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J. Agric. Food Chem., Just Accepted Manuscript • DOI: 10.1021/jf404776d • Publication Date (Web): 16 Jan 2014

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Characterization of Fruit Products by Capillary Zone Electrophoresis and Liquid Chromatography using the Compositional Profiles of Polyphenols. Application to Authentication of Natural Extracts.

Meritxell Navarro, Oscar Núñez*, Javier Saurina, Santiago Hernández-Cassou, Lluis Puignou

Department of Analytical Chemistry, University of Barcelona. Martí i Franquès, 1-11, E-08028 Barcelona. Spain.

* Corresponding author: Tel: +34-93-4033706; Fax: +34-93-4021233; E-mail: oscar.nunez@ub.edu
Abstract

Capillary zone electrophoresis (CZE) and high performance liquid chromatography (HPLC) were applied to the authentication of fruit products based on the compositional profiles of polyphenols. Various sample treatments were used to maximize the overall recovery of polyphenols or specific fractions such as phenolic acids or anthocyanins. The resulting CZE and HPLC data was treated with Principal Component Analysis (PCA) showing that samples were mainly clustered according to the fruit of origin, with cranberry- and grape-based products clearly separated in groups. A possible adulterated cranberry extract was analyzed more deeply by high resolution mass spectrometry (HRMS) in order to identify the presence of A-type proanthocyanidins which are characteristic and more abundant in cranberry-based products. In accordance with PCA interpretation, HRMS results indicated that the suspicious sample was not a cranberry-based product, allowing us to validate and demonstrate the suitability of both CZE and HPLC proposed methods for the characterization of fruit-based products.

Keywords: polyphenols; proanthocyanidins; capillary zone electrophoresis; liquid chromatography; principal component analysis; cranberry-based products.
INTRODUCTION

Berries are an excellent source of polyphenols, especially anthocyanins. The consumption of berry fruits associated to their contribution to improve human health is a subject of considerable interest. Berries contain natural antioxidants such as vitamins C and E, micronutrients such as folic acid, calcium, selenium, alpha and beta carotene and lutein, and polyphenols. Among polyphenols, high proportions of flavonoids including anthocyanins and ellagitannins have been found. Anthocyanins comprise the largest group of natural, water-soluble, plant pigments and they impart the bright colors to berry fruits and flowers. Approximately 400 anthocyanins have been identified in this kind of samples, especially in the skins. However, Red berry fruits, such as strawberries, cherries and cranberries, have also noticeable amounts of anthocyanins in their flesh.

Cranberry (Vaccinium macrocarpon) and its derived products, including juices and nutraceuticals, have shown some beneficial health effects including antioxidant activity, antimicrobial activity against bacteria involved in a wide range of diseases (dental caries, gastritis, enteritis, and infections), anti-inflammatory activity in periodontal disease, and antiproliferative activity on human oral, colon and prostate cancer cell lines, among others. However, the best known bioactivity of cranberry polyphenols deals with their capacity to inhibit the adhesion of pathogenic bacteria to uroepithelial cells of the urinary tract, thus, contributing to prevent urinary tract infections. This activity has also been extended to pathogens involved in diseases of the oral cavity.

The most common polyphenols found in cranberries comprise phenolic and benzoic acids, and flavonoids such as anthocyanins, flavonols, and flavan-3-ols. Flavan-3-ols in cranberry occur in both monomeric and polymeric forms, the so-called proanthocyanidins. These compounds can be classified according to the interflavan linkage as A-type and B-type molecules. B-type proanthocyanidins are those in which monomeric units are linked through the C4 position of the upper unit and the C6 or C8 positions of the lower unit. A-type proanthocyanidins contain an additional ether-type bond between the C2 position of the upper unit and the hydroxyl group at C7...
or C5 positions of the lower unit (C2-O-C7 or C2-O-C5) (Figure 1). Apart from their chemical structure, the most important difference between the two families is that only the A-type is capable of inhibiting the adhesion of bacteria to urinary tract tissues.\textsuperscript{3} Regarding the occurrence, almost 65\% of proanthocyanidins in cranberry are A-type ones.\textsuperscript{8} B-type proanthocyanidins are found in other food products like tea, chocolate, blueberry or grapes.\textsuperscript{9}

Recently some commercial products which claim to be manufactured from cranberry-based extracts have appeared in the market. These products are sold as if they had the same health properties of cranberries, but they do not contain the appropriate proanthocyanidins for having the desired bioactivity. This fact shows the importance of developing analytical methodologies for the characterization of natural extracts to achieve correct authentication regarding the fruit of origin.

Liquid chromatography (LC) with UV detection or coupled to mass spectrometry (LC-MS).\textsuperscript{10-13} are among the most common techniques used for the identification, characterization and determination of polyphenolic compounds.\textsuperscript{14-18} The first chromatographic studies on red berry fruits were focused on the correlation of polyphenolic profiles with some positive biological health effects.\textsuperscript{19, 20} In general, C18 reversed-phase separation conditions using water and methanol\textsuperscript{9, 19, 21, 22} or acetonitrile\textsuperscript{3, 12, 18, 23} as mobile phases were used. Regarding quantitative aspects, HPLC proved to be useful for the determination of B-type proanthocyanidins in several food products. However, the red-berry product analyses were less effective due to the presence of both A-type and B-type proanthocyanidins and the lack of adequate standards.\textsuperscript{24} The structural diversity of proanthocyanidins in red berry products led to complex profiles with poor chromatographic resolution. Furthermore, their analysis resulted in a great challenge due to the variety and the quantity of proanthocyanidin polymerized compounds.\textsuperscript{9, 21} High resolution mass spectrometry (HRMS) has also been proposed for the characterization of polyphenols in fruit products. Rockenbach \textit{et al.}\textsuperscript{18} used a Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) to characterize and assign the elemental composition of 251 different flavan-3-ol compounds.
Regarding cranberry-based products, Iswaldi et al. proposed the use of time-of-flight mass spectrometry (TOF-MS) for the characterization of the phenolic fraction in cranberry syrup. Capillary electrophoresis (CE) also appeared as an alternative technique for the analysis and characterization of polyphenolic compounds in fruit-based products. There are though only a few studies that apply these techniques to the analysis of red berry fruits. Because of the strong absorption of proanthocyanidins at 545 nm, simple colorimetric assays are still employed for the quantitative analysis of proanthocyanidins in cranberry-based products. The assay relying on the reaction of proanthocyanidins with 4-dimethylaminocinnamaldehyde is one of the most popular.

Characterization and classification of fruit-based products can be tackled from compositional profiles as a source of analytical information. Polyphenols, as well as other low molecular weight organic acids, alcohols, esters, etc., have been found to be efficient descriptors of some climatic, agricultural, and technological features. It has also been found that the variability of compounds will depend strongly on the fruit of origin. Therefore, polyphenolic profile can be a useful platform for reliable discrimination between fruit-based products via chemometric methods such as principal component analysis (PCA). Information recovered mathematically may be essential in order to prevent misuses in the production of commercial fruit-based extracts with health-promoting properties.

The aim of this work was the development of straightforward methods for the characterization of fruit products based on polyphenolic composition. The resulting instrumental profiles were exploited to carry out the authentication of natural extracts according to the fruit of origin. For that purpose, the potentiality of capillary zone electrophoresis (CZE) and HPLC combined with several sample extraction and treatment procedures was evaluated in order to obtain compositional data with high discriminant ability. Different kinds of samples were analyzed including fruits (cranberry, blueberry, grapes and raisins), fruit-based products such as grape-juice and cranberry-juice, as well as commercial cranberry-based products such as pharmaceutical natural...
extracts, powder capsules, syrup and sachets. CZE and HPLC data from each method was considered as a source of potential descriptors for the authentication of fruit-based products.

MATERIALS AND METHODS

Reagents and solutions

Unless otherwise stated, all reagents were of analytical grade. Acetic acid, formic acid, sodium tetraborate, Sephadex LH-20 and HPLC gradient grade acetone were obtained from Sigma-Aldrich (Steinhein, Germany). HPLC gradient grade acetonitrile and methanol were purchased from Panreac (Barcelona, Spain). Hydrochloric acid was from Merck (Darmstadt, Germany) and sodium hydroxide from Fluka (Steinhein, Germany).

Background electrolyte (BGE) for CE was prepared daily by diluting a 100 mM sodium tetraborate aqueous solution and by adding a 5% (v/v) of methanol. BGE solutions were filtered through 0.45 µm nylon filters (Whatman, Clifton, NJ) and sonicated before being used. Mobile phases for LC were prepared daily by adding 1% (v/v) acetic acid or 0.1% (v/v) formic acid to water, methanol or acetonitrile.

Water was purified using an Elix 3 coupled to a Mili-Q system (Millipore, Bedford, MA) and filtered through a 0.22 µm nylon filter integrated into the Milli-Q system.

Fruit products

Different kinds of fruits (cranberries, blueberries, grapes and raisins) and 6 juices (3 based on cranberry and 3 based on grapes) were purchased from Barcelona markets. In addition, a total of 10 raw extract materials and commercial cranberry products, presented as powder capsules, syrup, sachets and natural extracts were provided by Deiters, S.L. Company (Badalona, Spain). Prior to sample treatment, fruits and liquid samples (juices and cranberry pharmaceutical syrup) were freeze-dried to achieve a fully lyophilized product with a texture similar to that of natural extracts.
commercial pharmaceutical samples (powder samples). So, samples remained 24 h inside a lyophilizer from -80 °C to room temperature, and then were kept for 6.5 h at 40 °C.

**Sample treatment**

In order to extract phenolic compounds from fruit products different sample extraction procedures were evaluated as follows:

*Sample extraction method 1.1:*³ 0.5 g of sample were dispersed in 10 mL of MeOH:H₂O (10:90 v/v) with 0.2% HCl. Then, the mixture was sonicated for 30 min and centrifuged for 15 min at 3500 rpm.

*Sample extraction method 1.2:*³⁰ Same as sample extraction method 1.1 with an additional SPE step after centrifugation. SPE was carried out using two g-bed C18 cartridges (Scharlab, Barcelona, Spain) previously conditioned with 10 mL of methanol and 10 mL of water. After loading the extract, the cartridge was washed with 10 mL of water and, finally, phenolic compounds were eluted with 10 mL of methanol.

*Sample extraction method 2.1:*³¹ 0.5 g of sample were dispersed in 10 mL of MeOH:H₂O (85:15 v/v). Then, the mixture was sonicated for 30 min and centrifuged for 15 min at 3500 rpm.

*Sample extraction method 2.2:*³¹ Same as sample extraction method 2.1, except from the extraction solvent, which in this case was acetone:MeOH:H₂O (40:40:20 v/v/v).

*Sample extraction method 2.3:*³¹ Same as sample extraction method 2.1, except from the extraction solvent, which in this case was MeOH:H₂O:acetic acid (85:14.5:0.5 v/v/v).

*Sample extraction method 3:*⁹,¹³ 0.1 g of sample were dispersed in 10 mL of acetone:H₂O:HCl (70:29.9:0.1 v/v/v) and sonicated for 30 min. After that, the mixture was centrifuged for 15 min at 3500 rpm. The supernatant was then concentrated and semi-purified using a 3 g Sephadex LH-20 cartridge (Sigma-Aldrich) preconditioned overnight in 30% methanol solution. After sample loading, the cartridge was washed with 10 mL of the 30% methanol solution and eluted with 10 mL of acetone:H₂O:HCl (70:29.9:0.1 v/v/v).
All the extracts were stored at -4 °C until analyzed. Before injection extracts were filtered through 0.45 µm nylon filters (Whatman).

**Apparatus**

**Capillary zone electrophoresis**

CZE experiments were performed in an Agilent HP 3D-CE system (Santa Clara, CA) equipped with a diode-array detector. The electrophoretic separation was carried out using fused-silica capillaries with a total length of 60 cm (effective length of 50 cm), and a 75 µm i.d. Background electrolyte (BGE) consisted of a 35-mM sodium tetraborate aqueous solution (pH 9.4) containing 5% (v/v) methanol. Capillary temperature was held at 25 °C. The BGE was degassed by sonication before use. Samples were loaded by pressure-assisted hydrodynamic injection (10 s, 3.5 kPa). Electrophoretic separations were performed by applying a capillary voltage of +25 kV. Analyses were carried out for 40 min and direct UV absorption was recorded from 190 to 310 nm. Electrophoretic data was processed at 280 nm.

New capillaries for CE were pretreated with 0.1 M hydrochloric acid for 60 min, water for 60 min, 0.1 M sodium hydroxide for 60 min, and finally they were washed with water for 60 min. At the beginning of each working session, the capillary was rinsed with 0.1 M sodium hydroxide for 30 min, water for 30 min, and with the BGE for 60 min. The capillary was rinsed with BGE for 5 min between runs. At the end of each session, the capillary was stored after rinsing with water.

**Liquid Chromatography**

LC experiments were performed on an Agilent LC 1100 System (Santa Clara, CA) equipped with a diode array detection system. Separation was carried out on a 100 mm x 4.6 mm i.d., 2.6 µm, Kinetex C18 reversed-phase column, with a 4.0 mm x 3.0 mm i.d. guard column of the same material (Phenomenex, Torrance, CA). The injection volume was 10 µL and UV absorption was
recorded from 191 to 780 nm. Chromatographic data was processed at 280 nm. Three different gradient profiles were considered for the separation of polyphenolic fractions as follows:

**Gradient method A:** was created from solvent A (H$_2$O with 0.1% formic acid) and solvent B (MeOH) as follows: 0-3 min, linear gradient from 5-25% B; 3-6 min isocratic step at 25% B; 6-9 min linear gradient from 25-37% B; 9-13 min isocratic step at 37% B; 13-18 min linear gradient from 37-54% B; 18-22 min isocratic step at 54% B; 22-26 min linear gradient from 54-95% B; 26-29 min isocratic step at 95% B; 29-29.15 min back to initial conditions at 5% B; and from 29.15-36 min isocratic step at 5% B. The flow rate was 1 mL/min.

**Gradient method B:** was created from solvent A (H$_2$O with 1% acetic acid) and solvent B (acetonitrile with 1% acetic acid) as follows: 0-2 min linear gradient from 0-5% B; 2-4 min isocratic step at 5% B; 4-30 min linear gradient from 5-100% B; 30-35 min isocratic step at 100% B; and from 35-40 min back to initial conditions at 0% B. The flow rate was 1 mL/min.

**Gradient method C:** created from solvent A (H$_2$O with 1% acetic acid) and solvent B (acetonitrile with 1% acetic acid) as follows: 0-20 min linear gradient from 0-8% B; 20-24 min linear gradient from 8-15% B; 24-44 min linear gradient from 15-40% B; 44-45 min linear gradient from 40-100% B; and from 45-46 min back to initial conditions at 0% B. The flow rate was 1 mL/min.

**Data Analysis**

MATLAB (Version 6.5) was used for calculations. Principal component analysis (PCA) was from the PLS-Toolbox.$^{32}$ A detailed description of this method is given elsewhere.$^{33}$ The plot of scores showing the distribution of the samples on the principal components (PCs) revealed patterns that may be correlated to sample characteristics, which in this case was the fruit of origin. The study of the distribution of variables from the so-called loading plot provided information dealing with their correlations as well as dependences of proanthocyanidins and polyphenolic compounds on fruit product properties.
Data matrices to be treated were generated as a function of the separation technique, separation method and sample treatment procedure. Separation techniques were coded by CE for the CZE method, and by A, B, C for the three HPLC methods. Extraction procedures were coded numerically as 1.1, 1.2, etc. in the same way as described in the sample treatment section. As a result, data matrices were referred to as CE.1.1, CE.1.2, ..., A.1.1, ..., B.1.1, C.1.1, and so on. Dimensions of these matrices were number of samples × number of time (or area) points.

HRMS (Orbitrap) analysis

In order to identify and confirm the presence of proanthocyanidins in commercial natural extracts accurate mass measurements were carried out with an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source in negative mode. Mass spectra were acquired in profile mode with a setting resolution of 30000 at m/z 400. Operation parameters were as follows: source voltage, 3.5 kV; sheath gas flow rate, 40 arbitrary units (a.u.); auxiliary gas flow rate, 10 a.u.; sweep gas flow rate, 10 a.u.; and capillary temperature, 320 °C. Default values were used for other acquisition parameters (Fourier transform (FT) Automatic gain control (AGC) target 10^6 for MS mode). Maximum injection time was set to 100 ms with two microscans for MS analysis. Full scan mass range was from m/z 100 to 2000. XCalibur software was used for data acquisition and analysis.

HPLC method A was employed for LC-ESI-LTQ-Orbitrap-MS analysis by using an Accela chromatograph (Thermo Scientific) equipped with a quaternary pump, a photodiode-array detector and a thermostated autosampler. Commercial natural extracts analyzed by LC-ESI-LTQ-Orbitrap-MS were treated with sample extraction method 3. MS data was processed by ExactFinder 2.0 software (ThermoFisher) by applying a user target database list of polyphenolic compounds including proanthocyanidins. Several parameters such as retention time, accurate mass errors and isotopic pattern matches were used to confirm the identity of compounds.
RESULTS AND DISCUSSION

Capillary zone electrophoresis

In this study, both methanol and isopropanol were evaluated as organic modifiers to obtain better electrophoretic separations of polyphenolic profiles of cranberry products. For preliminary assays, a natural cranberry extract sample was homogenized and extracted according to method 1.1. The extract was then analyzed by CZE using a 35 mM sodium tetraborate solution containing 5% of each organic solvent. Electropherograms obtained using methanol provided more peak signals, with better resolution and peak efficiency than those obtained with isopropanol. As a result, 35 mM sodium tetraborate buffer with 5% methanol was selected as the optimum BGE, and it was used in further CZE studies.

The sample treatment procedures were investigated in term of capacity of classification/discrimination of fruit samples considering the polyphenolic profiles as the analytical information. Figure 2 shows, as an example, the electropherograms corresponding to each extraction procedure. Methods 1.1 and 1.2 were focused on obtaining a general polyphenolic profile with data of various families of compounds and they were adapted from references dealing with the analysis of phenolic compounds in berries. In sample extraction method 1.2 an additional SPE clean-up and preconcentration step using C18 cartridges was added. As it can be seen in electropherograms 2A and 2B, a high absorption pattern was observed from 20 to 45 min, which was attributed to the presence of some plant pigments such as anthocyanins.

Sample treatments 2.1, 2.2 and 2.3 differed in the polarity of solvents that were used. These procedures were adapted from those previously described in the literature Sample extraction method 2.1 employed a methanol:water 85:15 \( v/v \) solution and it was focused on the recovery of the most polar fractions of compounds. Extraction method 2.2 used acetone:methanol:water 40:40:20 \( v/v/v \) solution for the extraction of less polar compounds occurring in cranberries. Additionally, methanol:water:acetic acid 85:14.5:0.5 \( v/v/v \) solution was used in method 2.3 to obtain the
anthocyanin fraction of fruits. The corresponding electropherograms (Figures 2C, 2D and 2E) showed complex profiles with multiple peaks throughout the migration time.

Finally, sample extraction method 3 was developed for a more specific recovery of proanthocyanidins from cranberry products. For that purpose, after an extraction using acetone:water:hydrochloric acid 70:29.9:0.1 v/v/v solution, sonication and centrifugation, the supernatant was concentrated and purified using a lipophilic inorganic resin (Sephadex LH-20 cartridges). Electrophoretic profiles (Figure 2F) contained more characteristic information concerning proanthocyanidins as various broad peaks corresponding to these compounds were obtained.

Principal component analysis of electrophoretic data

Sample extracts resulting from the 6 treatment procedures were analyzed by the proposed CZE-UV method. The resulting electrophoretic profiles were used as a source of information for tackling characterization and classification of fruit products. An important issue in this study was finding which procedure was able to provide the most discriminant profiles for sample featuring.

In preliminary studies, analytical data to be treated consisted of the raw electrophoretic profiles at 280 nm recorded for the set of samples. In all the cases, electropherograms showed noticeable differences depending on the sample type. For instance, pharmaceutical products (syrup, capsules and sachets) displayed more intense signals than those of fruit and juice samples. This finding was attributed to the high contents of polyphenols in the pharmaceuticals.

Raw electropherograms displayed some imperfections such as baseline drifts and peak shifts that may hinder the interpretation of PCA results. When raw data was analyzed PCA models were highly complex. Furthermore, the first PCs were focused on modeling the electrophoretic variability while descriptive information regarding samples was hidden in further PCs. In these circumstances, the assessment of solid conclusions on the sample characteristics and classifications was hindered.
PCA was preliminarily applied to electropherogram profiles of fruits and juice samples. As an example, results showed that PC1 and PC2 for data matrix CE.1.2. were mainly focused on the description of the type of product (juice or fruit), and variance dealing with fruit of origin (cranberry or grapes) was captured by PC3 and PC4 (Figure 3A). As a result, fruits and juices were classified according to the kind of fruit that they contained. Similar results were obtained when using the other sample extraction methods.

For the simultaneous evaluation of pharmaceutical and fruit products, it was found that electrophoretic profiles of pharmaceuticals were much more intense that those obtained for fruits and juices, so data normalization was needed. Only the scatter plot of scores of PC4 versus PC5 generated with data from sample extraction method 2.1 (see Figure 3B) showed the same groups of samples than the ones achieved with the model built only with fruits and juices when considering also data related to cranberry syrup, sachets and natural extract, whereas the model completely changed when the electropherograms of cranberry commercial capsules were considered and then no groups were observed.

Up to this point, the results obtained by PCA using raw electrophoretic polyphenolic profiles indicated that none of the data sets was capable of characterizing all the targeted samples according to their fruit of origin. This drawback was solved using the peak areas of the most relevant peaks as a source of analytical information, thus avoiding variability of migration times and baseline drifts. For that purpose, loading plots were evaluated for choosing the most discriminant peak signals as shown in Figure 3C regarding CE.2.1 data set. After analyzing these results, eight peaks were selected as relevant ones. As an example, Figure 3D depicts the electropherogram of a cranberry commercial capsule where the eight discriminant peak signals selected are marked with an arrow. It should be mentioned that again normalization was applied to compensate differences in intensities between fruit or juice samples and commercial products.

Characterization models using the selected peaks indicated that information of the fruit of origin was captured by PC3 and PC4. The best descriptive models were obtained from the treatment
of data set CE.2.1 (see the corresponding scatter plot in Figure 3E). As can be seen, grape-based products were located on the right part, whereas cranberry-based products appeared on the left side, grouped independently of the kind of product (fruit, juice, syrup, natural extract, capsules or sachets). Sample products belonging to other fruits, such as blueberry and raisins, were plotted far away from the two main groups corresponding to grape- or cranberry-based products.

After analyzing these results it was observed that cranberry extract 1 was not located in the area corresponding to cranberry-based products. This natural extract, used on the production of pharmaceutical products to reduce urinary tract infections, was supposed to be elaborated from cranberries. However, our results suggested that the fruit of origin could be suspicious and, in general, it was more similar to grape products. This assumption was studied thoroughly to try to confirm the authenticity of this natural extract by high resolution mass spectrometry (HRMS).

These results show that the combination of CZE with sample extraction method 2.1 and PCA analysis using eight discriminant peak signal areas is a good strategy for the characterization of food products based on their fruit of origin and could be proposed for the authentication of commercial natural and pharmaceutical products.

**Liquid chromatography**

Chromatograms corresponding to polyphenolic fractions were exploited in order to tackle the classification and authentication of cranberry-based natural extracts. For that purpose, three reversed-phase HPLC methods, which differed in the elution gradient program, were applied to obtain the compositional profiles. As an example, Figure 4 shows the chromatograms of a cranberry natural extract analyzed with each HPLC method. For HPLC method A (Figure 4A), previously developed in our research group for the characterization of wines and fruits, the chromatographic profile was complex. In the time range from 4-28 min the elution of most of phenolic acids and flavonoids was produced so this data could be useful for the fruit product characterization. HPLC method B provided also complex chromatograms with a high number of peak signals that could be
useful for characterization purposes (Figure 4B). In contrast, chromatograms from HPLC method C (Figure 4C) were simpler, at least in terms of peak signals, but it provided important information of proanthocyanidins composition.

Sample extracts of a cranberry juice according to the six sample treatment procedures are compared in Figure 5. As it can be seen, very different chromatographic profiles were obtained depending on the sample extraction. On the other hand, in general, more peak signals were here observed than in the case of CZE. Chromatograms (A), (C) and (E) were similar in shape and corresponded to methods focused on the overall recovery of polyphenols of different families. In general, it was found that the extraction of polyphenols was better in the case (E). Conversely, chromatograms (B), (D) and (F) concerned the most extraction of polyphenols of some families. Amounts recovered from simple phenolic acids and flavonoids were lower so that more characteristic peaks related to complex polyphenols such as proanthocyanidins were observed.

Principal component analysis of chromatographic data

Analytical data to be treated by PCA consisted of raw chromatograms recorded at 280 nm. Data sets were arranged in matrix structures corresponding to each HPLC method/sample extraction combination. For more efficient modeling, specific time ranges were selected to work with the most discriminant zones of chromatograms while avoiding disturbing variance from non-retained components and cleaning steps. In particular, time ranges dealing with high influence of proanthocyanidins were chosen. Here, these chromatographic zones were established from the injection of cranberry-based pharmaceutical products, which reasonably contained high concentrations of proanthocyanidins. Since proanthocyanidin absorption was almost selective at 520 nm, as corresponded to the detection of colored components, the elution range of the proanthocyanidins fraction was deduced from chromatograms recorded at this specific wavelength. As a result, time ranges chosen were as follows: HPLC Method A from 7 to 15 min, HPLC Method B from 6 to 15 min, and HPLC Method C from 12 to 20 min. Another drawback to be considered
was that chromatograms showed noticeable differences on signal intensities depending on the sample type. In this study, data was preprocessed by normalization to equalize the influence of each sample in the PCA model for a more effective comparison of product features.

The results obtained by PCA for all data matrices indicated that PC1, PC2 and PC3 were mainly focused on the description of the type of product regardless the nature of the fruit of origin (i.e., fruit, juice, natural extract, sachet, capsule and syrup) since this was a main contribution to the variance. In contrast, information dealing with the fruit of origin, if existed, was captured by further PCs. In particular, for matrices A.1.1, A.1.2, A.2.1, A.2.3, B.1.1, B.3, C.2.1, and C.2.3, PC4 and PC5 showed a noticeable sample organization as a function of the fruit of origin. Such a correlation was not observed for the rest of matrices. The most favorable data set to tackle the description and classification of fruit product was achieved with HPLC method A and sample extraction procedure 3 which combined a high chromatographic resolution with a more specific recovery of proanthocyanidins. In the PCA results corresponding to matrix A.1.1. (Figure 6), grape-based products were located on the top, whereas cranberry-based products appeared below. In addition, sample products belonging to raisins and blueberry were plotted far from grape- and cranberry-based products areas.

One cranberry natural extract (referred to as extra1), was classified as a grape-based product. In order to confirm the authenticity of this extract HRMS experiments were carried out comparing the supposedly wrong with genuine samples.

**High resolution mass spectrometry (Orbitrap) study**

As described in the introduction section, cranberries are characterized for their high content of A-type proanthocyanidins. Sample extract 1 was analyzed by UHPLC-HRMS using an LTQ-Orbitrap mass analyzer. For comparison purposes, commercial sample extract 2, which was correctly assigned as a cranberry-based product with both CZE and HPLC methodologies, was also analyzed by UHPLC-HRMS as a reference of genuine products. Results obtained regarding the
presence of procyanidins and proanthocyanidins are shown in Table 1. It should be pointed out that many other polyphenolic compounds such as catechin, epicatechin, protocatechuic acid, 2,5-
dihydroxybenzoic acid, 4-hydroxybenzoic acid, caffeic acid, coumaric acid, cinnamic acid, vanillic acid, and quercetin, among others, were confirmed by HRMS in this kind of samples.

Table 1 shows the list of procyanidin and proanthocyanidin compounds detected and identified by HRMS Orbitrap MS analyzer. Experimental m/z values and mass errors observed (always lower than 3.4 ppm) are also indicated. A- and B-type procyanidins were found in both commercial extracts whereas C-type procyanidins were only detected and confirmed in commercial extract sample 2. Regarding proanthocyanidins, only trimer BA was detected in both samples, while the presence of higher polymeric proanthocyanidin compounds with A-type bonds such as trimer AA and tetramer BAA were only detected and confirmed in commercial extract sample 2. The HRMS analysis reporting the lack of presence of A-type proanthocyanidin compounds in commercial sample extract 1 allowed us to confirm that this sample was not mainly cranberry-based extract, as suspected.

The results obtained in this work proved that both CZE and HPLC combined with PCA analysis (selecting the most relevant peak signals in CZE, and using the polyphenolic chromatographic data within a delimited time range in HPLC) resulted in a cheap, straightforward and useful strategies for authentication of natural extracts according to the food of origin. PCA results from both CZE and HPLC showed that sample extract 1 was not a cranberry-based product, which was confirmed by HRMS analysis. By means of these results, the suitability of the proposed methods for the characterization of fruit-based products has been validated and demonstrated. So the information recovered with the proposed methods could be used to easily detect adulterations and prevent misuses.

ACKNOWLEDGEMENTS

Authors wished to thank Deiters S.L. Company for providing some cranberry-based raw material extracts and commercial cranberry-based products.
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catechin and procyanidins A2 and B2 for use as standards in the 4-


Figure captions

Figure 1. Representative structure of a trimeric proantocyanidin with both A-type and B-type linkages.

Figure 2. Electrophoretic polyphenolic profiles of a natural cranberry extract obtained after applying sample extraction methods 1.1 (A), 1.2 (B), 2.1 (C), 2.2 (D), 2.3 (E), and 3 (F). Electropherograms registered at 280 nm.

Figure 3. (A) PCA result (PC3 vs PC4 score plot) using electrophoretic polyphenolic profiles obtained with sample extraction method 1.2 for fruits and juice samples; (B) PCA results (PC4 vs PC5 score plot) using electrophoretic polyphenolic profiles obtained with sample extraction method 2.1 for all samples except pharmaceutical capsules. (C) Loading plot using peak signal areas and sample extraction method 2.1. (D) Electropherogram obtained for a cranberry commercial capsule using sample extraction method 2.1. Discriminant peak signals are indicated with an arrow. (E) PCA result (PC3 vs PC4 score plot) using eight discriminant peak signal areas and sample extraction method 2.1.

Figure 4. Chromatographic polyphenolic profiles of a natural cranberry extract obtained with (A) HPLC method A, (B) HPLC method B, and (C) HPLC method C, after applying sample extraction method 1.1. Chromatograms registered at 280 nm.

Figure 5. Chromatographic polyphenolic profiles of a cranberry juice sample obtained using HPLC method A after applying sample extraction methods 1.1 (A), 1.2 (B), 2.1 (C), 2.2 (D), 2.3 (E), and 3 (F). Chromatograms registered at 280 nm.

Figure 6. PCA results (PC4 vs PC5 score plot) using chromatographic polyphenolic profiles obtained using HPLC method C and sample extraction 3.
Table 1. Proanthocyanidin Detection and Confirmation in Commercial Extract Samples by LC-HRMS.

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</table>
Figure 1
Figure 2

(A) 

(B) 

(C) 

(D) 

(E) 

(F)
Figure 3
Figure 4

(A) [Graph showing data]

(B) [Graph showing data]

(C) [Graph showing data]
Figure 6
For Table of Contents Only

Cranberry-based products?
A-type proanthocyanidins?
CZE HPLC PCA characterization