Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 629–635

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Separation of quercetin, sexangularetin, kaempferol and isorhamnetin for simultaneous HPLC determination of flavonoid aglycones in inflorescences, leaves and fruits of three *Sorbus* species

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ARTICLE INFO

Article history:
Received 9 April 2008
Received in revised form 30 May 2008
Accepted 5 June 2008
Available online 11 June 2008

Keywords:
RP-HPLC
Flavonoids
Quercetin
Sexangularetin
Kaempferol
Isorhamnetin
Sorbus aucuparia L.
Sorbus aria (L.) Crantz.
Sorbus intermedia (Ehrh.) Pers

ABSTRACT

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous determination of four flavonol aglycones (quercetin, QU; sexangularetin, SX; kaempferol, KA; isorhamnetin, IS) in hydrolyzed extracts from different plant parts of *Sorbus aucuparia* L., *Sorbus aria* (L.) Crantz. and *Sorbus intermedia* (Ehrh.) Pers. Separation of the four compounds was accomplished on a C18 Lichrosphere 100 column (5 μm, 250 mm × 4.6 mm, i.d.) with a methanol gradient elution and recorded at 370 nm. The high resolution of critical bands – SX, KA and IS – was achieved with retention of the last peak (IS) in 19.5 min. The equilibration of the standard mixture by addition of HCl to an acid concentration equal that of hydrolyzed extracts injected was found to be necessary when minimizing calibration error. The correlation coefficients of all the calibration curves showed good linearity ($r > 0.9991$) over the test range. The relative standard deviation of the method was less than 2.8% for intra- and inter-day assays, and the average recoveries were between 95.5 and 102.5%. High sensitivity was demonstrated with detection limits between 0.050 and 0.085 μg/ml. The level of total aglycones was found to be in the range of 687–1515 mg/100 g of dry weight in the inflorescences, 424–1078 mg/100 g in the leaves and 20–60 mg/100 g in the fruits depending on the *Sorbus* species.

1. Introduction

The plant genus *Sorbus* comprises about 100–200 species of deciduous trees or shrubs in the subfamily Maloideae of the family Rosaceae [1,2]. Three of these – *Sorbus aucuparia* L. (rowan tree), *Sorbus aria* (L.) Crantz. (whitebeam) and *Sorbus intermedia* (Ehrh.) Pers. (Swedish mountain ash) – are most common in Polish flora. Fruits of several *Sorbus* species (*S. aucuparia*, *S. aucuparia edulis*, *S. aria*, *S. domestica* and *S. torminalis*) are used as food ingredients [3,4] and also as traditional diuretic, anti-inflammatory, antidiarrhoeal (dried fruits), vasodilatory and vitamin agents [5,6] with high antioxidant activity [4,7–9]. Like the fruits, the inflorescences of *S. aucuparia* are recommended in traditional medicine for similar disorders, i.e., as diuretic and anti-inflammatory agents [3,6]. Recent papers indicated a direct correlation between the antioxidant capacity of *Sorbus* extracts and high content of phenolic compounds [4,9]. So far, various polyphenols have been found in *Sorbus* [10], and among them, flavonoids have been reported as the main bioactive components in *S. aucuparia*, *S. aria* and *S. intermedia* [11–14]. The studies referred to several quercetin (QU) and kaempferol (KA) glycosides in different plant parts, such as inflorescences, leaves and fruits of the three species. Sexangularetin (SX) glucoside has additionally been found in *S. aucuparia* [15], while isorhamnetin (IS) conjugates have been identified in *S. intermedia* [16] and in the related species *S. domestica* [17]. However, no quantitative information is currently available regarding flavonoid content in *Sorbus*, with the exception of detailed studies of *S. aucuparia* fruit (rowanberries) [13,14,18]. The analyses of flavonol aglycones in this material have been accomplished by HPLC, but only QU and KA have been separated, and therefore, the elaborated methods are not suitable for standardization of other *Sorbus* products with more complicated aglycone patterns. Thus, with the wide-ranging use of *Sorbus* in food and traditional medicine, it is necessary to establish a more universal analytical method for quality control and determination of flavonoids.

Plant materials containing flavonoids are often standardized by hydrolysis of the glycosides (which reduces the number of analytes) and subsequent quantification of the released aglycones as prescribed in pharmacopeias [19,20]. Several methods have been used to separate and determine flavonoid aglycones in hydrolyzed media (plant extracts or other biological samples) by HPLC [21–27]. KA and IS have been reported as being especially difficult to

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doi:10.1016/j.jpba.2008.06.004

chromatographically separate, but good resolution has been achieved by several authors so fare [22–24,27–30]. There are, however, only a few reports discussing HPLC separation of SX [31–34]. Even in these cases, the reported resolution of co-occurring bands of SX, KA and IS was not satisfactory [32,33] or the separation required long analysis times [31,34]. Although SX is a relatively rare aglycone, it was found not only in *Sorbus* [15] but also in other rosaceous species like *Crataegus* [35], *Pyrus* [36], *Prunus* and *Dryas* [37], as well as in a number of other plant families, like Crassulaceae [32], Malvaceae [37], Fagaceae, Fabaceae or Cruciferae [38]. Moreover, SX was reported as constituent of numerous bee pollens and honeys with different floral origin [31,34]. The quantitation of SX should therefore be required for many plant materials.

The present work attempts to optimize the simultaneous HPLC separation of four flavonol aglycones, QU, SX, KA and IS. The data obtained enabled refinements in HPLC procedures to be made, providing enhanced resolution of the analytes during a relatively short run time. The optimized procedure was then applied to the quantification of flavonoids in hydrolyzed extracts of three Polish *Sorbus* species to evaluate the quality of their inflorescences, leaves and fruits.

2. Experimental

2.1. Plant material

All samples of inflorescences, leaves and fruits of *S. aucuparia* L., *S. intermedia* (Ehrh.) Pers. and *S. aria* (L.) Crantz. were collected in 2006 in the Botanical Garden in Łódź, and were prepared by air-drying in normal conditions and powdering. The inflorescences and leaves were collected in June, whereas the fruits were obtained in October. Voucher specimens (Nos. SAc001-SAc003, SIt001-SIt003, SAr001-SAr003) have been deposited at the Department of Pharmacognosy, Medical University of Łódź, Poland.

2.2. Chemicals, solvents and standards

Analytical-grade hydrochloric acid, methanol (POCh, Poland) and deionized water were used for hydrolysis and extraction procedures, while HPLC-grade methanol (POCh, Poland), water and orthophosphoric acid (Merck, Germany) were used in HPLC analyses. The standards of three aglycones (QU, KA and IS) were from Roth (Germany).

The fourth standard, SX, was isolated from inflorescences of *S. aucuparia*. The dried and powdered plant material (600 g) was pre-extracted with petrol and chloroform in a Soxhlet apparatus, then exhaustively extracted with boiling MeOH. The methanolic extract was evaporated, dissolved in water and re-extracted with diethyl ether (Et_2O). The Et_2O extract (5.5 g) thus obtained was separated by column chromatography on polyamide SC6 (50 g, Macherey-Nagel, Germany) (eluent: C_6H_6–MeOH, 9:1, v/v, isocratic elution) to yield two fractions of free aglycones (30 and 60 mg, respectively), which were separately purified by gel filtration on Sephadex LH-20 (25 g, Sigma–Aldrich, Germany) using MeOH as eluent. Two aglycones were isolated: SX (25 mg) and QU (50 mg). The quality of purification was verified by HPLC analysis and the purity was determined by injecting 20 μl of SX solution (at the concentration of about 0.1 mg/ml) into an analytical HPLC–UV under the conditions described in Section 2.3. As a result, the final purity was above 98% as determined by calculating the peak area percentage. The chemical structure of SX was also confirmed by ^{1}H and ^{13}C NMR studies and by physical properties.

Sexangularetin (8-*methoxykaempferol, herbacetin* 8-*methyl ether*) (*SX*): orange-yellow needles, mp 271–273 °C (MeOH) (Boetius apparatus); ^{1}H NMR δ, ppm: 12.15 (1H, s, OH-5), 8.05 (2H, d, J = 8.6 Hz, H-2′ and H-6′), 6.95 (2H, d, J = 8.6 Hz, H-3′ and H-5′), 6.26 (1H, s, H-6), 3.81 (3H, s, OMe-8). ^{13}C NMR δ, ppm: 176.11 (C-4), 159.29 (C-4′), 156.49 (C-7), 155.42 (C-5), 148.45 (C-9), 146.76 (C-2), 135.71 (C-3), 129.38 (C-2′ and C-6′), 127.46 (C-8), 121.85 (C-1′), 115.60 (C-5′), 115.33 (C-3′), 102.97 (C-10), 98.37 (C-6), 60.97 (8-OMe). The ^{1}H and ^{13}C NMR (125.7 MHz) spectra were recorded on Bruker 500 MHz instrument (in DMSO-d_6, TMS as int. standard). The data agree with those reported in the literature [35].

2.3. Chromatographic instrumentation and conditions

The analyses were carried out on a Waters 600E Multisolvent Delivery System (Waters Co., MA, USA) with a UV–vis dual wavelength absorbance detector (W 2487), a 20 μl sample injector (Rheodyne 7725 i) and a LC workstation equipped with Waters Millenium 32 version 4.0 software for data collection and acquisition. A C18 Lichrosphere 100 column (5 μm, 250 mm × 4.6 mm, i.d.) (Merck, Germany) guarded by a C18 pre-column and maintained at room temperature was used. The detection wavelength was set at 370 nm. The mobile phase consisted of solvent A (0.5%, v/v solution of orthophosphoric acid in water) and solvent B (MeOH) with the elution profile as follows: 0–10 min, 40–60% B (linear gradient, v/v); 10–21 min, 60% B; 21–23 min, 60–40% B (linear gradient); 23–26 min, 40% B; 26–30 min, 40% B (equilibration). The flow rate was 1.0 ml/min.

2.4. Sample preparation

The sample of plant material was powdered and sieved through a 0.315-mm sieve. An accurately weighed mass (200–400 mg for inflorescences, 300–500 mg for leaves and 1000 mg for fruits) was first defatted by pre-extraction with chloroform to remove waxes (after filtration, the chloroform extract was discarded) and refluxed for 1 h with 30 ml of 95% (v/v) aqueous MeOH and 9 ml of 25% (w/w) hydrochloric acid (7.7 M; 281 g/l). The hydrolysis solution thus obtained consisted of 1.8 M HCl (65 g/l) and 73% aqueous MeOH (v/v). After filtration, the sample was extracted twice with 20 ml of MeOH for 10 min. The combined hydrolysates were diluted with MeOH to 100 ml and filtered through a PTFE syringe filter (13 mm, 2 μm, Whatman, UK). The filtrate was directly injected (20 μl) into the HPLC system. Determinations were performed after three separate extractions of each sample, and each extract was injected in triplicate.

2.5. Optimization of the acid concentration in the standard solution

To evaluate the impact of acid concentration in the standard solution on the quality of separation (resolution and tailing factors), five acidified test solutions were prepared by diluting 1.6 ml of the standard stock solution (see Section 2.6.1.) with five different volumes of 15% (4.4 M; 160.9 g/l) hydrochloric acid (0, 100, 200, 300 and 400 μl, respectively) and MeOH to a final volume of 2 ml. The results are presented in Table 1.

2.6. Method validation

The analytical method was validated by determination of the linearity, precision, accuracy and stability of each analyte.

2.6.1. Standard solutions and calibration curves

The standard stock solution of the four aglycones was prepared in MeOH at the final concentrations of 35.70 μg/ml for QU, 19.15 μg/ml for SX, 20.89 μg/ml for KA and 21.26 μg/ml for IS,