Talanta 82 (2010) 1271–1280

Contents lists available at ScienceDirect

Talanta

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

Development and application of UHPLC–MS/MS method for the determination of phenolic compounds in Chamomile flowers and Chamomile tea extracts

Lucie Nováková [a,*], Anna Vildová [b], Joana Patricia Mateus [c], Tiago Gonçalves [d], Petr Solich [a]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Crop Production, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences, Kamýcká 129, 165 21 Prague 6, Czech Republic
[c] Instituto Superior Ciencias de Saúde Egas Moniz, Caparica, Portugal
[d] Faculty of Pharmacy, University of Porto, Portugal

ARTICLE INFO

Article history:
Received 26 March 2010
Received in revised form 4 June 2010
Accepted 28 June 2010
Available online 31 July 2010

Keywords:
UHPLC–MS/MS
Chamomile
Tea
Flowers
Phenolic compounds
Flavonoids

ABSTRACT

UHPLC–MS/MS method using BEH C18 analytical column was developed for the separation and quantitation of 12 phenolic compounds of Chamomile (*Matricaria recutita* L.). The separation was accomplished using gradient elution with mobile phase consisting of methanol and formic acid 0.1%. ESI in both positive and negative ion mode was optimized with the aim to reach high sensitivity and selectivity for quantitation using SRM experiment. ESI in negative ion mode was found to be more convenient for quantitative analysis of all phenolics except of chlorogenic acid and kaempherol, which demonstrated better results of linearity, accuracy and precision in ESI positive ion mode. The results of method validation confirmed, that developed UHPLC–MS/MS method was convenient and reliable for the determination of phenolic compounds in Chamomile extracts with linearity >0.9982, accuracy within 76.7–126.7% and precision within 2.2–12.7% at three spiked concentration levels. Method sensitivity expressed as LOQ was typically 5–20 nmol/l.

Extracts of Chamomile flowers and Chamomile tea were subjected to UHPLC–MS/MS analysis. The most abundant phenolic compounds in both Chamomile flowers and Chamomile tea extracts were chlorogenic acid, umbelliferone, apigenin and apigenin-7-glucoside. In Chamomile tea extracts there was greater abundance of flavonoid glycosides such as rutin or quercitrin, while the aglycone apigenin and its glycoside were present in lower amount.

1. Introduction

Chamomile (*Matricaria recutita* L.) is a medicinal plant often used for its analgesic, anti-allergic, anti-spasmodic, antibacterial, anti-inflammatory and sedative properties. Its essential oil containing volatile compounds including terpenoids such as azulene, chamazulene and α-bisabolol is the most commonly used [1]. Recently, the attention has been paid also to non-volatile fraction of the extract, especially phenolic compounds for their spasmolytic and antiphogistic activity [2] and for antioxidant activity. Antioxidant activity of Chamomile was found to be 0.42 of quercetin equivalent or 1.30 of trolox equivalent [3].

As described in literature, flavonoid glycosides represent the major fraction of water-soluble components in Chamomile. Apart from the glycosides, flavonoid aglycones were found in great variety among lipophilic constituents [2]. Apigenin and later apigenin-7-glucoside were the first flavonoid compounds isolated from Chamomile [4,5]. Phenolic fraction of Chamomile might further contain phenolic acids: chlorogenic acid, caffeic acid, vanillic acid, syringic acid and anisic acid, coumarins: umbelliferone and herniarin and flavonoids including aglycones and/or glycosides from: isorhamnetin, luteolin, quercetin, apigenin, patuletin and some others [2,3]. There are differences among individual Chamomile plant types and similarly, the process of drying and tea product preparation might influence the content of phenolic compounds.

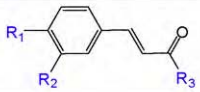
Only few analytical methods for qualitative and quantitative evaluation of the Chamomile extracts have been published so far. Phenolic fraction of Chamomile was previously analyzed by means of reversed phase chromatography with UV detection [6–10] or MS detection [6,7,11,12] or by capillary electrochromatography (CEC), capillary zone electrophoresis (CZE) and μ-HPLC, which were compared by Fonseca et al. [13]. CEC was found to be a powerful tool enabling high efficiency and resolution however on the other hand an impractical approach which requires very long column conditioning and use of fragile, often self-made capillary columns, that makes CEC time-consuming and non-robust method [13]. HPLC methods on the other hand were typically very time-consuming,

* Corresponding author. Tel.: +420 495067381; fax: +420 495067164.
E-mail address: nol@email.cz (L. Nováková).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.06.057

Flavonoids	R1	R2	R3	R4	R5
quercetin-3-glucoside	glucose	OH	OH	H	OH
rutin	rhamno-glucose	OH	OH	H	OH
apigenin-7-glucoside	H	H	OH	H	glucose
quercitrin	rhamnose	OH	OH	H	OH
quercetin	OH	OH	OH	H	OH
luteolin	H	OH	OH	H	OH
kempherol	OH	H	OH	H	OH
apigenin	H	H	OH	H	OH
isorhamnetin	OH	O-CH_3	OH	H	OH

Phenolic acids	R1	R2	R3
caffeic acid	OH	OH	OH
chlorogenic acid	OH	OH	quinate



Coumarin
umbelliferone

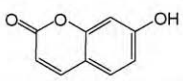


Fig. 1. Structures of phenolic compounds under study.

which means about 50 min for one analytical run [6,7,10,11] and they were not validated for quantitative purposes, which might be caused also by quite old origin of the methods [6,7,10,11]. A combination of two different methods was often used for the quantitation of various groups of phenolics such as phenolic acids and coumarins [7] or phenolic acids and flavonoids [14] or solely phenolic acids were determined in Chamomile samples [15].

Only identification and description of phenolic profile of the Chamomile tea was performed by means of HPLC–ESI-MS [3]. Some of developed methods were focused on the determination of one of Chamomile's the most abundant compounds – apigenin and its derivatives using CZE [16], HPLC [17] or HPLC–MS [18].

Sensitive, efficient and selective methods for the quantitative evaluation of phenolic fraction of extracts of Chamomile (*M. recutita*) are still missing in scientific literature as it is demonstrated by low number of given references and also by their old origin. The development of such method might be a difficult task due to variability of phenolic compounds present in phenolic fraction and due to great differences in their concentration and polarity. Moreover, individual Chamomile varieties may significantly differ in quantitative phenolic profile as well. High selectivity of detection, high separation efficiency and wide linear range of the determination are therefore necessary. In this paper, an original method using coupling of high separation efficiency of UHPLC with tandem mass spectrometry using triple quadrupole analyzer for its wide linearity range was developed. ESI in both positive and negative ion mode was compared in analysis of 12 phenolic compounds (Fig. 1). Finally, the method was applied for the comparison of phenolic content of Chamomile flowers and Chamomile tea extracts.

2. Experimental

2.1. Chemicals and reagents

Working standards of chlorogenic acid, caffeic acid, umbelliferone, rutin, quercetin-3-glucoside, apigenin-7-glucoside, quercitrin, quercetin, luteolin, kaempherol, apigenin and isorhamnetin were used for the purpose of this study. All compounds were obtained from Sigma–Aldrich (Prague, Czech Republic). Formic acid LC–MS grade was purchased by Sigma–Aldrich as was LC–MS grade methanol. Ultra-pure water was obtained with a Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and met the requirements of the European Pharmacopoeia.

2.2. Chromatography

The Acquity UPLC system (Waters, Prague, Czech Republic) was used for the purposes of this study. The system consisted of an ACQ-binary solvent manager, an ACQ-sample manager and an ACQ column thermostat, where the analytical column was kept at 30 °C. All injected solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill mode was set up to inject 3 μL. Acetonitrile was used as a strong wash, and 20% methanol in water was used as a weak wash solvent. The analytes were separated on Acquity BEH C18 (100 × 2.1 mm, 1.7 μm) analytical column using gradient elution by mobile phase consisting of 0.1% formic acid and methanol at flow-rate 0.45 ml/min.

An MS/MS triple quadrupole system Quattro Micro (Micromass, Manchester, GB) was equipped with a Multi-Mode Ionisation Source (ESCI). Ion source in ESI negative ion mode was set up as follows: capillary voltage: 2000 V, ion source temperature: 130 °C, extractor: 3.0 V and RF lens: 0.5 V. The desolvation gas was nitrogen at a flow of 400 l/h and a temperature of 450 °C. Cone voltage (CV) was set up individually for each analyte. Nitrogen was also used as a cone gas (70 l/h) to prevent contamination of the sample cone. Triple quadrupole was set up to the SRM (selected reaction monitoring) experiment. Argon was used as the collision gas, and collision energy (CE) was optimised for each analyte individually. MassLynx 4.1 software was used for MS control and data gathering. QuanLynx software was used for data processing, peak integration and linear regression.

2.3. Preparation of standard solutions and samples

Reference standard solutions of phenolic compounds were prepared as follows: stock solutions of chlorogenic acid, caffeic acid, umbelliferone, rutin, quercetin-3-glucoside, apigenin-7-glucoside, quercitrin, quercetin, luteolin and kaempherol were prepared at 0.01 mol/l concentration in methanol. Apigenin and isorhamnetin were prepared at 0.001 mol/l concentration in methanol for solubility reasons. The stock solutions were unified in one solution, which finally contained 60% methanol in water. Further dilutions for SST and calibration measurements were performed by 60% methanol. Stock solutions of phenolic compounds were kept in dark and cool ambient (4 °C).

Thirty-two samples of Chamomile anthodia (*M. recutita* L.) 2 g (with accuracy ± 0.001 g) were used as a plant material. 16 samples

of different kinds of bio and conventional Chamomile tea extracts from Czech Republic, Denmark, Great Britain, Italy and Poland and 16 samples of Chamomile anthodia in bio and conventional quality, concretely primary production of the Czech diploid variety ($2n = 18$ chromosomes) Bohemia and the Slovak tetraploid ($4n = 36$ chromosomes) variety Goral obtained from the experimental farm of the Department of Crop Production, Faculty of Agrobiology, Natural and Food Resources, University of Life Sciences in Prague.

The Czech diploid ($2n = 18$ chromosomes) variety Bohemia was licensed in 1952 and it belongs to chemocultivar A. Bohemia is classified as the bisabololoxid genotype. The Bohemia arose from selection of Chamomile autochthonic population, cultivated in the Czech Republic. The first name of the variety was *Matricaria chamomilla* L. form Culta Provincialis Bohemica. Later the variety was renamed to Bohemia. The Chamomile of the variety BOHEMIA has the certification trade mark no. CZ/00411/PDO – "Chamomilla Bohemica" Bohemia typically contains 1.2% of essential oil, 8–14% of farnesene, 0.3–5% of (−)-α-bisabolol, 36% of (−)-α-bisabolol oxide A 36%, 1–5% of (−)-α-bisabolol oxide B, 21% of chamazulene, 10–25% of cis-spiroether.

The Slovak tetraploid ($4n = 36$ chromosomes) variety Goral was bred at University of Pavel Josef Šafárik, Faculty of Natural Sciences, University of Pavol Josef Šafarik, Košice and licensed in 1990. This variety belongs to the bisabolol and bisabololoxide genotype group of varieties. The Goral variety contains up to 1–1.2% of essential oil, 21% of chamazulene, up to 25% of bisabolol and other components.

Methanolic extracts of 32 Chamomile samples were also provided by University of Life Sciences in Prague. Samples of Chamomile were ground and approximately 2 g (with accuracy ± 0.001 g) was weighed into 100 mL volumetric flasks. Samples were poured over with 60 mL methanol and inserted into a shaking apparatus (for 30 min). Then the samples were left to stand for 5 days in the darkness and afterwards they were adjusted to 100 mL with methanol. After the filtration through yellow filter (medium density) the extracts were used for subsequent analyses. They were filtrated through 0.20 μm PTFE filter and further diluted 5 times prior to analysis using 50% methanol in order to achieve 60% of methanol in water in final sample.

The one-factor analysis of variance (ANOVA) at the significance level $\alpha = 0.05$ and 0.01 (in the program SAS version 6.12., SAS Institute Inc., Cary, NC, USA) was used for statistical evaluation of comparison of active compounds in commercial teas samples and chamomile flowers.

2.4. System suitability test (SST) and validation

The method was validated according to the requirements of ICH guidelines [19] using standard mixtures (SST, linearity, LOD and LOQ) and Chamomile extract samples (accuracy and precision).

SST was performed under the optimized chromatographic conditions for the separation of 12 phenolics in order to verify method repeatability. The repeatability of the injection of reference standard solution (retention time and peak area) was established in UHPLC–MS/MS measurements. Details for determination and limits of individual parameters are given in Pharmacopoeias [20,21].

Following validation parameters were determined: range, linearity, LOD, LOQ, precision, accuracy and selectivity. Linearity was established using mixed standard solutions for each compound (typical tested range was 0.01–10 μmol/l). The range was extended for chlorogenic acid, apigenin-7-glucoside and apigenin, as they occurred at high concentrations in measured samples. Method precision was tested at three concentration levels in three replicates using Chamomile flowers and Chamomile tea extract samples in order to calculate % of RSD of the determination, which describes the closeness of agreement between series of measurements.

The Chamomile extract samples spiked with standard solution at three concentration levels were used for the determination of method accuracy, thus the method of standard addition was used. The samples were spiked in triplicates and they were run in three replicates. The agreement between theoretical and measured value was confronted with appropriate guidelines [19].

Method selectivity was evaluated using a comparison of standard calibration curves diluted in 60% methanol versus matrix calibration curves using Chamomile flower sample number 1 prepared according to Section 2.3 (the sample, which contained low amount of phenolics, therefore it simulated blank sample to be spiked). A significant difference between the slope of standard and matrix calibration curves would mark possible matrix effects. Good agreement between the slope of standard and matrix calibration curve means sufficient selectivity. No other approach for evaluation of selectivity was possible, as blank samples of Chamomile extracts without any content of phenolic compounds were not available. Carryover effects among individual samples were checked by the injection of blank sample – 60% methanol in water, the dissolution media for calibration solutions and dilution media for Chamomile extracts.

3. Results and discussion

3.1. Development of UHPLC–MS/MS method

Acquity BEH C18 analytical column was chosen for the separation of 12 selected phenolic compounds based on our previous experience [22,23]. Development of separation method addressed two important issues – the separation of luteolin and kaempherol, as the molecular weight 286 is the same for both structures and secondly, the separation of caffeic and chlorogenic acid, as in fact, chlorogenic acid contains caffeic acid in its structure and this ester bond might easily be fragmented. Efficient separation is not definitely necessary in MS/MS detection however it brings further enhancement of sensitivity and selectivity of the analysis. Gradient elution with mobile phase consisting of methanol and formic acid 0.1% was used. The formic acid as an additive also played an important role in the enhancement of ionization efficiency.

The phenolic compounds were eluted according to their polarity and molecule size – first phenolic acids, relatively small molecules with polar groups, which are not strongly retained on C18. The separation of chlorogenic and caffeic acid was better enabled when an isocratic step was applied within the first 4 min using mobile phase 88.5% formic acid 0.1% and 11.5% of methanol. Secondly, flavonoid glycosides were eluted, as they contain many polar groups including sugars and other hydroxy groups, however they are quite large molecules. Finally flavonoid aglycones were eluted as non-polar compounds according to the number of hydroxy groups and their position (see Fig. 1). Using gradient elution from 88.5 to 50% formic acid 0.1%/methanol with isocratic step the separation and elution of 12 phenolic compounds was achieved in reasonable time (19 min).

The key issue of UHPLC–MS/MS method optimization was the choice of standard and sample dissolution media as a great variability in response was observed (see Fig. 2). Based on the theory of chromatography, dissolution media of composition close to the mobile phase should be used, which in our case means approximately 90% of formic acid 0.1%/10% methanol. Such dissolution media enables appropriate peak shape of firstly eluted compounds such as caffeic acid and chlorogenic acid. However, 10% methanol did substantially decrease method sensitivity for non-polar aglycones of flavonoids such as quercetin, luteolin, kaempherol, apigenin and isorhamnetin. Method sensitivity was much less affected in intermediate-polarity compounds, while it was almost not affected in polar phenolic acids. The sensitivity

Table 1
Optimal conditions for individual SRM transitions of 12 phenolic compounds – the first one displayed in bold is the one used in polarity switching mode.

Compound	Ionization mode	Precursor ion > fragment ion	Dwell time	Cone *V*	Collision *E*	tR
Chlorogenic acid	**ESI+**	**354.8 > 163.1**	**0.1**	**30**	**15**	**4.16**
	ESI–	353.0 > 190.9		20	15	
Caffeic acid	**ESI–**	**178.9 > 135.0**	**0.1**	**30**	**15**	**4.69**
	ESI+	181.1 > 163.1		30	10	
Umbelliferone	**ESI–**	**161.1 > 133.0**	**0.1**	**35**	**20**	**7.67**
	ESI+	162.9 > 107.1		30	20	
Quercetin-3-glucoside	**ESI–**	**463.1 > 301.2**	**0.1**	**35**	**20**	**11.66**
	ESI+	464.9 > 303.1		30	15	
Rutin	**ESI–**	**609.2 > 300.5**	**0.1**	**50**	**35**	**11.72**
	ESI+	610.9 > 303.1		30	20	
Apigenin-7-glucoside	**ESI–**	**431.2 > 268.6**	**0.1**	**45**	**35**	**12.76**
	ESI+	432.9 > 271.1		30	20	
Quercitrin	**ESI–**	**447.1 > 300.5**	**0.1**	**35**	**25**	**13.19**
	ESI+	448.9 > 303.1		30	15	
Quercetin	**ESI–**	**301.1 > 151.2**	**0.1**	**30**	**25**	**15.22**
	ESI+	302.8 > 153.0		45	30	
Luteolin	**ESI–**	**285.2 > 133.1**	**0.1**	**45**	**35**	**16.14**
	ESI+	286.9 > 153.1		50	30	
Kaempherol	**ESI+**	**287.0 > 153.1**	**0.1**	**45**	**30**	**17.89**
	ESI–	285.2 > 151.1		45	20	
Apigenin	**ESI–**	**269.2 > 151.2**	**0.1**	**40**	**25**	**18.22**
	ESI+	271.0 > 153.1		50	30	
Isorhamnetin	**ESI–**	**315.0 > 300.3**	**0.1**	**40**	**20**	**18.53**
	ESI+	316.9 > 302.1		40	25	

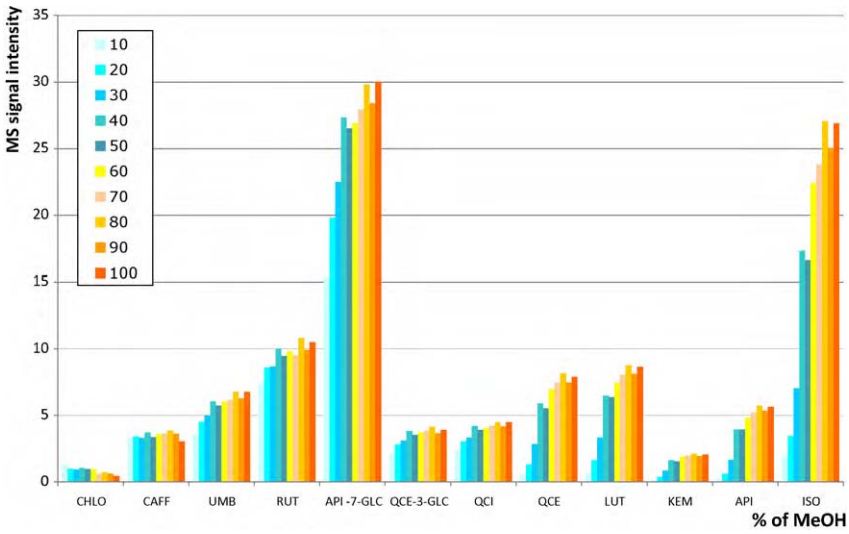


Fig. 2. Optimization of dissolution media for standard mixture and the Chamomile methanolic extract samples – MS response intensity in dependence on the composition of dissolution media, ESI negative data shown for all tested compounds.

for non-polar aglycones increased with increasing percentage of methanol. On the other hand, pure methanol as dissolution agent deteriorated the peak shape of phenolic acids. Therefore, as a compromise 60% methanol in ultra-pure water was chosen as dissolution media for both standard and sample solutions (see Fig. 2).

3.2. A comparison of ESI ionization in positive and negative ion mode

ESI conditions in both positive and negative ion mode were optimized (Section 2.2) with the aim to reach high sensitivity and selectivity for quantitation using SRM experiment. All tested phenolic compounds were possible to be ionized in both ESI positive and negative ion mode. In all cases protonated and deprotonated molecules were chosen as precursor ions. Both precursor ions $[M-H]^-$ in negative and $[M+H]^+$ in positive ion mode were further fragmented at various collision energies (10–40 V) and individual SRMs were optimized for each analyte. The results obtained for ESI negative and ESI positive ionization mode are displayed in Table 1.

However, some analytes, namely flavonoid glycosides such as rutin, quercetin-3-glucoside and quercitrin provided sodium adduct $[M+Na]^+$ in very high concentration. In case of quercitrin the adduct formation substantially decreased the concentration of precursor ion $[M+H]^+$ for further SRM quantitation and therefore

Table 2
SST parameters and validation results for linearity, range and sensitivity of UHPLC–MS/MS method.

Component		LOQ (μmol/l)	LOD (μmol/l)	Tested–linear range (μmol/l)	Correlation coefficient	Repeatability of calibration curve (%RSD)	Validation range	Retention time (%RDS)	Peak area (%RSD)
Chlorogenic acid	**ESI+**	**0.020**	**0.006**	**0.02–100**	**0.9998**	**12.24**	**0.5–100.0**	**0.39**	**3.44**
	ESI−	0.020	0.006	0.02–100	0.9983				
Caffeic acid	**ESI−**	**0.050**	**0.015**	**0.05–10**	**0.9995**	**0.41**	**0.1–10.0**	**0.20**	**1.84**
	ESI+	2.000	0.606	2.0–10	NA				
Umbelliferone	**ESI−**	**0.005**	**0.002**	**0.005–10**	**0.9999**	**3.89**	**0.2–10.0**	**0.06**	**1.87**
	ESI+	0.002	0.0006	0.002–10	0.9992				
Quercetin-3-glucoside	**ESI−**	**0.020**	**0.006**	**0.02–10**	**0.9997**	**5.83**	**0.1–5.0**	**0.06**	**2.26**
	ESI+	0.020	0.006	0.02–10	0.9993				
Rutin	**ESI−**	**0.050**	**0.015**	**0.05–10**	**0.9983**	**8.60**	**0.05–5.0**	**0.07**	**1.37**
	ESI+	0.020	0.006	0.02–10	0.9958				
Apigenin-7-glucoside	**ESI−**	**0.005**	**0.002**	**0.005–100**	**0.9998**	**3.46**	**1.0–50.0**	**0.07**	**1.46**
	ESI+	0.050	0.015	0.05–100	0.9987				
Quercitrin	**ESI−**	**0.020**	**0.006**	**0.02–10**	**0.9982**	**5.12**	**0.2–5.0**	**0.07**	**2.23**
	ESI+	1.00	0.303	1.0–10	NA				
Quercetin	**ESI−**	**0.050**	**0.015**	**0.05–10**	**0.9993**	**8.07**	**0.1–5.0**	**0.04**	**2.23**
	ESI+	0.050	0.015	0.05–10	0.9993				
Luteolin	**ESI−**	**0.050**	**0.015**	**0.05–10**	**0.9997**	**7.45**	**0.1–5.0**	**0.08**	**2.99**
	ESI+	0.100	0.030	0.1–10	0.9993				
Kaempferol	**ESI+**	**0.020**	**0.006**	**0.02–10**	**0.9994**	**7.62**	**0.1–5.0**	**0.09**	**3.03**
	ESI−	0.200	0.061	0.5–10	0.9989				
Apigenin	**ESI−**	**0.020**	**0.006**	**0.02–100**	**0.9996**	**2.23**	**0.2–50.0**	**0.08**	**1.75**
	ESI+	0.020	0.006	0.02–10	0.9997				
Isorhamnetin	**ESI−**	**0.010**	**0.003**	**0.01–100**	**0.9990**	**7.50**	**0.02–5.0**	**0.05**	**2.38**
	ESI+	0.020	0.006	0.02–10	0.9993				

Bold value indicates the results finally used in polarity switching mode.

the sensitivity in ESI positive ion mode was found to be very low. Analogically, ESI positive was not convenient for caffeic acid as carboxylic acid easily lose proton and forms $[M-H]^-$, therefore the sensitivity in ESI negative was much higher (see Table 2).

For quercitrin, caffeic acid and others, such as apigenin-7-glucoside, luteolin and isorhamnetin thus ESI negative was the method of choice because of higher sensitivity. Some phenolics were well ionized in both ion modes with adequate sensitivity for both ESI positive and negative (chlorogenic acid, quercetin-3-glucoside, quercetin, apigenin, rutin and umbelliferone). For these phenolics ionization in ESI negative was preferred because it is more selective. Kaempherol was the only one compound, which provided substantially higher response in ESI positive. In real samples it was present in very low amounts therefore the sensitivity in ESI negative would not be sufficient. Finally, polarity switching mode between ESI positive and negative was applied.

Method validation was performed in both ESI positive and ESI negative ion mode. Based on validation results and method sensitivity, ESI negative was found to be more convenient for quantitative analysis of phenolic compounds except of kaempherol and chlorogenic acid, which was finally analyzed in ESI positive due to validation results. Polarity switching was further used for the simultaneous analysis of 12 phenolic compounds – Fig. 3, the conditions are described in Table 1.

3.3. System suitability test and method validation

The method was validated using standard mixtures (SST, linearity, LOD and LOQ) and Chamomile extract samples (accuracy and precision). System suitability test was performed by 10 times injecting of mixed standard solutions of phenolics at optimum found chromatography conditions (see Section 2.2). Method repeatability was satisfactory for both peak area (RSD < 4%) and retention time (RSD < 1%) (Table 2).

3.3.1. Linearity – calibration range

Calibration curves of all 12 phenolics were measured in the concentration range 0.001–10 μmol/l in both ESI positive and ESI negative ion mode in order to define well linearity range and limits of detection and quantitation. Further, after application to real samples the calibration range must have been extended for three analytes: chlorogenic acid, apigenin and apigenin-7-glucoside up to 100 μmol/l. For all analytes the response was linear within tested concentration range at chosen ionization mode ($r^2 > 0.9982$) as it can be seen in Table 2 – comparison of ESI positive and negative ion mode is also shown. The intra-day repeatability of calibration curve was expressed as the repeatability of calibration curve slope in % of RSD of three measurements at the condition of polarity switching mode. The repeatability was always within 12% RSD for all analytes. Validation range was defined for each compound in accordance with the concentration of phenolics in Chamomile extracts (Table 2).

3.3.2. Limits of detection and quantitation

(LOD and LOQ) were calculated based on S/N ratio. They were established first using standard solutions in 60% methanol by the injection of the smallest amounts which provided S/N = 3 for LOD and S/N = 10 for LOQ. Subsequently this was confirmed by measurements in real matrix, which provided adequate values. The limits are displayed in Table 2. The method had appropriate sensitivity to be able to perform the quantification of phenolics in Chamomile flowers and Chamomile tea extract samples. Method sensitivity expressed as LOQ was typically 5–20 nmol/l.

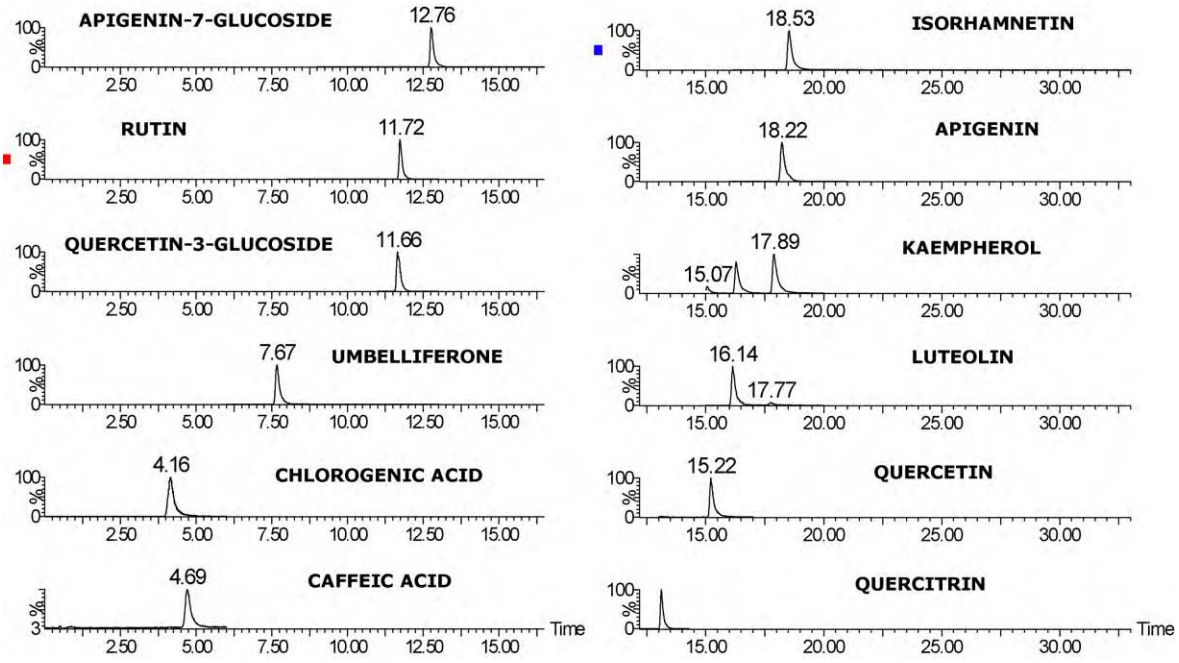


Fig. 3. Chromatogram of the separation in standard mixture – SRM record is displayed from calibration level 10^{-6} mol/l, each chromatogram displays SRM transition for individual compound according to Table 1.

3.3.3. Accuracy and precision

Method accuracy and precision were established by spiking Chamomile flowers and Chamomile tea extract samples at three concentration levels (high medium and low, specified in Table 3) within linearity range. Method precision was determined as intra-day variability of three determinations at three different levels expressed as % RSD – see Table 3, data shown for Chamomile flower samples measured by the method using polarity switching at optimum conditions for each analyte. Intra-day precision was generally within 12% RSD for all analytes.

Accuracy was determined by the method of standard addition. The values for method accuracy ranged from 77% to 127%, data shown again for optimized method using polarity switching, Chamomile flowers extract.

3.3.4. Selectivity

Selectivity of UHPLC–MS/MS method was tested using a comparison of standard calibration curve versus matrix calibration curve. The slopes of both calibration curves were in good correlation for all analytes. The variability of standard and matrix calibration curves was within the range of 15% RSD, which was in a good agreement with the repeatability of the slope of calibration curves (Table 2).

3.4. Applicability of the method

The developed and validated UHPLC–MS/MS method was applied for the measurement of concentration of 12 phenolic compounds in 16 methanolic extracts of Chamomile flowers and 16 methanolic extracts of Chamomile tea (Tables 4 and 5). The calibration curves in the validation range defined in Table 2 were used for quantitation. The comparison of average concentration of tested phenolic compounds is displayed in Fig. 4. The concentration of 12 phenolic compounds in 16 methanolic extracts of Chamomile flowers and 16 methanolic extracts of Chamomile tea was evaluated in dependence on following parameters: primary production

Table 3
The results of method validation: precision and accuracy for the Chamomile flower methanolic extract obtained at optimum UHPLC–MS/MS conditions.

Component	Level 1 – high[a] (5;50)		Level 2 – medium[a] (1;5)		Level 3 – low[a] (0,1;1)	
	Precision [% RSD]	Accuracy [%]	Precision [% RSD]	Accuracy [%]	Precision [% RSD]	Accuracy [%]
Chlorogenic acid	6.09	86.55	2.92	87.31	0.03	76.65
Caffeic acid	0.83	79.99	4.94	116.65	12.67	85.49
Umbelliferone	6.16	105.42	4.38	108.33	6.76	113.21
Quercetin-3-glucoside	2.79	105.60	6.43	96.20	6.63	99.34
Rutin	1.32	103.86	1.84	103.42	6.76	105.98
Apigenin-7-glucoside	2.23	98.32	1.51	113.66	6.60	109.67
Quercitrin	0.63	105.82	3.93	97.61	5.28	101.28
Quercetin	3.20	114.50	1.47	101.16	9.86	91.47
Luteolin	3.01	107.04	4.81	109.93	8.05	111.15
Kaempferol	4.41	90.87	7.09	126.71	9.15	95.35
Apigenin	7.91	115.86	4.51	121.27	7.07	111.05
Isorhamnetin	2.04	92.61	5.88	91.95	2.68	93.19

[a] High level means 5 μmol/l for caffeic acid, rutin, quercetin-3-glucoside, quercitrin, luteolin, kaempherol and isorhamnetin (group 1) and 50 μmol/l for chlorogenic acid, umbelliferone, apigenin-7-glucoside and apigenin (group 2). Medium level means 1 μmol/l for group 1 and 5 μmol/l for group 2. Low level means 0.1 μmol/l for group 1 and 1 μmol/l for group 2.

Table 4
Quantification of phenolic compounds (μmol/l) in the methanolic extracts of Chamomile flower samples.

	CHLO ± Sds	CAFF ± Sds	UMB ± Sds	RUT ± Sds	API-7-G ± Sds	QCE-3-G ± Sds	QCI ± Sds	QCE ± Sds	LUT ± Sds	KEM ± Sds	API ± Sds	ISORH ± Sds
CH1	12.1 ± 0.066	1.7 ± 0.044	20.8 ± 0.018	0.9 ± 0.022	94.1 ± 0.021	2.3 ± 0.039	LOD ± 0.00	1.1 ± 0.032	1.5 ± 0.012	0.6 ± 0.043	38.8 ± 0.045	1.5 ± 0.022
CH3	14.3 ± 0.026	2.7 ± 0.029	38.6 ± 0.034	0.9 ± 0.04	119.4 ± 0.05	2.1 ± 0.025	LOD ± 0.00	2.1 ± 0.026	4.3 ± 0.018	0.3 ± 0.027	56.1 ± 0.008	3.6 ± 0.034
CH5	124.9 ± 0.025	3.4 ± 0.018	16.8 ± 0.047	1.9 ± 0.018	187.1 ± 0.014	5.0 ± 0.11	LOD ± 0.00	0.6 ± 0.025	0.7 ± 0.044	0.4 ± 0.026	20.6 ± 0.049	0.9 ± 0.05
CH7	93.2 ± 0.022	2.9 ± 0.013	14.4 ± 0.028	1.2 ± 0.029	153.4 ± 0.014	4.3 ± 0.026	LOD ± 0.00	0.5 ± 0.024	0.6 ± 0.015	0.3 ± 0.038	18.1 ± 0.09	0.7 ± 0.021
CH10	59.6 ± 0.016	3.7 ± 0.05	19.4 ± 0.018	1.0 ± 0.064	158.3 ± 0.062	5.9 ± 0.025	LOD ± 0.00	0.8 ± 0.026	2.0 ± 0.258	0.6 ± 0.047	17.7 ± 0.018	0.4 ± 0.022
CH12	254.8 ± 0.022	3.0 ± 0.024	2.9 ± 0.017	1.5 ± 0.013	211.8 ± 0.017	8.4 ± 0.021	LOD ± 0.00	1.3 ± 0.025	0.6 ± 0.051	0.8 ± 0.033	4.0 ± 0.1	0.5 ± 0.024
CH14	212.2 ± 0.018	5.1 ± 0.026	13.0 ± 0.28	2.9 ± 0.22	209.4 ± 0.082	7.9 ± 0.29	LOD ± 0.00	1.6 ± 0.21	4.3 ± 0.24	0.8 ± 0.032	9.6 ± 0.024	0.8 ± 0.022
CH16	97.8 ± 0.045	3.4 ± 0.29	13.5 ± 0.042	1.6 ± 0.18	206.6 ± 0.22	6.5 ± 0.36	LOD ± 0.00	1.4 ± 0.18	2.9 ± 0.14	0.6 ± 0.18	18.6 ± 0.18	0.6 ± 0.037
CH2	11.4 ± 0.021	2.2 ± 0.018	53.1 ± 0.022	0.9 ± 0.014	105.0 ± 0.082	2.0 ± 0.125	LOD ± 0.00	1.7 ± 0.022	3.3 ± 0.032	0.8 ± 0.042	56.7 ± 0.021	2.9 ± 0.026
CH4	9.2 ± 0.021	1.2 ± 0.032	32.6 ± 0.029	1.0 ± 0.021	166.3 ± 0.032	1.7 ± 0.027	LOD ± 0.00	0.8 ± 0.022	3.2 ± 0.035	0.3 ± 0.008	77.6 ± 0.042	1.1 ± 0.008
CH6	121.1 ± 0.026	4.4 ± 0.029	31.0 ± 0.082	1.1 ± 0.024	216.2 ± 0.043	8.3 ± 0.033	LOD ± 0.00	0.9 ± 0.021	2.0 ± 0.22	0.6 ± 0.032	21.2 ± 0.026	0.9 ± 0.032
CH8	7.3 ± 0.039	2.0 ± 0.081	46.4 ± 0.008	0.7 ± 0.029	152.1 ± 0.012	1.7 ± 0.014	LOD ± 0.00	1.3 ± 0.032	4.1 ± 0.008	0.3 ± 0.014	95.1 ± 0.022	2.1 ± 0.041
CH9	25.4 ± 0.041	2.5 ± 0.025	11.1 ± 0.082	1.4 ± 0.040	138.2 ± 0.008	3.2 ± 0.034	LOD ± 0.00	0.5 ± 0.008	1.2 ± 0.025	0.2 ± 0.033	30.1 ± 0.045	0.1 ± 0.008
CH11	310.3 ± 0.018	3.5 ± 0.008	1.1 ± 0.018	2.2 ± 0.032	206.4 ± 0.029	10.6 ± 0.042	LOD ± 0.00	6.5 ± 0.037	9.2 ± 0.026	0.9 ± 0.047	4.2 ± 0.029	2.1 ± 0.024
CH13	271.8 ± 0.021	2.9 ± 0.052	1.0 ± 0.008	2.5 ± 0.016	189.6 ± 0.042	9.9 ± 0.026	LOD ± 0.00	2.4 ± 0.008	3.2 ± 0.018	0.5 ± 0.008	3.0 ± 0.115	1.2 ± 0.025
CH15	123.4 ± 0.029	3.8 ± 0.029	11.9 ± 0.042	2.5 ± 0.022	207.6 ± 0.008	8.3 ± 0.029	LOD ± 0.00	1.4 ± 0.034	2.2 ± 0.037	0.8 ± 0.042	11.9 ± 0.021	0.5 ± 0.037

CH1, CH3, CH5, CH7: eco primary production; variety BOHEMIA; 1st harvest 2007. CH10, CH12, CH14, CH16: conventional primary production; variety BOHEMIA; 1st harvest 2007. CH2, CH4, CH6, CH8: eco primary production; variety GORAL; 1st harvest 2007. CH9, CH11, CH13, CH15: conventional primary production; variety GORAL; 1st harvest 2007.

Table 5
Quantification of phenolic compounds (μmol/l) in the methanolic extracts of Chamomile tea samples.

	CHLO ± Sds	CAFF ± Sds	UMB ± Sds	RUT ± Sds	API-7-G ± Sds	QCE-3-G ± Sds	QCI ± Sds	QCE ± Sds	LUT ± Sds	KEM ± Sds	API ± Sds	ISORH ± Sds
mTEA1	37.0 ± 0.039	1.8 ± 0.032	6.3 ± 0.045	3.6 ± 0.008	59.7 ± 0.024	3.6 ± 0.042	LOD ± 0.00	0.8 ± 0.042	4.2 ± 0.014	1.8 ± 0.045	16.2 ± 0.045	0.1 ± 0.02
mTEA2	13.2 ± 0.045	1.0 ± 0.034	2.8 ± 0.042	1.8 ± 0.07	7.3 ± 0.035	1.5 ± 0.039	LOD ± 0.00	0.2 ± 0.018	1.1 ± 0.026	0.4 ± 0.035	2.0 ± 0.022	0.1 ± 0.034
mTEA3	68.4 ± 0.027	4.5 ± 0.036	7.5 ± 0.036	15.9 ± 0.032	15.4 ± 0.02	10.0 ± 0.029	1.7 ± 0.037	1.7 ± 0.036	1.5 ± 0.032	0.7 ± 0.022	10.9 ± 0.008	1.0 ± 0.041
mTEA4	116.0 ± 0.008	5.9 ± 0.035	20.9 ± 0.032	2.4 ± 0.029	93.4 ± 0.026	7.2 ± 0.027	8.5 ± 0.035	1.2 ± 0.029	3.9 ± 0.047	0.6 ± 0.029	52.5 ± 0.029	0.9 ± 0.037
mTEA5	114.3 ± 0.036	5.8 ± 0.022	14.5 ± 0.034	0.9 ± 0.041	67.0 ± 0.082	9.0 ± 0.048	LOD ± 0.00	3.4 ± 0.022	1.1 ± 0.022	0.9 ± 0.052	11.7 ± 0.037	3.4 ± 0.008
mTEA6	73.5 ± 0.039	3.5 ± 0.018	18.1 ± 0.041	1.6 ± 0.037	48.2 ± 0.026	3.3 ± 0.029	LOD ± 0.00	0.7 ± 0.021	2.4 ± 0.036	0.4 ± 0.024	17.2 ± 0.022	0.5 ± 0.034
mTEA7	23.7 ± 0.052	2.7 ± 0.044	9.0 ± 0.02	1.0 ± 0.036	16.2 ± 0.034	3.1 ± 0.035	LOD ± 0.00	1.5 ± 0.029	1.2 ± 0.037	LOD ± 0.00	2.7 ± 0.038	1.6 ± 0.034
mTEA8	110.4 ± 0.026	7.1 ± 0.018	10.8 ± 0.035	1.0 ± 0.041	87.2 ± 0.028	7.2 ± 0.026	LOD ± 0.00	2.2 ± 0.041	0.7 ± 0.032	1.2 ± 0.008	6.3 ± 0.035	2.7 ± 0.028
mTEA9	68.0 ± 0.021	4.2 ± 0.036	12.0 ± 0.032	1.1 ± 0.027	16.9 ± 0.014	7.1 ± 0.029	LOD ± 0.00	2.9 ± 0.035	1.4 ± 0.027	1.8 ± 0.029	8.6 ± 0.035	2.5 ± 0.026
mTEA10	11.0 ± 0.052	0.6 ± 0.021	1.2 ± 0.026	0.8 ± 0.029	21.1 ± 0.026	0.9 ± 0.052	LOD ± 0.00	0.7 ± 0.018	0.7 ± 0.027	LOD ± 0.00	2.5 ± 0.029	0.03 ± 0.003
mTEA11	24.1 ± 0.034	2.5 ± 0.055	5.4 ± 0.037	11.2 ± 0.034	127.1 ± 0.037	2.3 ± 0.039	LOD ± 0.00	0.7 ± 0.032	3.5 ± 0.056	LOD ± 0.00	24.1 ± 0.029	0.03 ± 0.001
mTEA12	34.5 ± 0.035	2.2 ± 0.048	2.4 ± 0.024	3.1 ± 0.039	8.3 ± 0.029	2.8 ± 0.036	LOD ± 0.00	0.5 ± 0.014	2.1 ± 0.042	LOD ± 0.00	3.0 ± 0.041	0.1 ± 0.034
mTEA13	107.5 ± 0.033	4.1 ± 0.058	12.7 ± 0.022	1.3 ± 0.014	48.0 ± 0.017	5.1 ± 0.021	LOD ± 0.00	3.2 ± 0.034	2.2 ± 0.031	LOD ± 0.00	8.7 ± 0.018	1.2 ± 0.048
mTEA14	79.6 ± 0.014	3.6 ± 0.021	6.5 ± 0.026	2.6 ± 0.029	29.0 ± 0.042	5.0 ± 0.043	LOD ± 0.00	1.3 ± 0.022	3.4 ± 0.052	LOD ± 0.00	8.9 ± 0.008	0.2 ± 0.041
mTEA15	104.7 ± 0.029	5.4 ± 0.041	6.1 ± 0.012	1.1 ± 0.032	82.9 ± 0.052	6.1 ± 0.036	LOD ± 0.00	1.5 ± 0.037	0.5 ± 0.032	LOD ± 0.00	5.7 ± 0.021	1.0 ± 0.026
mTEA16	55.5 ± 0.029	5.8 ± 0.037	8.8 ± 0.016	1.0 ± 0.075	57.9 ± 0.029	4.7 ± 0.029	LOD ± 0.00	2.4 ± 0.036	1.3 ± 0.029	LOD ± 0.00	5.6 ± 0.026	1.5 ± 0.037

mTEA1: methanolic extract of eco tea; loose tea type; made in PL, mTEA2: methanolic extract of eco tea; tea bag type; made in GB, mTEA3: methanolic extract of eco tea; tea bag type; made in DK, mTEA4: methanolic extract of conventional tea; loose tea type; made in CZ, mTEA5: methanolic extract of eco tea; loose tea type; made in CZ, mTEA6: methanolic extract of conventional tea; loose tea type; made in DK, mTEA7: methanolic extract of eco tea; loose tea type; made in DK, mTEA8: methanolic extract of conventional tea; tea bag type; made in GB, mTEA9: methanolic extract of eco tea; tea bag type; made in IT, mTEA10: methanolic extract of conventional tea; loose tea type; made in IT, mTEA11: methanolic extract of conventional tea; loose tea type; made in PL, mTEA12: methanolic extract of conventional tea; tea bag type; made in CZ, mTEA13: methanolic extract of eco tea; tea bag type; made in CZ, mTEA14: methanolic extract of eco tea; tea bag type; made in CZ, mTEA15: methanolic extract of conventional tea; loose tea type; made in CZ, mTEA16: methanolic extract of conventional tea; loose tea type; made in CZ.

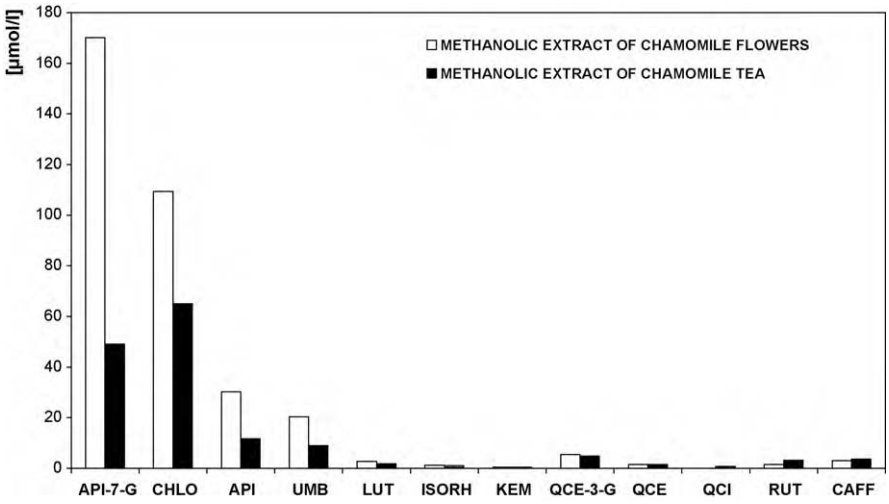


Fig. 4. A summary of average content of phenolic compounds in the methanolic extracts of Chamomile flowers and Chamomile tea extracts.

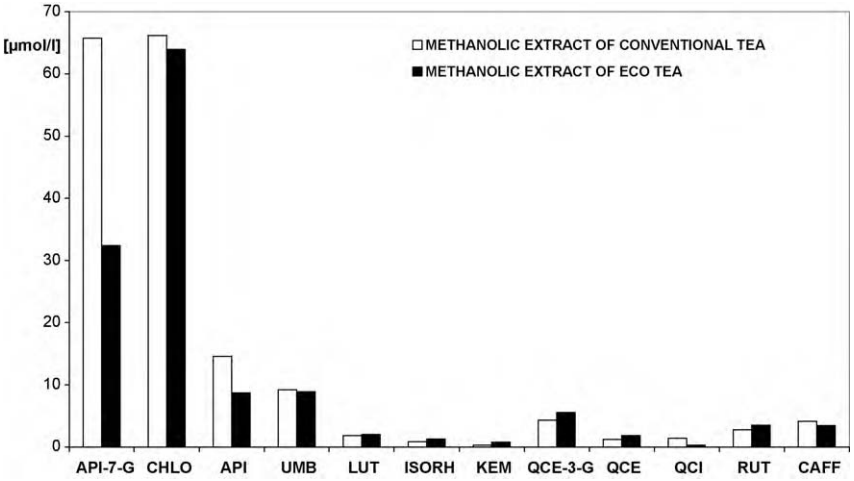


Fig. 5. A comparison of average content of phenolic compounds in the methanolic extracts of conventional and ecological Chamomile tea extracts.

(chamomile flowers)/final product (chamomile tea), the manufacturing procedure – tea bag/loose tea and source of production BIO/conventional commodity.

In both methanolic extracts from Chamomile (tea samples and Chamomile flowers) apigenin-7-glucoside and chlorogenic acid were present at the highest average concentrations (Tables 4 and 5). In the Chamomile extract the large amounts (39.1%) of the cinnamic acid, derivatives of ferulic and caffeic acid, as well as other unidentified phenolic derivatives (25.8% of the total flower) were also previously determined [6]. The highest values of chlorogenic acid

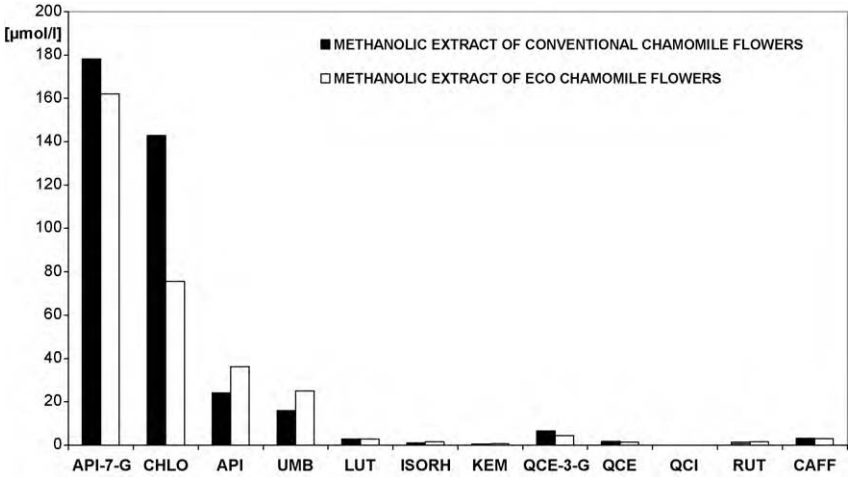


Fig. 6. A comparison of average content of phenolic compounds in the methanolic extracts of conventional and ecological Chamomile flowers.

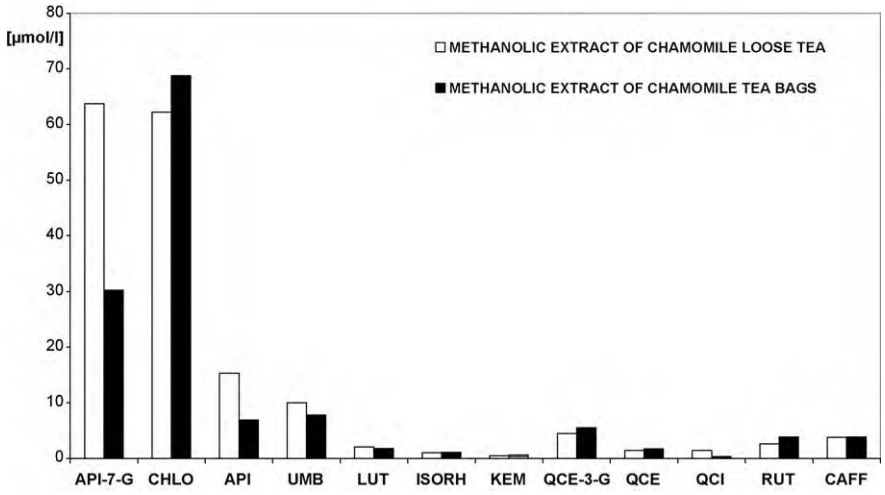


Fig. 7. A comparison of average content of phenolic compounds in dependence to the manufacturing procedure (the methanolic extracts of tea bag/loose tea).

(310.3 μmol/l), umbelliferone (53.1 μmol/l), apigenin-7-glucoside (216.2 μmol/l), quercetin-3-glucoside (10.6 μmol/l), quercetin (6.5 μmol/l), luteolin (9.2 μmol/l), apigenin (95.1 μmol/l) and isorhamnetin (3.6 μmol/l) were determined in methanolic extract of samples from Chamomile flowers. The components are genetically manifested in quality, but the quantitative composition depends on external factors [24]. On the contrary, the highest values of caffeic acid (7.1 μmol/l), rutin (15.9 μmol/l), quercitrin (15.9 μmol/l) and kaempferol (1.8 μmol/l) were observed in methanolic extract of samples from Chamomile tea. The average highest content of 12 phenolic compounds was found in Chamomile flowers (Fig. 4). The most noticeable difference between Chamomile flowers and Chamomile teas is in average values of apigenin-7-glucoside, chlorogenic acid, apigenin and umbelliferone. The contents of chlorogenic acid (109.3 μmol/l), apigenin-7-glucoside (170.09 μmol/l), apigenin (30.21 μmol/l) and umbelliferone (20.47 μmol/l) were significantly higher in samples of methanolic extract of Chamomile flowers (at the significance levels $\alpha = 0.05$ and 0.01). The average concentration of apigenin-7-glucoside (49.1 μmol/l), umbelliferone (20.9 μmol/l) and apigenin (11.66 μmol/l) were more than three times lower and also the average concentration of chlorogenic acid (65.09 μmol/l) is nearly about 1/3 lower in methanolic extract from Chamomile tea samples. The Chamomile flavonoids were recognized to be spasmolytic and antiphlogistic and are therefore of great interest [2].

The average concentration of 12 phenolic compounds in dependence on the source of production (bio/conventional commodity) was next evaluated. The presence of two main flavonoids apigenin-7-glucoside (64.22 μmol/l) and apigenin (17.6 μmol/l) was in average significantly higher in methanolic extract of conventional tea samples (at the significance levels $\alpha = 0.05$ and 0.01). The average values of concentration of chlorogenic acid (65.09 μmol/l), umbelliferone (9.21 μmol/l), quercitrin (1.42 μmol/l) and caffeic acid (4.12 μmol/l) were also higher in methanolic extract of conventional tea samples (Fig. 5). On the other hand in the average values of concentration of chlorogenic acid in methanolic extract of conventional tea samples (61.58 μmol/l), and ecological tea samples (67.19 μmol/l) the significant variance was not proved at the significance levels $\alpha = 0.05$ and $\alpha = 0.01$.

Significantly higher average concentration of umbelliferone (18.88 μmol/l) and apigenin (25.84 μmol/l) was determined in samples of methanolic extract of organic Chamomile flowers (at the significance levels $\alpha = 0.05$ and 0.01) (Fig. 6). On the other hand the higher average values of concentration of chlorogenic acid (123.2 μmol/l) and apigenin-7-glucoside (136.66 μmol/l) were determined in samples of methanolic extract of organic Chamomile flowers (at the significance levels $\alpha = 0.05$ and 0.01).

Scientific papers comparing quality of products from organic and conventional agriculture exist only in a limited range. In many cases their conclusions differ considerably. The quality of bio products and conventional products is very difficult to review exactly [25].

The values of content of apigenin-7-glucoside (63.72 μmol/l) and apigenin (15.26 μmol/l) were in average more about 1/2 higher due to the way of manufacturing process (loose or tea bags) (Fig. 7). The contents of apigenin-7-glucoside (30.3 μmol/l) and apigenin (6.91 μmol/l) were significantly lower in samples of a tea bag type of tea (at the significance levels $\alpha = 0.05$ and 0.01). In samples of methanolic extract of a loose type of tea there was also in average higher concentration of luteolin (2.09 μmol/l) and umbelliferone (10.03 μmol/l). Nevertheless the average concentrations of luteolin and umbelliferone in samples of a loose type of tea and samples of a tea bag type are not varied significantly in the significance level $\alpha = 0.05$ and 0.01. The average content of other analyzed phenolic compound was higher in samples of methanolic extract of tea bag type of tea. The study confirmed considerable differences also in the content and composition of essential oil of the Chamomile herb tea [26].

Different choices in agricultural practice, storage and processing of the plant material and the final manufacturing procedures will inevitably influence the composition and content of active compounds in the final product. This subsequently results in quite incomparable preparations that are being sold to customers although they originate from the same plant species. As a consequence it may be very difficult to draw conclusions about their activity. In pharmaceutical and cosmetic industry, Chamomile flower drugs of different origin and completely diverse therapeutic quality are processed and utilized [27]. One of the favourite and popular remedies to cure common cold – Chamomile (*M. recutita* L.) is used as an example to highlight the challenges in quality assurance of herbal medicinal products.

4. Conclusion

A novel UHPLC–MS/MS method for the quantitation of 12 phenolic compounds in methanolic extracts of Chamomile flowers and Chamomile tea was developed. The method combined high separation efficiency of UHPLC together with wide linear range of triple quadrupole mass analyzer therefore it possessed high sensitivity and selectivity. One of the key issues of the method development

was the right choice of dissolution media for standard mixtures and dilution of Chamomile samples. 60% methanol in water met the requirements.

Careful optimization of UHPLC–MS/MS conditions revealed that ESI in negative ion mode was more convenient for the analysis of phenolic compounds except of chlorogenic acid and keampherol that provided better results in ESI positive ion mode. Complete method validation was performed and the applicability of the method was verified on real samples of Chamomile flowers and Chamomile tea extracts, which were compared in terms of content of 12 phenolic compounds. The content of phenolic compounds was correlated with factors of primary production, the manufacturing process and a source of production.

Acknowledgements

The authors gratefully acknowledge research projects *MSM0021620822, MSM6046070901* and technical support of *Waters*, Czech Republic.

References

[1] M. Adams, C. Berset, M. Kessler, M. Hamburger, J. Ethnopharmacol. 121 (2009) 343–359.
[2] R. Franke, H. Schilcher, Chamomile Industrial Profiles, CRC Press, USA, 2005.
[3] A.K. Atoui, A. Mansouri, G. Boskou, P. Kefalas, Food Chem. 89 (2005) 27–36.
[4] F. Power, J. Browning, J. Chem. Soc. 105 (1914) 2280–2291.
[5] W. Lang, K. Schwandt, Dtsch. Apoth. Ztg. 97 (1957) 149s.
[6] N. Mullinaci, A. Romani, P. Pinelli, F.F. Vinvieri, D. Prucher, Chromatographia 51 (2000) 301–307.
[7] J. Kováčik, J. Grúz, M. Bačkor, J. Tomko, M. Strnad, M. Řepčák, Environ. Exp. Bot. 62 (2008) 145–152.
[8] Z. Zekovic, B. Pekic, Z. Lepojevic, L. Petrovic, Chromatographia 39 (1994) 587–590.
[9] M. Řepčák, T. Krausová, Food Chem. 116 (2009) 19–22.
[10] A. Carnat, A.P. Carnat, D. Fraisse, L. Ricoux, J.L. Lamaison, Fitoterapia 75 (2004) 32–38.
[11] F.A. Ayaz, S. Hayirlioglu-Ayaz, J. Gruz, O. Novak, M. Strnad, J. Agric. Food Chem. 53 (2005) 8118–8122.
[12] R. Avallone, P. Zanoli, G. Puia, M. Kleinshnitz, P. Schreier, M. Baraldi, Biochem. Pharmacol. 59 (2000) 1387–1394.
[13] F.N. Fonseca, M.F.M. Tavares, C. Horváth, J. Chromatogr. A 1154 (2007) 390–399.
[14] J. Kováčik, B. Klejdus, M. Bačkor, J. Plant Physiol. 166 (2009) 1460–1464.
[15] J. Kováčik, J. Grúz, J. Hedbavny, B. Klejdus, M. Strnad, J. Agric. Food Chem. 57 (2009) 9848–9855.
[16] F.N. Fonseca, M.F.M. Tavares, Phytochem. Anal. 15 (2004) 65–70.
[17] V. Švehlíková, M. Řepčák, Biochem. Syst. Ecol. 34 (2006) 654–657.
[18] V. Švehlíková, R.N. Bennett, F.A. Mellon, P.W. Needs, S. Piacente, P.A. Kroon, Y. Bao, Phytochemistry 65 (2004) 2323–2332.
[19] International Conference on Harmonization (ICH), Q2(R1): Text on Validation of Analytical Procedures, US FDA Federal Register, 2003.
[20] European Pharmacopoeia, 6th edition (Ph. Eur. 6), Council of Europe, Strasbourg, 2007.
[21] United States Pharmacopoeia 32, United States Pharmacopoeial Convention, Rockville, MD 20852, 2009.
[22] L. Nováková, Z. Spáčil, M. Seifrtová, L. Opletal, P. Solich, Talanta 80 (2010) 1979–11979.
[23] Z. Spáčil, L. Nováková, P. Solich, Talanta 76 (2008) 189–199.
[24] C.H. Franz, D. Frit, F.-J. Schröder, Planta Med. 27 (1975) 46–52.
[25] J. Hajšlová, V. Schulzová, Porovnání produktů ekologického a konvenčního zemědělství, in: Odborná studie VŠCHT, UZPI, Praha, 2006, pp. 1–24.
[26] I. Šalamon, I. Sudimáková, Acta Hortic. 749 (2007) 181–187.
[27] H. Schilcher, Die Kamille, Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart, 1987, ISBN: 3-8047-0939-7, 152p.