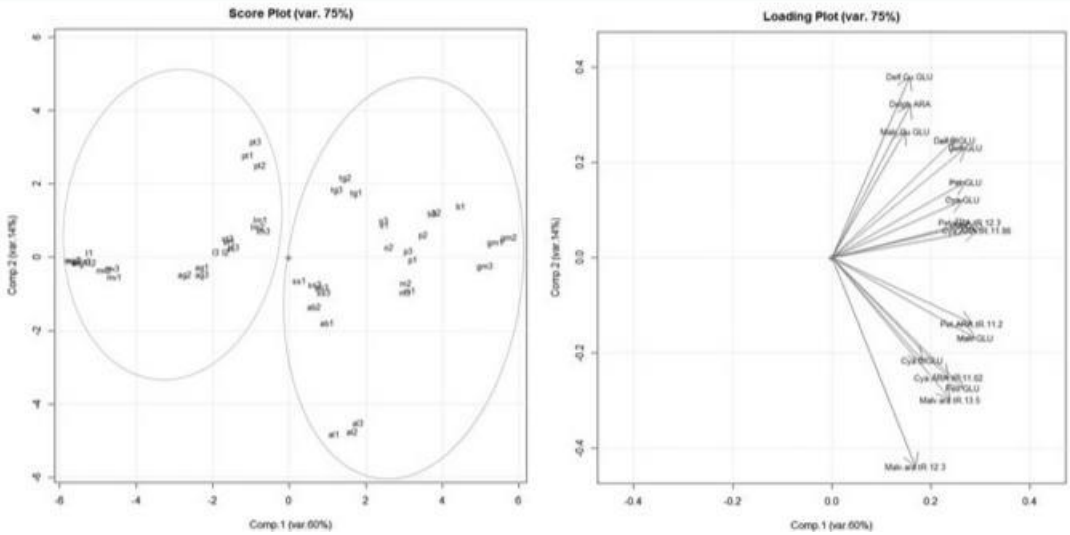

Figure 2. Principal component analysis score plot (a) and principal component analysis loading plot (b).

and to identify individual anthocyanins contributing to the classification.

PCA transforms a number of related variables into a smaller set of uncorrelated variables, which are called principal compo-nents (PCs).

The scores plot of the first PC (PC1) captures 60% of the total variance in the dataset, while the second PC (PC2) captures 14% of the total variance (Figure 2(a)). This plot demonstrates that 17 samples are grouped into two different classes: one class consists of the berries from Montevecchio (mv), Morgongiori (mg), Trinità d'Agultu (t), Sant'Antonio di Gallura (ag), Stintino (st), Luras (l), La Maddalena (lm) and Porto Torres (pt) with both PC1 and PC2 positive scores, and the second class includes the berries from Sassari (ss), Abbasanta (ab), Santa Teresa di Gallura (tg), Olbia (o), Bortigiadas (b) Paulilatino (p), Mara (m), San Gavino Monreale (gm) and Alghero (al) with both PC1 and PC2 negative scores. To evaluate the influence of each variable on the classification of the samples, the loading plot obtained for the same dataset was then studied, and this plot is presented in Figure 2(b). The loading plot shows which anthocyanins contribute most to the differentiation of the samples, and the location of the anthocya-nins in a specific area of the space can be highlighted. Interest-ingly, anthocyanins are localised only in the area corresponding to the myrtle samples collected from Santa Teresa di Gallura (tg), Olbia (o), Bortigiadas (b) Paulilatino (p), Mara (m), San Gavino Monreale (gm) and Alghero (al), Sassari (ss), and Abbasanta (ab). The loading plot indicates that these samples are characterised by higher concentrations of anthocyanins. Quantitative differ-ences in the content of anthocyanin derivatives among samples collected in different geographical areas in Sardinia could be as-cribed not only to geoclimatic factors but also to genetic and/or environmental factors.

Conclusion

In conclusion, in the present study, a complete and specific snap-shot of the anthocyanins that occur in the extracts of myrtle berries collected from different sites in Sardinia was obtained by develop-ing a platform that integrates UHPLC–MS/MS analysis with multi-variate data analysis allowing the main information on a large number of samples to be obtained rapidly. UHPLC-ESI-QTRAP-MS/ MS was a highly informative analysis technique for the identification of individual anthocyanins. A UHPLC-ESI/triple quadrupole-MS/MS method, which was based on an MRM technique, was developed for the quantitative determination of all of the anthocyanins identi-fied in different samples of Myrtus communis berries. The method de-veloped was validated according to ICH and found to be accurate, selective and precise in the applied range of concentrations. The method was specific and sensitive for the studied extracts.

The application of the quantitative method developed here is suitable for the quality control of Myrtus communis authenticity. However, differences in the content of these compounds were found, depending on the harvesting site, and a high concentration of anthocyanins was found in the berries from Alghero.

Furthermore, the quantitative data obtained from the HPLC– MS/MS MRM analyses coupled with the PCA approach proved to be a potentially useful and effective tool for rapidly providing both visual and statistical evaluations of the similarities and differences among berries of Myrtus communis collected from different areas of Sardinia. The data obtained have an immedi-ate commercial value due to the current interest in developing antioxidant nutraceuticals from Mediterranean plants, including Sardinian myrtle.

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