Journal of Chromatography A, 1216 (2009) 4736–4744

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Use of high-performance liquid chromatography with diode array detection coupled to electrospray-Qq-time-of-flight mass spectrometry for the direct characterization of the phenolic fraction in organic commercial juices

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ARTICLE INFO

Article history:
Received 27 October 2008
Received in revised form 2 April 2009
Accepted 9 April 2009
Available online 16 April 2009

Keywords:
Polyphenols
HPLC
DAD
ESI-Qq-TOF-MS

ABSTRACT

We have developed a direct method for the qualitative analysis of polyphenols in commercial organic fruit juices. The juices were diluted with water (50/50), filtered and directly injected. The analysis of phenolic compounds was carried out by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to photodiode array detection (DAD) and electrospray ionisation-Qq-time-of-flight mass spectrometry (ESI-Qq-TOF-MS). A unique gradient program has been optimized for the separation of several phenolic classes and the analysis time was only 5 min. The fruit juice samples were successfully analysed in positive and negative ionisation modes. In positive mode the anthocyanins were identified whereas the vast majority of polyphenols were identified using the negative ionisation mode. The sensitivity, together with mass accuracy and true isotopic pattern of the Qq-TOF-MS, allowed the identification of the phenolic compounds. Moreover, the advantage of the proposed method is the combined search of MS and MS/MS spectra, which improves the identification of compounds considerably, reducing ambiguities and false positive hits. Therefore the total fragmentation of the compound ion leading to the aglycone ion or other fragments was corroborated by MS–MS. The method was successfully employed to characterize diverse phenolic families in commercially available organic juices from four different fruits and consequently could be used in the future for the quantification purposes to compare different content of polyphenols in juices.

1. Introduction

Polyphenols, widely distributed in fruits, have great importance in the nutritional, organoleptic and commercial properties of these fruits and their derived products through their contributions to sensory-attributes of them (colour, bitterness and astringency) [1]. They affect oxidative stability of plant-derived food products, so they are also continually investigated by food technologists.

During the last few decades, the consumption of fruits has received growing interest because many epidemiological and biochemical studies have demonstrated that they possess beneficial effects on human health. Polyphenolic compounds have antioxidant activity, free-radical scavenging capacity, coronary heart disease prevention, and anticarcinogenic properties [2]. Fruit juices are an excellent source of phenolic compounds and its study is interesting as juices are easily available and affordable products for most of the population. Health is the principal concern of modern society and the food habits are part of a good health. Organic food has been giving answers to consumers and also to the attention paid to environment. Organic foods are produced according to certain production standards, meaning they are grown without the use of conventional pesticides, artificial fertilizers, human waste, or sewage sludge, and that they were processed without ionizing radiation or food additives. There are studies detailing the effects and side effects of pesticides upon the health. Even when pesticides are used correctly, they still end up in the air. The biggest study ever of organic food was completed in 2007 [3] and found that organic fruit and vegetables contain up to 40% more antioxidants than conventional equivalents.

The methodology used to analyze these phenolic compounds in fruits and fruit products, generally includes a series of steps ranging from exhaustive solvent extraction, clean-up of extracts and pre-concentration procedures to simple filtration and centrifugation in liquid samples. After the extraction procedures, the phenolic compounds are characterized and quantified. Various analytical methods have been published for the determination of these compounds in food samples. The most widely used are based on reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to UV–vis [4] detection and/or mass or tandem mass spectrometry. These latter methods, liquid chromatography–mass

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doi:10.1016/j.chroma.2009.04.022

spectrometry (LC–MS) [5] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [6], have become the best method for separation, identification and quantification of these compounds in fruits and their derived products.

In the present work, a very simple methodology to separate and identify simultaneously the most representative phenolic compounds in organic fruit juices consisting in diluting and filtering the juice followed by reversed-phase HPLC coupled to the photodiode array detection (DAD) and ESI-Qq-TOF-MS is reported for all the compounds.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were purchased from MERCK (Darmstadt, Germany). Deionized water was prepared using a Simplicity 185 system (Millipore, Bedford, MA, USA). The Tunning Mix solution to optimize the TOF parameters was purchased from Agilent Technologies.

2.2. Sample preparation

Four different organic juices, blueberry, cranberry, apple and grape, all of them, commercially available, were filtered, diluted (1:1) and injected directly into the HPLC system.

2.3. HPLC–MS/MS instrumentation and conditions

The separation and analysis of the polyphenolic compounds was performed using an HPLC Agilent 1100 Series with Phenomenex Synergy Fusion RP100A 50 mm × 2 mm (2.5 μm) column and precolumn equipped with DAD and coupled to a Qq-TOF mass spectrometer equipped with an ESI interface operating in negative and positive ion mode. (MS-Instrument: microQTOF-^{QTM} ESI-Qq-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). After several attempts for the optimal separation of the compounds using different mobile phases and also different gradients, the two mobile phases chosen consisted in A: water and B: acetonitrile, both with 0.1% (v/v) formic acid. The addition of formic acid gave the best results for the ionisation of the compounds. On the other hand, the optimized linear gradient elution program was chosen as it provided short analysis times and good chromatographic separations. The mentioned gradient was run from 1% of B at 100% in 5 min and returned to initial conditions in 0.5, maintained for another 0.5 min with a post run time of 3 min in order to equilibrate the column and for the baseline to return to the normal and initial working conditions. The flow rate was established at 0.5 ml/min. At this stage the use of a splitter was required to the coupling with the MS detector as the flow which arrived to the ESI-Qq-TOF detector had to be 0.25 ml/min or less in order to obtain repeatable results and stable spray. This splitter (1:2) provided the required separation of the working flow needed to reach the detector. The injection volume in the HPLC system was 5 μl and the temperature of the column was set at 35 °C. As above stated, the HPLC system was coupled to a TOF mass spectrometer equipped with an ESI interface operating in negative and positive ion polarity. TOF-MS transfer parameters were optimized by direct infusion with Tuning mix (Agilent Technologies). The other optimum values of the ESI-MS parameters were drying gas heater temperature, 200 °C; drying gas flow, 7 L/min; nebulizing gas pressure, 2 bar and the spectra rate was 1 Hz. The trigger time was set to 50 ms, corresponding to a mass range of 50–800 *m*/*z*. The acquisition in automated MS/MS mode was of one precursor ion. To tune the detector to optimal conditions calibration was performed with sodium formate acetate clusters (5 mM sodium hydroxide in water/isopropanol 1/1 (v/v), with 0.2% (v/v) of formic and acetic acids) in quadratic + high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior to the polyphenol identification.

The accurate mass data for the molecular ions were processed using the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formulas by using the GenerateMolecularFormula^{TM} editor. The GenerateMolecularFormula^{TM} editor uses the sigmaFit^{TM} algorithm, a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue^{TM}) for increased confidence in the suggested molecular formula [7].

3. Results and discussion

3.1. Profile and polyphenols characterization

Fig. 1a and b shows the ESI-Qq-TOF chromatograms obtained using the optimum gradient elution program and the optimal MS conditions in negative and positive polarity for the blueberry juice. The peak showed in the segment between 0 and 0.25 min corresponded to the mentioned calibrant.

In Tables 1 and 2 (negative and positive polarity modes) we have represented the *m*/*z* experimental versus the *m*/*z* calculated values of the different polyphenolic compounds at their retention times. UV data are included only for the compounds which presented absorbance in the working range. Some shifts of the absorbance bands for some of the compounds due to coelution and overlapping are observed. It is needed to point out that the given names for the polyphenolic compounds are just proposed ones after the information from MS/MS fragmentation and bearing in mind all the data reported in the literature. The later also apply for the isomers of the mentioned compounds in the way it was not possible to elucidate between them. We have expressed in bold the precursor ions and below and in plain text the product ions.

The exact masses of the ions found in the fragmentation pattern together with the errors and sigma values for both the principal/precursors ions and their corresponding fragments/products have been included. We have also quoted which juices contain these proposed compounds or their isomers.

The fragments of each product ion confirmed the proposed structures. Fragmentation patterns for all the compounds are commented in this section. The fragmentation pattern for the quercetin 3-glucuronide and quercetin and delphinidin 3-glycoside and delphinidin are shown in Figs. 2 and 3.

3.2. Interpretation of results from Table 1 corresponding to the negative polarity mode

The ions found at *m*/*z* 133 at 0.57 and 0.91 min correspond to either (L) or (D) malic acid and the latter was proposed as *m*/*z* 133 was found and its fragment at *m*/*z* 115 was consistent with the loss of a molecule of water giving the correspondent fumaric acid, also corroborated with the exact mass M−1 ($C_4H_3O_4$). Malic acid is also an organic acid existing naturally in many fruits [8]. The presence of tartaric acid was proposed in the grape juice as the ion found at *m*/*z* 149 presented a fragment at *m*/*z* 85 which is consistent with the fragmentation pattern of the mentioned organic acid. The high content of this organic acid in grapes and normally mentioned in the literature [9–11] is widely known. The presence of quinic acid was proposed as at 0.47 min and the ion was found