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Determination of phenolic compounds in fennel by HPLC and HPLC–MS using a monolithic reversed-phase column

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method for analyzing phenolic compounds in fennel (*Foeniculum vulgare*) has been developed. The use of a monolithic column with short dimensions in combination with optimized chromatographic conditions allows over 100 samples per day to be analyzed. Chromatographic parameters such as column temperature and injection volume, were found to be crucial in obtaining adequate selectivity and resolution, consequently allowing short run times. The method was validated for the major phenolic compounds present in fennel plant material: 3-*O*-caffeoylquinic acid (3-CQA), chlorogenic acid, 4-*O*-caffeoylquinic acid (4-CQA), eriocitrin, rutin, miquelianin, 1,3-*O*-dicaffeoylquinic acid (1,3-diCQA), 1,5-*O*-dicaffeoylquinic acid (1,5-diCQA), 1,4-*O*-dicaffeoylquinic acid (1,4-diCQA) and rosmarinic acid. The limits of detection (LOD) and the limits of quantitation (LOQ) ranged from 0.05 to 1.0 μg/mL and from 0.15 to 2.5 μg/mL, respectively. With some adaptation, the extraction procedure could be even less invasive, which is useful in screening work.

Keywords: Fennel; *Foeniculum vulgare*; High-performance liquid chromatography; Phenolic compounds; *Apiaceae*

1. Introduction

Fennel (*Foeniculum vulgare*) has been used for centuries in the Mediterranean area as an aromatic herb and also in folk medicine, due to the pharmacological properties of its essential oil. Typically, fennel and its preparations are used to cure various disorders, acting as a carminative, digestive, lactogoge and diuretic agent [1]. The antioxidant and antimicrobial properties of the essential oil have been also recognized [2]. Most of the papers published are related to the analysis of essential oil constituents [3–6] and chemosystematic studies of fennel [7,8], involving the use of gas chromatography (GC). Much less work has been done using high-performance liquid chromatography (HPLC) in the analysis of fennel teas and essential oil [1,9]. In contrast, other constituents of fennel, namely the phenolic compounds, have received much less attention.

Oktay et al. [10] reported a high phenolic content of both aqueous and ethanolic fennel extracts which exhibit a strong antioxidant activity. Parejo et al. [11] identified 42 phenolic compounds, 27 of which were found in fennel for the first time. Antioxidant activities of the major phenolic compounds were determined as well [12]. A reversed-phase HPLC method for the determination of the major phenolic compounds has also been published. 3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid, 5-*O*-caffeoylquinic acid (5-CQA) or chlorogenic acid, 4-*O*-caffeoylquinic acid (4-CQA) or cryptochlorogenic acid, eriodictyol-7-*O*-rutinoside (eriocitrin), quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-glucuronide (miquelianin), 1,3-*O*-dicaffeoylquinic acid (1,3-diCQA), 1,5-*O*-dicaffeoylquinic acid (1,5-diCQA), 1,4-*O*-dicaffeoylquinic acid (1,4-diCQA) and rosmarinic acid were the analytes of interest [13]. From the mentioned compounds, dicaffeoylquinic acids have given rise to particular interest in recent years. Besides their known antioxidant and radical scavenging activity [14–16], the effect of reducing serum and liver triglycerides has been also observed [17] as well as anti-HIV activity of dicaffeoylquinic acids [18,19], making fennel a potentially good source of compounds with pharmacological activity. There are several papers published about the determination of dicaffeoylquinic acids and other phenolic compounds from different

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plant materials [20–28], to name a few. Some of the work on phenolic compounds was also done on fennel samples [11–13], as mentioned.

There are many factors, which affect the biosynthesis of compounds in plants, growth conditions, plant development stage and genotype being the most significant. As a consequence, the content of any compound in a plant varies among the growth location, harvest time and of course, the genotype. Suitable analytical methods should be chosen or developed in order to characterize the plant material for further pharmacological studies or applications. Important features of such methods are also reliability and high sample throughput. Proper analytical methods are also very useful in the selection of genotypes of a plant species for purpose-targetted traits, for example high phenolic content. Minimally invasive methods are preferred in the monitoring process during the growth season, requiring minimal sample amount in order to provoke minimal plant damage. Sensitivity is thus a key feature of such methods.

Due to the complexity of phenolic content in fennel samples, a gradient elution, as employed in the work of Parejo et al. [13], is a logical choice. In the mentioned work the separation took place on a long (250 mm), packed-bed analytical column, requiring 40 min to separate the 10 peaks involved, plus the conditioning time needed between injections, due to gradient elution. The aim of the present work was to develop a more efficient analytical method for the determination of major phenolic compounds in fennel samples. It was mainly achieved by using a monolithic analytical column. Monolithic columns generate significantly less backpressure in comparison to packed-bed columns of the same dimensions, allowing a higher mobile phase linear velocity. In addition, the height equivalent to a theoretical plate (HETP) in monolithic columns is less dependent to mobile phase linear velocity as it is in packed-bed columns [29], meaning less separation performance loss at high-speed analyses. Peak identity and purity was checked and confirmed by fragmentation spectra using tandem mass spectrometry. Other method characteristics like reliability and sensitivity were also considered and confirmed by validation data.

2. Materials and methods

2.1. Instrumentation and analytical conditions

The analyses were performed using a Finnigan Surveyor HPLC system equipped with a UV–vis detector (Thermo Electron Corporation, San Jose, CA, USA) using a 50 mm light-pipe flow cell and data acquisition software Excalibur version 1.3 (Thermo Electron Corporation). The monolithic column in use was Chromolith Performance RP-18e (octadecylsilyl silica) with dimensions 100 mm × 4.6 mm i.d. (Merck, Darmstadt, Germany). UV detection was performed at 330 nm. The flow rate during analysis was constant with 3.0 mL/min and the injection volume was 5 μL. Mobile phase A consisted of 0.1% formic acid and 5% acetonitrile (v/v), while mobile phase B consisted of 0.1% formic acid and 90% acetonitrile (v/v). The mobile phase gradient is displayed in Table 1.

Table 1
Mobile phase composition

Time (min)	A (%)	B (%)	Flow (mL/min)
Analysis			
0.0	100	0	3.0
10.0	80	20	3.0
Conditioning			
10.1	70	30	5.0
11.0	70	30	5.0
11.1	100	0	5.0
13.5	100	0	5.0

For peak purity and identification the HPLC system was coupled to the ion-trap mass spectrometer (MS) Finnigan Mat LCQ (Thermo), equipped with an ESI ion source in negative-ion mode. The mobile phase flow was split before the MS in order to give an effective flow of 0.6 mL/min to the MS. The spray voltage was set to 4.0 kV and capillary temperature to 250 °C. The sheath and auxiliary gas flow rates were set to 65 and 10 (arbitrary units), respectively. During MS/MS experiments the collision energy was set between 20 and 35% (arbitrary units).

2.2. Chemicals and materials

Acetonitrile (HPLC grade), formic acid (p.a. grade) and reference standards of chromatographic purity (chlorogenic acid, quercetin-3-*O*-rutinoside and rosmarinic acid) were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q apparatus (Millipore, Milford, MA, USA). Eriodictyol-7-*O*-rutinoside was purchased from Fluka (Buchs, Switzerland). Quercetin-3-*O*-glucuronide and 1,5-*O*-dicaffeoylquinic acid were isolated in the laboratory from fennel plants using preparative HPLC and their purity was determined by NMR. Fennel plant samples were collected on various locations in the region of Istria (Slovenia and Croatia) during the flowering period (July 2005).

2.3. Preparation of standards

Stock solutions of individual standards were prepared in the concentration of 1.0 mg/mL in 50% methanol (v/v). The solutions were properly mixed and diluted in 20% methanol (v/v) in various concentrations for external standard calibration, linearity check and determination of limits of detection (LOD) and limits of quantitation (LOQ). For standard solution, the individual analyte concentrations were 20 μg/mL. For linearity check, the analyte concentrations ranged up to 100 μg/mL while, for LOD determination, the standards were diluted down to 0.01 μg/mL.

2.4. Sample preparation

Fennel plants were dried for 48 h at 50 °C in a forced-flow dessicator. After dessication, the plants were finely ground prior to extraction. Two-hundred and fifty milligrams of dried sample were extracted in 25 mL of 20% methanol (v/v) in a ultrasonic

bath at room temperature for 30 min. After extraction the sample solutions were filtered through a 0.45 μm membrane filter (Millipore, Milford, MA, USA) and injected in the chromatograph. The dried samples were also assayed for their water content by toluene distillation according to European Pharmacopoeia [30].

For recovery (extraction efficiency) tests used in accuracy determination, the sample solutions after extraction were centrifuged at $3000 \times g$ for 15 min, the supernatant filtered and analyzed. The pellet was then resuspended, reextracted and analyzed as in the case of the first extraction.

2.5. Validation of the method

Quantitation and method validation were performed using UV detection. For sample quantitation purposes, 3-CQA and 4-CQA were determined as chlorogenic acid, while 1,3-diCQA and 1,4-diCQA were determined as 1,5-diCQA.

Injection precision was determined by six injections of standard solution. For accuracy, six replicates of a homogeneous leaf sample were extracted two consecutive times and the extracts analyzed. Accuracy was calculated as the ratio between analyte concentration from the first extraction step and the sum of analyte concentrations from both extraction steps, and expressed as a mean recovery or extraction efficiency. Repeatability and intermediate precision were also tested on a homogeneous leaf sample. Six replicates were assayed for repeatability, while three replicates were assayed on each of the three consecutive days for intermediate precision. The determination of the limits of detection and quantitation was based on the signal-to-noise ratios of 3 and 10, respectively. Linearity was checked in three replicates in the range between the limit of quantitation and 100 μg/mL of each analyte, at least in six points. Correlation coefficients were calculated with intercept values set at zero. Validation of injection precision, sensitivity and linearity was performed on analytes with available standards: chlorogenic acid, eriocitrin, rutin, miquelianin, 1,5-diCQA and rosmarinic acid. For accuracy, repeatability and intermediate precision the samples were spiked with eriocitrin and rosmarinic acid at 20 μg/mL each, due to the lack of those compounds in the samples examined.

3. Results and discussion

3.1. Peak separation and identification

Reversed-phase liquid chromatography using acetonitrile gradient under acidic mobile phase conditions is a common practice in the separation of complex samples. In the case of phenolic compounds in fennel it is an appropriate type of selectivity, as evidenced by the elution pattern in the paper published by Parejo et al. [13]. However, in that case the separation was quite time-consuming, as already mentioned, owing to a moderate mobile phase linear velocity, probably dictated by the backpressure generated by the long analytical column and/or the separation performance loss at higher velocities. In addition, relatively large injection volumes were used, generating excessive peak broadening. The injection volume could be an important determinant of the peak width and consequently of the

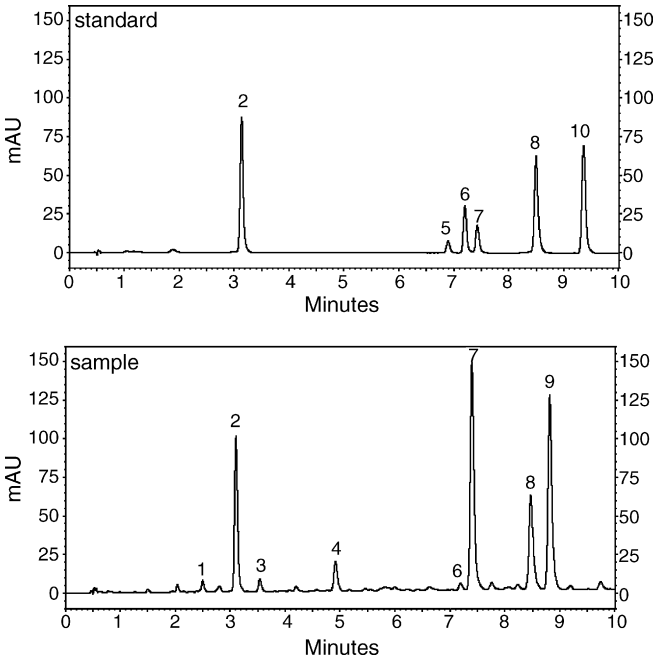

Fig. 1. Chromatograms from standard solution and fennel sample. Peak identification: (1) 3-caffeoylquinic acid, (2) chlorogenic acid, (3) 4-caffeoylquinic acid, (4) 1,3-dicaffeoylquinic acid, (5) eriocitrin, (6) rutin, (7) miquelianin, (8) 1,5-dicaffeoylquinic acid, (9) 1,4-dicaffeoylquinic acid and (10) rosmarinic acid.

overall chromatographic resolution, especially when the solution injected is a stronger-eluting solvent than the mobile phase [31]. Hence, the injection volumes in the work presented were kept as low as 5 μL, avoiding unnecessary peak broadening. The lack of sensitivity was compensated using a 50 mm light-pipe flow cell in the UV detector. The effectiveness of the separation presented (Fig. 1) was confirmed by MS and MS/MS data (Table 2). The data was in agreement with previously published papers [11,28,32–34] regarding the identity and purity of the peaks involved, based on their fragmentation patterns.

The application of a monolithic column of short dimensions (100 mm) made the separation performing at a low backpressure possible. Even at a flow rate of 5.0 mL/min during column conditioning the backpressure never exceeded 2000 psi, which in turn shortened the conditioning time between analyses. Indeed, during analysis the flow rate was kept at 3.0 mL/min because peak resolution was decreasing at higher flow rates.

Table 2
Peaks of value from negative ESI MS and MS/MS data of the analytes

Analyte	MS parent ion (*m/z*)	MS/MS product ions (*m/z*)
3-Caffeoylquinic acid	353	191, 179
Chlorogenic acid	353	191, 179
4-Caffeoylquinic acid	353	191, 173
1,3-Dicaffeoylquinic acid	515	353, 191, 179
Eriocitrin	595	459, 287, 151
Rutin	609	301, 151
Miquelianin	477	301, 151, 135
1,5-Dicaffeoylquinic acid	515	353, 191
1,4-Dicaffeoylquinic acid	515	353, 191, 173
Rosmarinic acid	359	197, 179, 161

Table 3
Method validation parameters

Analyte	Injection precision (% R.S.D., $n=6$)	Accuracy (%)	Repeatability (% R.S.D., $n=6$)	Intermediate precision (% R.S.D., $n=9$)	LOD (μg/mL)	LOQ (μg/mL)	Linear range (μg/mL)	Regression coefficient (R^2)
3-Caffeoylquinic acid	–	97.3	3.1	3.6	–	–	–	–
Chlorogenic acid	0.1	97.1	1.8	2.8	0.05	0.15	0.15–100	0.9997
4-Caffeoylquinic acid	–	96.9	3.7	3.4	–	–	–	–
1,3-Dicaffeoylquinic acid	–	97.6	2.8	2.3	–	–	–	–
Eriocitrin	0.6	99.1	1.9	2.8	1.0	2.5	2.5–100	0.9998
Rutin	0.6	98.4	1.7	2.6	0.15	0.7	0.7–100	0.9993
Miquelianin	0.9	96.1	2.8	4.3	0.4	1.2	1.2–100	0.9996
1,5-Dicaffeoylquinic acid	0.2	98.8	3.4	2.4	0.1	0.3	0.3–100	0.9994
1,4-Dicaffeoylquinic acid	–	98.5	3.5	3.7	–	–	–	–
Rosmarinic acid	0.4	98.2	4.0	4.6	0.1	0.3	0.3–100	0.9997

LOD, limit of detection; LOQ, limit of quantitation.

Besides the stationary and mobile phase, column temperature also affects analyte partition [35]. The optimal column temperature was found to be 22 °C. At higher temperatures some resolution problems arose, especially between rutin and miquelianin, while at lower temperatures no particular improvement was observed and the backpressure slightly increased.

3.2. Method validation

The validation data (Table 3) confirm the feasibility of the method for its purpose. Injection precision, accuracy and linearity by far exceed the primary acceptance criteria, which were R.S.D. $\leq 3\%$, recovery $\geq 90.0\%$ and $R^2 \geq 0.995$, respectively. Sensitivity for most analytes has also proved to be very good, because for all analytes involved in the study the sensitivity was much higher in comparison to the method previously published [13]. The use of a sensitive UV detector with a long optical path and the prevention of peak broadening by means of low injection volumes are most likely the main reasons for a good method sensitivity.

Despite the good injection precision, the results for repeatability and intermediate precision exhibited more variation. A feasible explanation for the fluctuations in replicate analyses is the degree of sample (in)homogeneity and also the conspicuous amount of essential oil present in dried fennel plant material which could impair sample wettability and consequently the extraction.

3.3. Sample analyses

Several fennel plants from different locations were analyzed for their phenolic content. Some of the most representative results are shown in Table 4. The content of individual analytes in fennel plant material ranged from below 10 to over 1700 mg/100 g of dry weight, showing significant differences in comparison to previously published results regarding the content range [13], where the individual analytes approximately ranged between 30 and 200 mg/100 g of dry weight. Eriocitrin and rosmarinic acid were not found in any of the samples analyzed, while miquelianin content was substantially higher. A feasible explanation for such differences among fennel samples are the differences in growing conditions in different regions and also the differences in the plant genotypes.

Table 4
Sample analyses. The values are expressed in mg/100 g of sample dry weight

Analyte	Sample no.		
	1	2	3
3-Caffeoylquinic acid[a]	13.7	15.6	9.6
Chlorogenic acid	232.5	75.6	61.4
4-Caffeoylquinic acid[a]	22.1	6.7	7.9
1,3-Dicaffeoylquinic acid[b]	58.1	24.6	15.2
Eriocitrin	n.d.	n.d.	n.d.
Rutin	35.6	19.4	18.0
Miquelianin	1752.0	915.8	985.2
1,5-Dicaffeoylquinic acid	205.1	67.7	27.1
1,4-Dicaffeoylquinic acid[b]	377.8	190.8	110.5
Rosmarinic acid	n.d.	n.d.	n.d.

n.d., not detected.
[a] Quantified as chlorogenic acid.
[b] Quantified as 1,5-dicaffeoylquinic acid.

4. Conclusions

A high-throughput reversed-phase HPLC method for the determination of phenolic compounds in fennel plants has been developed by exploiting the benefits of a monolithic column. Higher mobile phase linear velocities are appliable without a visible performance loss at that conditions in comparison to packed-bed columns. Injection volume and column temperature were also important parameters in keeping appropriate peak width and selectivity, respectively. The separation effectiveness was confirmed by MS and MS/MS data. As a result, the whole chromatographic cycle including separation and column conditioning requires only 13.5 min, allowing over 100 runs to be performed in a 24-h period.

Although satisfactory, repeatability and intermediate precision for most analytes exhibited more variation than expected. The most feasible explanation for such phenomenon are problems associated with sample wettability due to the presence of essential oil, besides sample (in)homogeneity. For a more thorough and thus more robust extraction procedure, more exper-

imental investigation is needed. However, the simplicity of the presented extraction procedure outweighs its drawbacks. Although 250 mg sample aliquots were used during the experimental work, the sample weights and extraction volumes could be proportionally diminished by over an order of magnitude or sample aliquots only could be even further reduced (i.e. 10 mg of sample per 2 mL of extraction solvent), since injection volumes and method sensitivity do not represent an issue. In such a case a much less invasive sampling of plants during the growing period could be performed, which is often an important feature in screening work.

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