Talanta 159 (2016) 181–188

Contents lists available at ScienceDirect

Talanta

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

Development and validation of a RP-UHPLC-ESI-MS/MS method for the chiral separation and determination of flavanone, naringenin and hesperetin enantiomers

Irena Baranowska*, Judyta Hejniak, Sylwia Magiera

Department of Inorganic, Analytical Chemistry and Electrochemistry, Silesian University of Technology, 7 M. Strzody Str., 44-100 Gliwice, Poland

ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form
9 June 2016
Accepted 10 June 2016
Available online 11 June 2016

Keywords:
Flavanone
Naringenin
Hesperetin
Chiral separation
UHPLC-MS/MS
Plant sample

ABSTRACT

A quick and sensitive RP-UHPLC-ESI-MS/MS method for the separation of flavanone, naringenin and hesperetin enantiomers was developed. The separation of analytes was performed using a Chiralpak AD-3R column, and methanol was used as the mobile phase. Detection was carried out using a triple quadrupole tandem mass spectrometer with an electrospray ionisation source. Positive ionisation and multiple reaction monitoring (MRM) were used. The developed method showed satisfactory linearity with determination coefficients greater than 0.996 in the concentration ranges of 2.5–100.0 ng mL^{-1} for naringenin and flavanone enantiomers and 0.5–100.0 ng mL^{-1} for hesperetin enantiomers. The limits of quantification varied from 0.1 to 2.0 ng mL^{-1}. The intra-day and inter-day precisions were below 15%, and the accuracy varied from −13.6% to 13.5%. The described method was successfully applied for the chiral separation and determination of flavonoid enantiomers in real samples of spices and herbal root. Due to the occurrence of the natural compounds in the forms of free aglycones and their glycosides, these samples were subjected to hydrolysis in order to obtain free aglycones from the glycosylated forms. Acid and enzymatic hydrolysis techniques were also compared. In the course of this study, the enzymatic hydrolysis technique was selected.

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1. Introduction

Polyphenolic compounds have been identified as one of the most interesting families of natural compounds. Their structures and biological properties are widely studied because of their potential benefits for multiple aspects of human life. Some examples of the uses of flavonoids include their addition to dietary supplements and their application as components of cosmetics. However, the most significant application of these compounds seems to be in medicine. A main consequence of the polyphenol structure is antioxidant activity. Some of these compounds also have the ability to inhibit some enzymes [1]. Moreover, they have a wide spectrum of biological activities including the following: antimicrobial, antiviral, anti-allergic, immunostimulatory, estrogenic, antihepatotoxic, anti-ulcerogenic, hypolipidemic, vasodilatory, antihypertensive, antiplatelet, anti-atherogenic, cytotoxic, antineoplastic, and anticancerogenic [1,2].

Plant materials are rich sources of polyphenolic compounds. These plant metabolites play important roles in the organisms of their origin. Flavonoids take part in the reproduction process because they are responsible for pollen and sex organ development; they are also pigments that attract pollinating animals. Polyphenols support plants in temperature acclimation, protect plants from UV radiation damage and defend plants against the influence of pathogenic microbes. On the other hand, they can also support symbiotic interactions with beneficial microbes by inducing or inhibiting the expression of appropriate genes [3,4]. The function of each polyphenol is related to its distribution in the plant and is dependent on the type of compound.

The basic flavonoid structure consists of two phenyls and a heterocyclic ring with an oxygen as a heteroatom. As was previously mentioned, there are several subclasses of flavonoids that are distinguished by the presence of a double bond in the heterocyclic ring and additional functional groups. The substitution pattern of the basic structure is not the only thing affecting the biological activity; it is essential that the lack of a double bond in the heterocyclic ring results in compound chirality. This feature may be crucial for compound metabolism by determining its fit into the active sites of enzymes, which makes enantioseparation as important as the separation of flavonoids and necessitates the

* Corresponding author.
E-mail addresses: irena.baranowska@polsl.pl (I. Baranowska), judyta.hejniak@polsl.pl (J. Hejniak), sylwia.magiera@polsl.pl (S. Magiera).

http://dx.doi.org/10.1016/j.talanta.2016.06.020
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identification of individual enantiomers.

One of the chiral subclasses of flavonoids are the flavanones, which are characterized by the absence of the double bond in the heterocyclic ring and the presence of a carbonyl group. The basic compound of this group is the synthetically obtained flavanone. Naturally occurring flavanones are substituted derivatives such as naringenin and hesperetin.

Some of the flavanone aglycones occur in the bark, roots or fruits of certain plant species [5]. In plant material, flavonoids often occur as their glycosides. Quantitative determination of free aglycones requires hydrolysis. There are two methods for flavonoid glycoside hydrolysis described in the literature: chemically, as in the case of acid hydrolysis [6], or biochemically, as in the case of enzymatic hydrolysis [7].

The majority of publications in the area of flavonoid enantioseparation describe the separation of the enantiomers of a single compound. There are some papers on the chromatographic separation of the enantiomers of flavanone [8–10], naringenin [7,8,10–21] and hesperetin [8,10,19,22–24]. Nevertheless, no information was given about the separation method for these three compounds. There have also been publications describing the separation of enantiomers of more than one of the compounds; however, the separation was carried out using different conditions, e.g., hesperetin was separated on a Chiralpak AD-RH column and naringenin was separated on a Chiralcel OD-RH column [19]. Thus, there is a need for methods enabling the chiral separation of more than one flavonoid. These compounds often occur in the presence of each other, and therefore, such a method will be time- and cost-saving.

The previously developed RP-UHPLC-DAD method [25] solves the problem with the determination of naringenin and hesperetin in citrus juices. Due to the structural similarity of these compounds, sample preparation methods still leave a large number of similar compounds, especially for samples with a complex flavonoid matrix, such as herbs, vegetables and several fruits. Moreover, the structural similarity among flavonoids results in similar Vis spectra, in particular within one structural group [26]. That fact could generate the risk of misinterpretation. If not all of the compounds are defined, interferences may occur. Some authors have described the use of molecularly imprinted polymers for the extraction of selected polyphenols [27], while others have used a more selective detection system. Previously, a mass spectrometry detector was successfully used for the determination of the selected compounds in such samples [28,29].

There is a need for a more sensitive and selective method for the separation of the selected analytes (flavanone, naringenin and hesperetin). Thus, the aim of this paper is the development and validation of the first RP-UHPLC-MS/MS method for the detection and quantification of flavanone, hesperetin and naringenin enantiomers.

2. Material and methods

2.1. Chemicals and reagents

The chiral flavonoid standards are the racemates of flavanone (FLV), naringenin (NAR), and hesperetin (HST). Chrysin (CHS) was used as the internal standard (IS). All of the standards as well as the enzyme β-glucuronidase/sulfatase (crude solution from Helix pomatia, type HP-2) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). Analytical-grade hydrochloric acid and methanol were purchased from POCH (Gliwice, Poland).

The following plant materials were obtained from local stores in their dried forms: Glycyrrhizae radix (liquorice root) as well as sweet, hot and chilli peppers (as spices).

2.2. Preparation of standard solutions, calibration standards and quality control samples

Standard stock solutions were prepared at the concentration of 1 mg mL^{-1} by dissolving each compound separately in methanol. Solutions were stored at 4 °C. A working solutions were prepared by the dilution of standard stock solutions of all of the chiral flavonoids to obtain concentrations of 1 μg mL^{-1}. The IS solution was prepared analogously to obtain a concentration of 100 ng mL^{-1}. Calibration standards (CS) and quality control samples (QC) were prepared by appropriate dilution of the 1 μg mL^{-1} working solutions, and they were spiked with the IS. QC samples were prepared at the following three concentrations: 50 ng mL^{-1} (high HQC), 10 ng mL^{-1} (medium MQC) and 2.5 ng mL^{-1} (low LQC). The IS concentration was 10 ng mL^{-1}.

2.3. Instrumentation and analytical conditions

The analysis was carried out with the use of an UHPLC-MS/MS system consisting of a Dionex UHPLC system (Dionex Corporation, Sunnyvale, CA, USA), which included an UltiMate 3000 RS (Rapid Separation) pump, an UltiMate 3000 autosampler, and an UltiMate 3000 thermostable column compartment. The UHPLC system was operated with Dionex Chromeleon™ 6.8 software and coupled to an API 4000 Q TRAP tandem mass spectrometer with a TurboIon Spray source equipped with an electrospray ionisation (ESI) source (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). The data were acquired using Analyst 1.4 software.

The chiral separation was performed using the UHPLC method, which was developed and described previously [25]. A Chiralpak AD-3R column (150 × 2.1 mm; 3 μm; Daicel/Chiral Technologies, Illkirch, France) was used with methanol as the mobile phase at a flow rate of 0.5 mL min^{-1}. The separation was carried out at a temperature of 40 °C. The injection volume was 2 μL.

For the detection of the analysed compounds, the ion source operating parameters were optimized to achieve the best instrumental response.

The compound-dependent parameters, i.e., the declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP), were optimized by direct infusion of a 1.0 μg mL^{-1} standard solution of each analyte into the ion source. A Harvard syringe pump was used with the flow rate set at 10 μL min^{-1}. Continuous mass spectra were obtained by scanning from *m/z* 50 to 350.

The source-dependent parameters, such as the curtain gas (CUR), collision assisted dissociation gas (CAD), ionisation voltage (IS), source temperature (TEM), nebulizer gas (GS1), and heater gas (GS2), were optimized by flow injection analysis (FIA). The parameters were optimized by making a series of injections with different parameter values. Values that gave higher responses were chosen for the best method sensitivity. Nitrogen was used as the curtain gas, collision gas, and ion source gas.

Multiple reaction monitoring (MRM) in negative and positive ionisation modes was evaluated to choose the mode that provided better sensitivity. Two MRM pairs for each compound were chosen in accordance with the European Union (EU) standard criteria. Each pair was the transition of a precursor ion into the corresponding product ion. The product ion for which the response was higher was used as the quantitative ion, and the second one was the qualitative ion.

2.4. Sample preparation

Approximately 2.5 g of each plant material (sweet, hot and

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