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A rapid validated UHPLC–PDA method for anthocyanins quantification from *Euterpe oleracea* fruits

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ABSTRACT

The aim of this work is to develop the first validated UHPLC–PDA method for major anthocyanins quantification in *Euterpe oleracea* fruits after fast extraction procedures and samples preparation. The separation was performed on HSS C18 column (1.8 μm) using a gradient elution with acetonitrile and 5% formic acid in a total run time of only 17 min. Total error and accuracy profiles were used as criteria for the validation process. Calibration in the matrix was found to be more accurate than calibration without matrix. Trueness (<6.76% relative bias), repeatability (<4.6% RSD), intermediate precision (<5.3% RSD), selectivity, response function and linearity for major anthocyanins, cyanidin-3-glucoside and cyanidin-3-rutinoside, were evaluated. The concentration range validated was 1–48 μg/mL for both compounds. In addition two cyanidin-di-O-glycosides were detected for the fist time in this fruit. We also showed that a first extraction of the fruits with ethyl acetate removes the lipophilic compounds and allows an easier extraction by methanol and quantification of anthocyanins in this extract.

1. Introduction

It is generally accepted that anthocyanins are the most important pigments in vascular plants. These pigments are responsible for the blue, purple, red colors and intermediate hues present in many plant tissues. They belong to the very large class of molecules called flavonoids synthesized via the phenylpropanoid pathway. There are hundreds of anthocyanins (glycosides) in nature that are derived from several anthocyanidins (aglycones). The main differences between them are the number of hydroxylated groups, the nature and the number of bonded sugars, the aliphatic or aromatic carboxylates bonded to the sugar and the position of these bonds [1,2]. Anthocyanins extracts from fruits are of great interest for the food industry and health sector. In fact many biological activities of the anthocyanins related to their potent antioxidant properties are described in the literature [3,4].

The palm *Euterpe oleracea* is widely distributed in northern South America. It is particularly abundant in the floodplains of the Amazonian delta where it has high economic importance. The fruits are mainly harvested between July and December. They are round-shaped drupes (diameter of about 12 mm) and associated in racemes [5]. The seed represents around 85% of the volume of the fruit. The epicarp is a thin layer and the mesocarp is 1–2 mm thick [6]. The fruit color goes from green to black during the ripening process. In addition fully ripen fruits are covered with a wax cuticle [5]. The ripen fruits have a very high content in phenolic compounds and notably anthocyanins. Cyanidin-3-rutinoside (cy3rut) and cyanidin-3-glucoside (cy3glu) are the major anthocyanins in *E. oleracea* fruits (EOF) [5,7,8] and as a consequence the content of anthocyanins attributed to fruit or fruit juices are basically based on this two compounds [9,10]. Some other minor anthocyanins have been identified in trace levels, like pelargonidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-sambubioside, and peonidin-3-rutinoside [11,12]. The fruits have also presented substantially higher antioxidant activity than most other fruits [10,13]. This contributed for the huge increase of the exports from Brazil and internal consumption of this fruit observed recently [14].

EOF are harvested at different maturity stages. Recently special attention has been given to the analysis of phenolic compounds of EOF during ripening [9,15] and to fingerprinting analysis of

Abbreviations: CID, collision-induced dissociation; cy3glu, cyanidin-3-glucoside; cy3rut, cyanidin-3-rutinoside; EOF, *Euterpe oleracea* fruits; EtOAc, ethyl acetate; ME, matrix effect; MeOH, methanol; MeOH 50%, methanol/water (50/50, v/v); PE, process efficiency; RE, recovery; RSD, relative standard deviation.

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dietary supplement raw materials derived from this fruit [16]. In fact accurate characterizations of these compounds from reliable analytical methods are very important for the post-harvest and food industry. In addition the validation of analytical methods gives to laboratories as well as to regulatory bodies "*guarantees*" of the reliability of the measures performed and minimize the consumer and the producer risks [17]. To our knowledge no article describing a (U)HPLC–UV method for the quantification of the major anthocyanins from EOF has been validated [6,9–12,15,18–20], probably for some of them, because they are not so recent. One poster was found about method validation and analysis of the major anthocyanins from EOF using UPLC and HPLC methods, but no validation data was available [21]. The run times of these HPLC methods are very long. Nowadays the use of UHPLC methods has become a trend because of improved resolution, higher sensitivity and reduction of the run time and solvent volumes [22]. A few validated UHPLC methods have been reported for the quantification of cy3glu and/or cy3rut [23–26]. But these methods analyzed milk-based products or biological fluids and tissues for bioavailability and pharmacokinetic studies. They use MS detectors for the quantification in trace levels. Therefore the sample matrix of these methods is very different of a plant matrix. In addition these compounds are present in EOF in high and variable concentrations [9,10]. In this case the validation of a wide dosing range with concentration levels covering also high concentrations is more suitable. The use of highly sensitive MS detectors is adapted for trace analysis. However the PDA detectors are sensitive enough for the concentration of cy3glu and cy3rut generally found in EOF [6,8,10–12,18]. The aim of this work is to develop and validate a UHPLC–PDA method for the cy3glu and cy3rut quantification in EOF after fast extraction procedures and samples preparation.

2. Experimental

2.1. Chemicals and plant material

Cy3glu (98.0%), cy3rut (97.2%), pelargonidin-3-glucoside (100%), peonidin-3-glucoside (95.0%), peonidin-3-rutinoside (95.5%) and cyanidin-3,5-diglucoside were purchased from Extrasynthèse (Genay, France). HPLC grade acetonitrile from Fisher Scientific (Tournai, Belgium) and formic acid from Prolabo, VWR (Leuven, Belgium) were used for the UHPLC runs. The Chromabond® C18 cartridges (1 g/6 mL) were acquired from Macherey-Nagel (Düren, Germany).

The EOF were harvested in the municipal district of Abaetetuba between July and December 2010 at four different maturity stages, identified as A, B, C, and D, corresponding to the maturity stages 2, 6, 8, and 11, respectively according to Rogez et al. [9]. The following fruits characteristics were used to classify the maturity stages: 20% of black fruits and 80% of green fruits (A), 100% of black and bright fruits (B), 60% of black fruits and 40% of opaque fruits (C), and finally 100% of black with intense gray coverage due to the presence of the intense wax cuticle (D). The samples were quickly transported to the laboratory in Belém (Pará State, Brazil) and immediately processed.

2.2. Extraction and clean-up procedure

The fruit samples were weighed (49.1–50.3 g) and introduced in 150 mL brown glass bottle. The fruits were extracted sequentially by EtOAc (0.1% HCl), MeOH (0.1% HCl) and MeOH/H_2O (50/50, v/v) (0.1% HCl). Each extraction was carried out twice with a ratio fruits/solution of 1:3, for 30 min at ambient temperature and without shaking. The extracts were called MeOH, EtOAc and MeOH 50% extracts respectively. The extracts were centrifuged for 10 min at 4300 × *g*. The residue of the EtOAc extract was re-extracted in MeOH/HCOOH (99/1, v/v) (10 mL) by ultrasound extraction (5 min), the other residues were re-extracted in their respective extraction solution. After centrifugation the supernatants of each extraction were mixed before drying. The EtOAc and MeOH extracts supernatants were evaporated to dryness with an acid-resistant CentriVap® vacuum concentrators (Labconco, Kansas City, MO) at 40 °C. The MeOH 50% extract supernatant was concentrated in the same conditions and then freeze dried. They were stored at −21 °C and sent to Belgium to be analyzed.

As the EtOAc extract was a little viscous, probably due to the presence of nonpolar compounds like lipids and chlorophylls, it was purified by solid phase extraction (SPE). First, the extract was reconstituted in MeOH/H_2O/HCOOH (90/9/1) to the concentration of 14 mg/mL (for all EtOAc extracts) and 0.8 mL was applied on a C18 SPE cartridge (1 g/6 mL) (Chromabond, Düren, Germany). This cartridge was previously conditioned in MeOH/H_2O/HCOOH (90/9/1). Then the anthocyanins were eluted with 9 mL of MeOH/H_2O/HCOOH (90/9/1) and recovered in a test tube. Afterwards the anthocyanins fraction was evaporated to dryness with a RapidVap multi-tube evaporator (Labconco) adjusted at 40 °C. The dried fraction was called the "anthocyanins fraction" and was stored at −21 °C until analysis.

The day of the analysis the totality of the anthocyanins fraction obtained from each SPE was solubilized with 1 mL of MeOH/ACN/H_2O/HCOOH (15/5/75.25/4.75) (Solution S). The solubilized fraction was then filtered on a 0.2 μm filter (Interchrom, Montluçon, France) and immediately injected into UHPLC.

The solutions from dried MeOH and MeOH 50% extracts were prepared at 0.25 mg of dried extract/mL. The final solvent composition of this solution was the same that the solvent composition of the Solution S. The solutions were also filtered and directly injected into UHPLC.

2.3. Apparatus and UHPLC–PDA–ESI-MS/MS analysis

The analysis were performed on a Accela UHPLC system acquired from Fisher Scientific (Thermo Fisher Scientific, Bremen, Germany) that consisted of a PDA detector, an autosampler equipped with a column oven and a tray compartment cooler and a quaternary pump with a built-in solvent degasser, all piloted by ChemoQuest software. The chromatographic separation was performed on an Acquity UPLC HSS C18 column 1.8 μm, 100 mm × 2.1 mm ID (Waters, Belfast, Ireland) equipped with a VanGuard UPLC HSS C18 pre-column 1.8 μm, 5 mm × 2.1 mm ID from the same supplier. 5 μL of samples was injected in a full loop injection mode. The separations were performed with a constant flow rate of 400 μL/min with the eluents being (A) H_2O/HCOOH (95/5) and (B) ACN with the following gradients: 0–10 min: 5–15% B; 10–10.1 min: 15–95% B; 10.1–12 min: 95% B; 12–12.1 min: 95–5% B; 12.1–17 min: 5% B. The column oven and tray cooler temperatures were set to 30 and 4 °C respectively. For quantitative analysis the PDA detector was used at 515 nm. The quantification was performed using the external calibration method.

The Accela UHPLC system was hyphenated with a LTQ-Orbitrap XL mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany) from UCL MASSMET platform. This system was employed to perform the identifications and evaluation of the selectivity of the method for all extracts and all maturity stages using the same separation conditions aforementioned. The system was equipped with an ESI interface that was used in positive ionization mode with the following conditions: capillary temperature and voltage at 270 °C and 9 V respectively, ion spray voltage at 5 kV and tube lens voltage at 95 V. Nitrogen was used as the sheath gas and helium as auxiliary gas with a flow rate of 20 and 10 arbitrary units. The compounds were identified by comparison with

reference compounds from retention times, MS and MS/MS analysis. For the MS experiments two scan events were applied. The first was a full MS scan, for which the spectra were recorded in the range of m/z 100–1000 with a resolution of 30,000. The second was a data dependent scan that selected the most intense ion or specified ions in another setting from the first scan event for the acquisition of MS/MS spectra. The collision-induced dissociation (CID) activation was set to a normalized collision energy of 20% or 15%. An external calibration of the equipment for mass accuracy was carried out the day before the analysis according to the manufacturer's guidelines.

2.4. Standard solutions

The Stock solution containing cy3glu and cy3rut at 0.98 and 0.972 mg/mL respectively was prepared in MeOH/HCOOH (99/1) and stored at −21 °C. Good stability of anthocyanins is known in similar storage conditions [27]. An aliquot of this solution was diluted to 98.0 and 97.2 μg/mL (cy3glu and cy3rut respectively) to have the same final solvent composition as Solution S. This solution, called "Standards Intermediate Solution 1" was diluted to obtain the calibration standards at 5 concentration levels (m = 5) ranging from 1.0 to 49.0 for cy3glu and from 1.0 to 48.6 μg/mL for cy3rut. Each concentration was analyzed three times (n = 3) for 3 series of experiments (k = 3). Another set of calibration standards was prepared in the MeOH extract itself in order to compare the calibration with and without matrix. A solution of MeOH extract (maturity stage B) of 0.5 mg/mL was prepared (final solvent composition = Solution S composition) and was used to prepare the calibration standards in the matrix. They were prepared by diluting in the extract the same volumes of Standards Intermediate Solution 1 that were employed for the preparation of the calibration standards without matrix. The signals from the cy3glu and cy3rut initially present in the extract was subtracted from the peaks obtained with these solutions. The final concentration of the extract in each solution was the same (0.25 mg of extract/mL). The number of concentration levels, series and repetitions per series were the same than without matrix calibrations (m = 5, k = 3 and n = 3).

The validation standards were prepared in the same way as the matrix based calibration standards. Five concentrations were analyzed (m = 5) and the dilutions from the stock solutions of standards and extract were independents for each sample (n = 3). Three series (k = 3) were performed.

2.5. Evaluation of the SPE clean-up procedure and the matrix effect

For the *clean-up* procedure of the EtOAc extract the recovery (RE) of the SPE process was determined along with the overall "process efficiency" (PE) and the matrix effect (ME). It was calculated for both cy3glu and cy3rut according to an adaptation of the procedures of Matuszewski [28]. From the standards stock solution an intermediate solution called "Standards Intermediate Solution 2" was prepared at 196.0 and 194.4 μg/mL (cy3glu and cy3rut respectively) for a final solvent composition of MeOH/H_2O/HCOOH (90/9/1). Three different SPE called SPE 1, SPE 2 and SPE 3 were performed, each one in triplicate, as described below from a solution of EtOAc extract of the maturity stage B.

Along with the extract solution volume an additional volume of 0.125 μL was applied on the SPE cartridges. This volume consisted of a blank solution of MeOH/H_2O/HCOOH (90/9/1) that was applied for SPE 1 and SPE 2 cartridges while it consisted of the Standards Intermediate Solution 2 that was applied for SPE 3 cartridges (spiked with standards *before* SPE extraction). After SPE elution and drying of the fraction the same volume of 0.125 μL of the blank solution was applied in the tubes containing the anthocyanins fraction obtained from SPE 1 and SPE 3 while 0.125 μL of Standards Intermediate Solution 2 was applied in the tubes containing the anthocyanins fraction obtained from SPE 2 (spiked with standards *after* SPE extraction). The solutions in all tubes were adjusted to 1 mL and the final solvent composition was the same that the solvent composition of the Solution S cited in Section 2.2. Finally, in order to have a reference of the standard responses without SPE, 0.125 μL of the Standards Intermediate Solution 2 was diluted in triplicate in new tubes to 1 mL. The final concentrations of this solution were 24.5 and 24.3 μg/mL for cy3glu and cy3rut respectively and the final solvent composition was also the same of the Solution S. All solutions of anthocyanins fraction obtained from SPE 1, SPE 2 and SPE 3 as well as the diluted Standards Intermediate Solution 2 (reference without SPE) were filtered and injected into the UHPLC for peak area recording.

The ME, RE and PE values for the cy3glu and cy3rut were calculated as follows:

$$\text{ME}\ (\%) = \frac{\text{MPA2} - \text{MPA1}}{\text{MPAs}} \times 100 \quad (1)$$

$$\text{RE}\ (\%) = \frac{\text{MPA3} - \text{MPA1}}{\text{MPA2} - \text{MPA1}} \times 100 \quad (2)$$

$$\text{PE}\ (\%) = \frac{\text{MPA3} - \text{MPA1}}{\text{MPAs}} \times 100 \quad (3)$$

where MPA1, MPA2 and MPA3 are the mean peak areas of the compounds of the anthocyanins fraction obtained from SPE 1, SPE 2 and SPE 3 respectively, and MPAs are the mean peak areas of reference without SPE.

The response of the cy3glu and cy3rut spiked *after* SPE extraction (MPA2 − MPA1) were back-calculated from a calibration curve prepared in the matrix (MeOH extract) whose preparation was explained in Section 2.4. Eq. (1) and the back-calculated concentrations were used to evaluate the influence of the matrix on the detection and on the quantification from a calibration curve prepared in the matrix.

For the MeOH 50% extract of maturity stage B only the matrix effect was evaluated as it did not undergo a SPE process. The procedure for this evaluation is the same as previously described. The extract solution was prepared at 0.5 mg/mL and had the same final solvent composition of the Solution S. In order to measure MPA 2 (spiked solution referred to in Eq. (1)), the extract solution was spiked with the intermediate solution 1 cited in Section 2.4 with a final concentration of 24.5 and 24.3 μg/mL for cy3glu and cy3rut respectively. To measure MPA 1 (not spiked), the extract solution was diluted twice with blank solution S. Finally, the Standards Intermediate Solution 1 was diluted to the same concentration but in blank solution S in order to have the reference without matrix (MPAs in Eq. (1)). All solutions were prepared in triplicate and injected into UHPLC to record the peak areas to calculate ME and evaluate the back-calculated concentrations as for the EtOAc extract.

2.6. Validation of the method

The validation of the method was realized from three series of experiments. For each series the eluents of the mobile phase were renewed and 78 injections were performed. From these series the following criteria were tested: response function, linearity, selectivity, precision (repeatability and intermediate precision), trueness, accuracy, limits of detection (LOD) and quantification (LOQ) and matrix effect. Total error was used as decision criterion for the validation process [17,29–31]. The acceptance limits were set at $\lambda = \pm 20\%$ and the minimum probability to obtain future results within these limits was set at $\beta = 95\%$ (β-expectation limits). Statistical analyses were performed using the e-noval V3.0 (Arlenda, Liège, Belgium) software.

3. Results and discussion

3.1. Extraction, SPE clean-up procedure and matrix effect

A sequential extraction method with different solvents was developed for the extraction of anthocyanins. On the surface of the fruits a thin layer of lipids could interfere with the transfer of anthocyanins from the cells to any polar extraction solution in the conditions of extraction used in this study. Thus before applying quite polar extraction solutions as MeOH, a less polar solution EtOAc (0.1%HCl) was applied to remove the lipids and other apolar compounds like chlorophylls and to begin the extraction of the anthocyanins.

We developed a *clean-up* procedure for the EtOAc extract by SPE from C18 cartridges and observed that a complete elution of the anthocyanins was obtained with 7 mL of MeOH/H_2O/HCOOH (90/9/1) for the different maturity stages. No further anthocyanin elution was observed after an additional elution with 6 mL of acidified MeOH (data not shown).

The matrix effect (ME ± SD) was calculated by Eq. (1) and gave 94.5% (±2.0) and 96.7% (±1.9) for cy3glu and cy3rut respectively, indicating no significant influence of the matrix on the response. The back-calculated concentrations of the spiked solution from a calibration curve in the MeOH extract matrix as described in Section 2.5 were 23.7 (SD ± 0.5) and 24.2 μg/mL (SD ± 0.5) for cy3glu and cy3rut respectively which were not significantly different from to the spiked concentrations of cy3glu and cy3rut (24.5 and 24.3 μg/mL respectively). These results confirm that the responses of the "anthocyanins fraction" can be analyzed from the calibration curve in the MeOH extract matrix.

The recovery (RE ± SD) was calculated by Eq. (2) as described in Section 2.5. The RE were 58.7% ± 4.9 and 74.6% ± 3.6 for cy3glu and cy3rut respectively. These values were taken into account for the calculation of their concentrations in the EtOAc extract. Recoveries of these orders can be used to correct the final results [32]. In addition the recoveries obtained have a good repeatability. Similar values of "process efficiency" (PE ± SD) were obtained according to Eq. (3) which corresponded to 55.4% ± 4.0 and 72.0% ± 2.4 for cy3glu and cy3rut respectively, which is logical as no matrix effect was observed. The recovery obtained was inferior to the one obtained by Pacheco-Palencia et al. [19] who used a C18 cartridge and an acidified MeOH as eluent for anthocyanin extraction from the clarified *E. oleracea* juice. Considering that the SPE conditions were relatively similar, the inferior recovery we obtained could be explained by the different lipid content of the samples or by the degradation of compounds during drying procedures which probably was more time consuming because of the presence of water. We also have to note that the amount of cy3glu and cy3rut in the EtOAc extract is very low compared to the total concentrations in the fruits.

No significant matrix effect was also observed for the MeOH 50% extract (ME = 98.8% ± 3.2) and back calculated concentrations were 24.6 μg/mL ± 0.8 for both compounds. This confirms that the anthocyanin quantification from this MeOH 50% extract can be performed from the calibration curve in the matrix constructed with MeOH extract.

3.2. Development of the UHPLC method

The UHPLC method that we developed allowed separating the major and minor anthocyanins detected in the extracts (Fig. 1a) in only 10 min on a total run time of 17 min. Some of these compounds (peaks 3–7) are the dominant anthocyanins of many other berry fruits [4] for which this separation method can be applied. Good resolution of the peaks is very important when UV detector is used. Another work using UHPLC C18 column [16] did not provides the complete chromatographic separation of the major anthocyanins of EOF, because it used MS detector for which no separation of peaks that have different molecular ions is necessary. In addition the total run time of our method was 2.1× faster that this one. Regular HPLC methods for *E. oleracea* anthocyanins have a total run time between 30 and 80 min [6,8–12,15,18–20,33] so that our UHPLC method is 1.8–4.7× faster, which shows the interest of UHPLC analysis for these compounds. In terms of organic mobile phase, ACN, MeOH and a mixture of ACN/MeOH (1/1) were tested. A better resolution and a faster elution of the chromatographic peaks were obtained with ACN. Moreover its lower viscosity and consequently lower back pressure promoted a better system stability. The acidified mobile phase allowed a predominance of the flavylium cation species and a better separation, as reported previously [27]. We used 5% formic acid in the aqueous solution of the mobile phase which corresponds to a quite low pH (1.8) because we observed that smaller acid percentages provided an increase of the retention times and decreases peaks resolution. In addition, the predominance of the flavylium cation forms provided better MS detection in positive ionization mode that we applied for the identification studies. It was necessary to apply a linear gradient with low slope (5–15% ACN in 10 min) to separate all the peaks. From this gradient interval, convex and concave gradients were tested but the conditions aforementioned remained the better compromise between the run time and resolution of the peaks. The samples were very well solubilized in ACN/H_2O/MeOH/HCOOH (5/75/15/5). In fact, the dissolution solvent is an important parameter for UHPLC where it has more influence on the chromatographic separation than with conventional HPLC analysis [34].

The final UHPLC conditions were: acetonitrile and 5% formic acid in water as mobile phase, eluted at a constant flow rate of 400 μL/min with a gradient of 5–15% ACN in the first 10 min, after that a washing of the column was performed with 95% ACN and an equilibration of 4.9 min to the initial condition.

3.3. Validation of the method

3.3.1. Identification and selectivity

From the full high resolution MS scan analysis in positive mode, the anthocyanins molecular ions were detected in their oxonium form and their molecular weight were directly obtained. Peak 1 showed a molecular ion at *m/z* 611.15839 $[M]^+$ (calculated mass: 611.16066) from which the formula $[C_{27}H_{31}O_{16}]^+$ was deduced. From the data-dependent MS/MS acquisition (CID = 15%) the molecular ion showed two fragment ion at *m/z* 449,10614 $[M-162]^+$ and *m/z* 287.05411 $[M-162-162]^+$ due the loss of two hexoses sequentially (Fig. 2a). The fragment ion at *m/z* 287 indicates the presence of cyanidin or an isomer in the structure of the molecule. According to the literature [35] the only disaccharide linked to anthocyanins losing a sugar after MS/MS is the rutinoside. The presence of rutinose in the structure is excluded because its fragmentation did not correspond to a sequential loss of two *m/z* 162 fragments. As we observed the fragment ion at *m/z* 449 $[M]^+$, the two hexoses must be likely attached at different positions, probably to oxygen atom because a carbon-carbon bond of a C-glycosilation is more difficult to be broken, but the exact position of the sugars cannot be ensured by MS. Peak 2 showed similar MS data than peak 1 (Fig. 2b and Table 1) indicating that these compounds are isomers. Nevertheless retention time of these two compounds was different from standard of cyanidin 3,5-diglucoside. Peaks 1 and 2 were partially identified for the first time in EOF. The retention times and the MS data (CID = 20%) of peaks 3–7 were compared with those of standards allowing their identification (Table 1) confirming other studies [5–12]. All peaks were present in the MeOH extract of all evaluated maturity stages, but in the EtOAc and MeOH 50% extract only cy3glu and cy3rut were always observed at detectable levels.

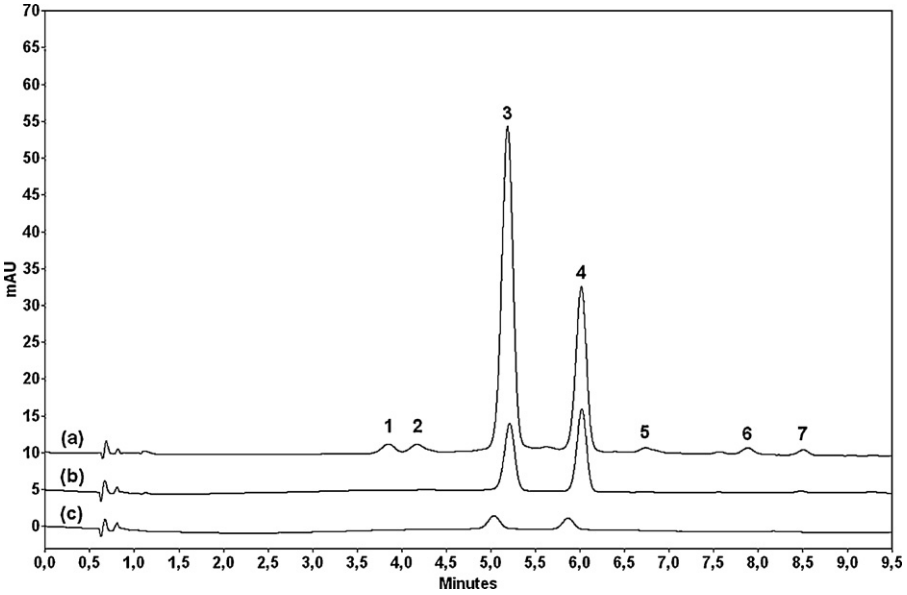

Fig. 1. UHPLC–PDA chromatograms recorded at 520 nm from (a) MeOH, (b) MeOH50% extracts and (c) anthocyanins fraction of the EtOAc extract. 1: cyanidin-di-O-glycoside, 2: cyanidin-di-O-glycoside (isomer of the first), 3: cyanidin-3-glucoside, 4: cyanidin-3-rutinoside, 5: pelargonidin-3-glucoside, 6: peonidin-3-glucoside, 7: peonidin-3-rutinoside.

Special attention was dedicated in the anthocyanin identifications because some studies reported different profiles with respect to the major anthocyanins in EOF. Cyanidin-3-arabinoside and cyanidin-3-arabinosylarabinoside were reported as major anthocyanins in [36]. In another study peonidin-3-(6″-malonylglucoside) and delphinidin 3-(6″-acetoyl)glucoside in addition to cy3glu and cy3rut were reported as major anthocyanins [33]. These other major anthocyanins were not detected in our study.

The selectivity of the analytical method was assessed by peak purity verification of the cy3glu and cy3rut from the UHPLC–MS analysis. Mass spectra were recorded at three retention times corresponding to the beginning, the middle and the end of the peaks and were found to be similar for the different extracts (anthocyanins fraction of the EtOAc extract, MeOH and MeOH 50% extracts) and for all maturity stages studied. Fig. 3 gives one example of MS spectra for cy3glu (Fig. 3a–c) and cy3rut (Fig. 3d–f) from the MeOH extract of the maturity stage C which is the most commercialized [5].

3.3.2. Response function and accuracy

For both compounds, cy3glu and cy3rut, different regression models were tested such as linear, quadratic (weighted or unweighted), with or without transformations. Total error (systematic + random error) was the main decision criterion for our validation process. The suitability of the different models was assessed by plotting the accuracy profiles using 95% β-expectation tolerance intervals and ±20% acceptance limits. For cy3glu calibration in the matrix using a linear regression was chosen for the quantification. With this calibration model all concentrations, except the lowest one, gave results within the acceptance limits (Fig. 4a). The same linear regression but in a calibration without matrix showed higher values of total error for the two lowest concentration levels (Fig. 4b). Improved accuracy profiles for low concentration levels were also obtained in a calibration in the matrix for other phenolic compounds in a HPLC–UV method [37].

Several calibration models for cy3rut showed relatively good accuracy profiles, with the tolerance intervals within the ±20% acceptance limits. The weighted ($1/X$) linear regression in the matrix was chosen for the quantification because it gave the best accuracy profile. We also observed, as for cy3glu, lower total error when calibration was made in the matrix (Fig. 4c and d). Accuracy values obtained for cy3glu and cy3rut are summarized in Table 2. This work was the first that evaluated an external calibration in the matrix extract. In other quantification studies from EOF, external calibrations without matrix were applied from non-validated methods [6,9–11,15,18–20].

3.3.3. Trueness, precision and linearity

The values obtained are also summarized in Table 2. Trueness expressed in terms of relative bias was generally inferior to 6.76% for cy3glu to 3.6% for cy3rut which shows the good trueness of

Table 1
Anthocyanins identification of *Euterpe oleracea* fruits by UHPLC–PDA–MS/MS method.

Peak[a]	RT UHPLC–PDA (min)	Experimental mass m/z $[M]^+$ (error: ppm)	Formula $[M]^+$	MS/MS (m/z)	Identification
1	3.85	611.15839 (−3.72)	$C_{27}H_{31}O_{16}$ [b]	287.05411, 449.10614	Cyanidin-di-O-glycoside[c]
2	4.18	611.15845 (−2.21)	$C_{27}H_{31}O_{16}$ [b]	287.05426, 449.10645	Cyanidin-di-O-glycoside[c]
3	5.19	449.10654 (−2.89)	$C_{21}H_{21}O_{11}$	287.05423	Cyanidin-3-glucoside[d]
4	6.01	595.16418 (−2.63)	$C_{27}H_{31}O_{15}$	287.05411, 449.10620	Cyanidin-3-rutinoside[d]
5	6.75	433.11191 (−2.34)	$C_{21}H_{21}O_{10}$	271.05939	Pelargonidin-3-glucoside[d]
6	7.90	463.12198 (−3.26)	$C_{22}H_{23}O_{11}$	301.07053	Peonidin-3-glucoside[d]
7	8.51	609.17908 (−3.803)	$C_{28}H_{33}O_{15}$	301.07056, 463.12305	Peonidin-3-rutinoside[d]

[a] The numbers correspond to Fig. 1.
[b] Best fitted molecular formula obtained by HRMS.
[c] Not fully identified on the basis of the MS data.
[d] Identification were confirmed by analysis of standards.

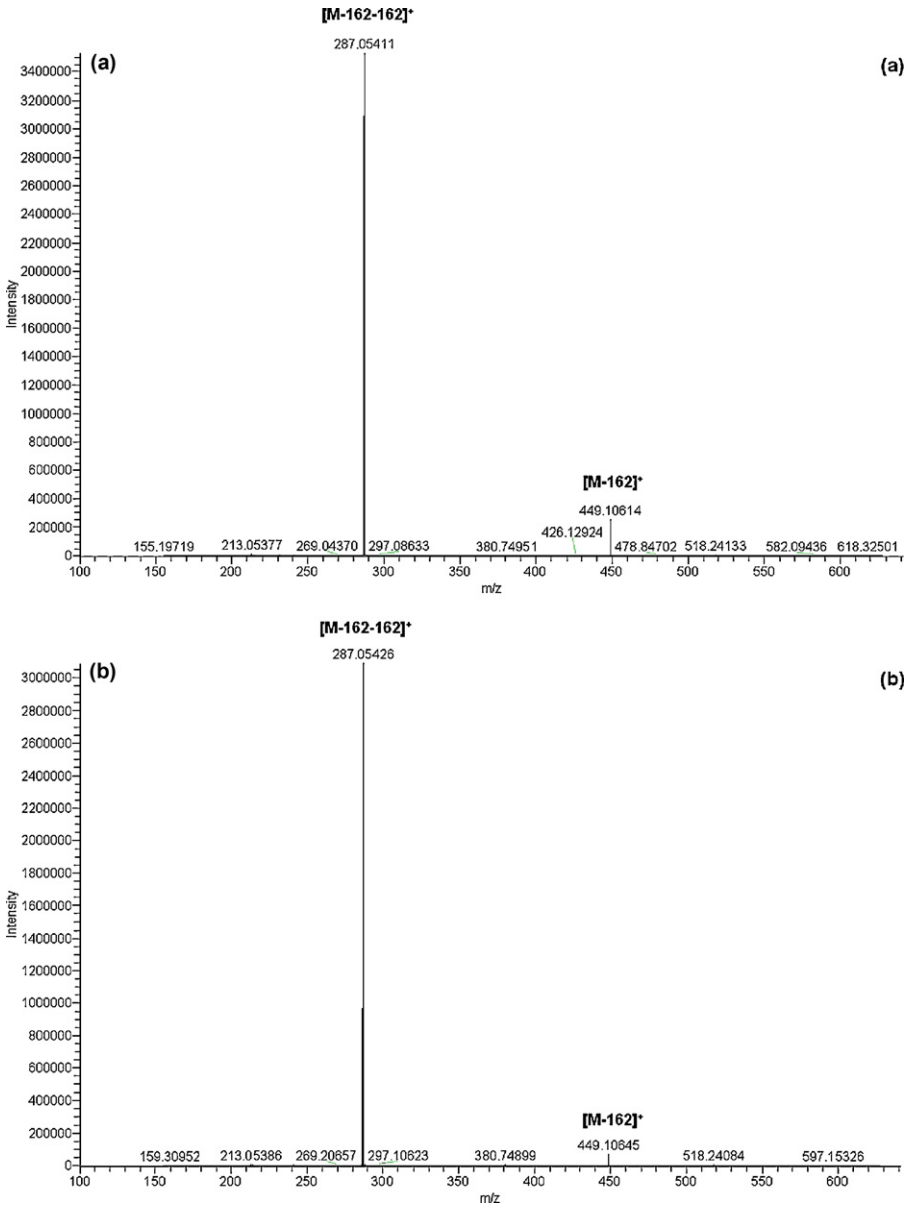

Fig. 2. (a) MS/MS spectra of the molecular ion at *m/z* 611.15839 $[M]^+$ corresponding to peak 1 (Fig. 1). (b) MS/MS spectra of *m/z* 611.15845 $[M]^+$ corresponding to peak 2 (Fig. 1) (MeOH extract obtained from *Euterpe oleracea* fruits at maturity stage C).

the method. The precision is evaluated at two levels, repeatability and intermediate precision and were expressed in terms of relative standard deviation (RSD) values. The repeatability and intermediate precision for both compounds were very good and inferior to 5.3% (cy3glu) and 3.8% (cy3rut). In addition they showed similar variance along the dosing range indicating that the effect of the series did no provide significant additional variability for each compound. The linearity is the ability within a definite range to obtain results directly proportional to the concentration of the analyte. The concentrations of the validation standards were back-calculated from the calibration curve. A linear regression model was fitted on the back-calculated concentrations as a function of the introduced concentrations. The intercept, the slope and the coefficient of determination of the equations obtained for cy3glu and cy3rut are presented in Table 2. The slopes values close to 1 demonstrate the linearity of the method. The linearity was also demonstrated because the absolute β-expectation tolerance limits were within the absolute acceptance limits [31,37,38].

3.3.4. Limits of detection and of quantification

The limits of detection were calculated from the residual standard-deviation and the slope of the calibration curve. The results obtained were 0.32 and 0.12 μg/mL for cy3glu and cy3rut respectively. The lower (LLOQ) and upper (ULOQ) limits of quantification were obtained by calculating the lowest and highest concentration of the targeted substance that can be assayed under experimental conditions for which the β-expectation limits remain inside the acceptance limits. For cy3glu the LLOQ and the ULOQ were 1.07 and 48.99 μg/mL respectively and for cy3rut they were 0.97 and 48.59 μg/mL respectively.

3.4. Application of the quantification method to samples of E. oleracea fruits

Three EOF samples corresponding to the most commercialized maturity stage (C) were harvested in a same location of the municipal district of Abaetetuba (harvest season: 2010) and analyzed.

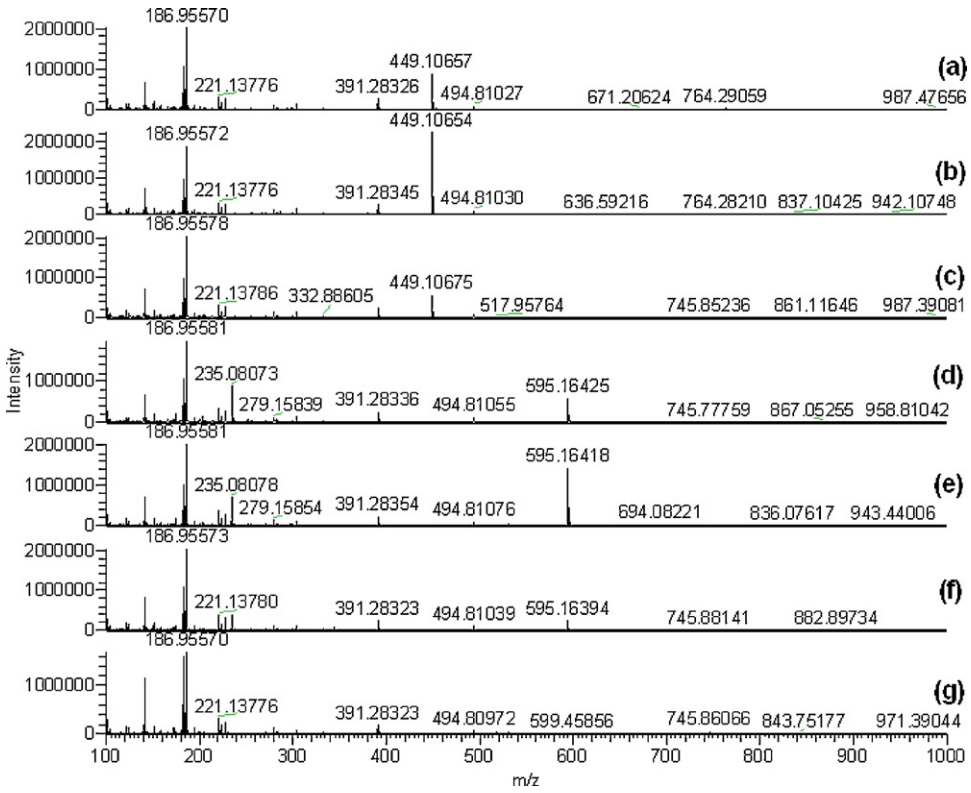

Fig. 3. Positive ion mode MS spectra of cyanidin-3-glucoside (*m*/*z* 449 $[M]^+$) recorded at (a) the beginning (b) the middle and (c) the end of the peak, and MS spectra of cyanidin-3-rutinoside (*m*/*z* 595 $[M]^+$) corresponding to (d) the beginning (e) the middle and (f) the end of the peak, and (g) MS spectrum of a blank analysis.

Table 2
Validation results for the quantification method of cyanidin-3-glucoside and cyanidin-3-rutinoside obtained in MeOH extract of *Euterpe oleracea* fruits.

Validation criteria	Cyanindin-3-glucoside		Cyanidin-3-rutinoside	
Response function	Linear regression		Weighted (1/*X*) linear regression	
	Calibration in the matrix (5 points)		Calibration in the matrix (5 points)	
	Range: 1.0–49.0 μg/mL		Range: 1.0–48.6 μg/mL	
Trueness	Concentration (μg/mL)	Relative bias (%)	Concentration (μg/mL)	Relative bias (%)
	1.0	6.76	1.0	3.60
	2.0	2.54	1.9	0.51
	4.9	0.67	4.9	0.38
	24.5	−0.07	24.3	−0.24
	49.0	−0.83	48.6	−1.29
Precision	Repeatability (RSD%)	Intermediate precision (RSD%)	Repeatability (RSD%)	Intermediate precision (RSD%)
	4.63	5.30	3.10	3.80
	2.49	2.49	1.86	2.85
	1.90	1.90	1.41	1.41
	2.99	2.99	2.47	2.47
	1.46	1.46	1.35	1.35
Accuracy	Relative β-expectation lower and upper tolerance limits (%)		Relative β-expectation lower and upper tolerance limits (%)	
	−7.6, 21.1		−7.2, 14.4	
	−3.8, 8.9		−8.8, 9.8	
	−4.1, 5.5		−3.2, 4.0	
	−7.6, 7.5		−6.5, 6.0	
	−4.7, 3.0		−4.9, 2.3	
Linearity				
Slope	0.9910		Slope	0.9873
Intercept	0.0910		Intercept	0.0829
r^2	0.9994		r^2	0.9995

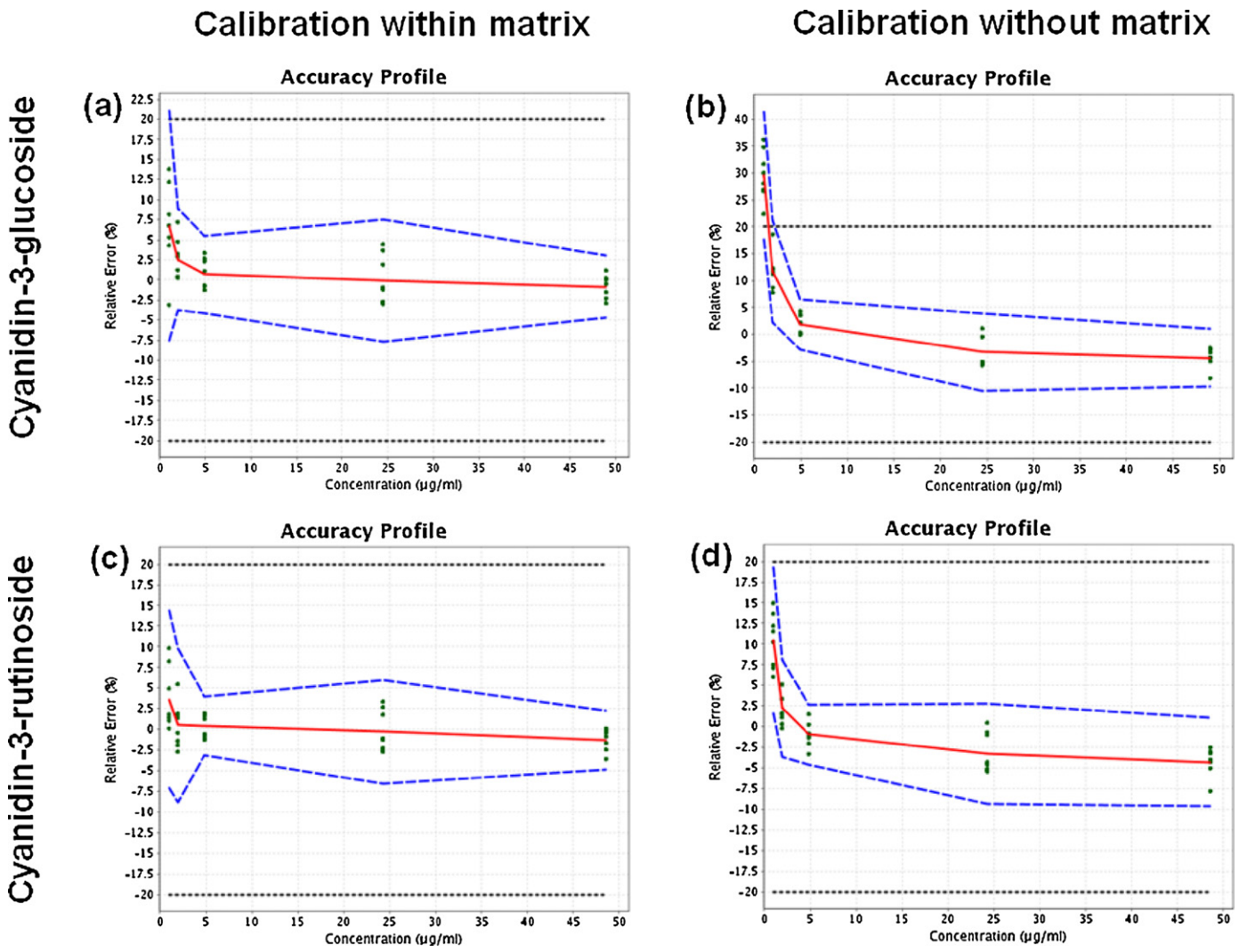

Fig. 4. Accuracy profiles in relative values for different regression models using 5 points calibrations. (a) Linear regression in the matrix (MeOH extract) and (b) without matrix for cyanidin-3-glucoside, (c) weighted (1/*X*) linear regression in the matrix and (d) without matrix for cyanidin-3-rutinoside. The continuous line represents the relative bias, the dashed lines the 95% β-expectation tolerance limits and the dotted lines the ±20% acceptance limits.

This is the maturity stage recommended to the harvest of these fruits [9]. The calibration curves in the matrix were applied for the quantification of the major anthocyanins.

The concentrations obtained for cy3glu and cy3rut as well as the sum of these concentrations for each extract are presented in Table 3. The lowest concentration of the sum of the major anthocyanins was obtained in the EtOAc extract (0.02%) due to the low solubility of anthocyanins in this solvent. This can suggest that an EtOAc extraction can be applied to remove the wax cuticle present on the fruits before the anthocyanins extraction by methanolic solutions without considerable loss of the anthocyanins. This procedure can optimize the mass transfer of the

Table 3
Anthocyanins quantification results from EtOAc, MeOH and MeOH50% extracts obtained from *Euterpe oleracea* fruits at maturity stage C.

Sample	Extract	Cyanidin-3-glucoside (mg/kg fruits) ± SD	Cyanidin-3-rutinoside (mg/kg fruits) ± SD	∑Major anthocyanins (mg/kg fruits)
Sample 1	EtOAc	0.06 ± 0.00	0.05 ± 0.00	0.11 ± 0.00
	MeOH	277.45 ± 1.33	192.64 ± 0.95	470.09 ± 2.26
	MeOH50%	8.14 ± 0.88	10.16 ± 0.90	18.31 ± 1.51
	Total	285.65 ± 0.47	202.85 ± 1.09	488.50 ± 1.24
Sample 2	EtOAc	0.05 ± 0.00	0.04 ± 0.00	0.09 ± 0.00
	MeOH	328.56 ± 7.77	231.41 ± 5.38	559.97 ± 13.14
	MeOH50%	9.94 ± 0.92	13.67 ± 1.10	23.61 ± 2.01
	Total	338.55 ± 8.62	245.12 ± 6.45	583.67 ± 15.04
Sample 3	EtOAc	0.05 ± 0.00	0.05 ± 0.00	0.11 ± 0.00
	MeOH	283.37 ± 21.58	240.66 ± 17.47	524.03 ± 39.05
	MeOH50%	9.83 ± 0.19	12.68 ± 0.64	22.51 ± 0.82
	Total	293.25 ± 21.59	253.39 ± 17.39	546.65 ± 38.98

anthocyanins, promote the obtainment of a methanolic extract without lipids and other apolar compounds as chlorophylls that can damage C18 columns and avoid SPE *clean-up* procedures. Almost 95% of the major anthocyanins are present in the MeOH extract. This percentage can be considered as the extraction yield because of the low content of anthocyanins in the MeOH 50% extract obtained on the residue, showing the great extraction efficiency of the MeOH solution. Thus the last extraction can be eliminated of the protocol reducing the time for extraction and samples preparation.

The sum of the concentrations of cy3glu and cy3rut for the three samples varied between 488 and 583 mg/kg fruits and were inferior to the concentrations obtained in a previous study on the same maturity stage C from two different locations of the same region of Abaetetuba (800 and 1200 mg/kg fruits) [9] demonstrating the great variability of the anthocyanins content in the *E. oleracea* fruits. This variability was also reported from different juices samples [10].

Therefore considering the economic importance of the EOF as a rich anthocyanin source and its great concentration variability it is of crucial importance to have a validated analytical method for their quantification for producers and consumers.

4. Conclusions

We developed a fast protocol of anthocyanins extraction and sample preparation without *clean up* procedures from EOF. Elimination of lipophilic compounds can be done by an EtOAc extraction, while anthocyanins are extracted from the residue by MeOH. Calibrations in the matrix were used for cy3glu and cy3rut quantifications and showed better accuracy profiles in comparison to calibrations without matrix. To our knowledge this is the first article that describes a validated UHPLC–PDA method for the quantification of the major anthocyanins from *E. oleracea* fruits. It was found to be faster (17 min) that other HPLC–UV methods and allowed the separation of 3 other anthocyanins that are dominant in other common berry fruits. Furthermore, this method was selective and gave good estimators of linearity, accuracy, trueness and precision from 1 to 48 μg/mL of cy3glu and cy3rut. In addition minor diglycosilated anthocyanins were found in EOF for the first time.

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