8. Přílohy – publikace zahrnuté v disertační práci

8.1 Development and validation of UHPLC-MS/MS method for determination of eight naturally occurring catechin derivatives in various tea samples and the role of matrix effects

Journal of Pharmaceutical and Biomedical Analysis 114 (2015) 62–70

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Development and validation of UHPLC–MS/MS method for determination of eight naturally occurring catechin derivatives in various tea samples and the role of matrix effects

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ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form 15 April 2015
Accepted 17 April 2015
Available online 24 April 2015

Keywords:
Catechins
Matrix effects
Tea
Method validation
UHPLC–MS/MS

ABSTRACT

A complete analytical procedure combining optimized tea infusion preparation and validated UHPLC–MS/MS method was developed for routine quantification of eight naturally occurring catechin derivatives in various tea samples. The preparation of tea infusions was optimized in terms of temperature, time and water-to-tea ratio in green, white and black teas. The catechins were analyzed using ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry in a run of only 4 min including equilibration of the system. The UHPLC–MS/MS method was fully validated in terms of inter/intra-day precision, accuracy, linearity ($r^2 > 0.9991$), range (50–5000 ng/ml), LOD (1.5–7.5 ng/ml) and LOQ (5–25 ng/ml). Validation of the method included also the determination of the matrix effects that were evaluated in both flavored and unflavored green, white and black teas. Dilution of the resulting tea infusions appeared to be crucial for the matrix effects and also for subsequent catechin quantification in real tea samples in order to fit into the linear range of the UHPLC–MS/MS method. This complete procedure for catechin quantification was finally applied to real sample analysis represented by 70 commercial tea samples.

1. Introduction

Besides water, tea is the most widely consumed beverage in the world, which is due to its health benefits, gustatory properties, stimulant effects and cultural dimension [1]. Post-harvest processing of leaves of *Camellia sinensis* (L.) (Theaceae) is an important factor determining a type of tea and affecting tea polyphenol content and also polyphenol composition. According to the level of fermentation, three basic types of tea are distinguished i.e. non-fermented green tea, partially fermented oolong tea and fully-fermented black tea. Fermentation, an enzymatic oxidation process, in the case of tea, converts monomeric phenolic compounds into dimers, oligomers and polymers [2]. To avoid oxidation in post-harvest processing, tea leaves resulting in green tea are subjected to fixing process that inactivates enzymes such as polyphenol oxidase, polyphenol peroxidase, and ascorbic acid oxidase [1,3]. Therefore, monomeric catechins predominate in green tea whereas dimeric theaflavins and polymeric thearubigins in black tea [4]. Black tea is the most popular type of tea representing approximately 76–78% of the worldwide tea production and consumption, green tea 20–22% and oolong tea less than 2% [5]. Black tea is consumed primarily in North America, Europe and India, whereas green tea in Japan and China [6].

Catechins, flavan-3-ol derivatives, are colorless and water-soluble compounds. The major catechins contained in fresh tea leaves include (−)-epigallocatechin gallate, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epicatechin [7]. During post-harvest processing and storage of tea leaves, catechins are prone to oxidation, epimerization, polymerization and degradation. Temperature, humidity, oxygen, metal ions, pH of the system and tea ingredients are responsible for these chemical changes [8]. Tea catechins show various health beneficial effects and they are best known for their antioxidant activity [9,10]. Theaflavins contained in black tea leaves also act as antioxidants [11]. Similar activity to catechins was reported by Leung et al. [12]. Anti-microbial [13,14], anti-viral [15,16] and anti-fungal [17,18] effects were observed as well. Due to anti-oxidant, anti-inflammatory, anti-proliferative and anti-platelet activity catechins are assumed to decrease cardiovascular risk [19] and they are also hypothesized to reduce the risk of several types of cancer [6].

Analysis of catechins in biological matrices is typically performed using liquid chromatography coupled to mass spectrometry

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http://dx.doi.org/10.1016/j.jpba.2015.04.026
0731-7085/

(MS), UV or PDA detector. The major drawback of most of the published HPLC methods is a long chromatographic run of about 20–45 min [20–26]. It results in high costs of analysis, high solvent consumption and also potential analyte degradation [27]. Only few faster HPLC methods [28–30] were published in the past years taking still about 10 min. Use of columns packed with sub-2 μm particles in ultra-high performance liquid chromatography (UHPLC) allows to reduce time of chromatographic separation while maintaining the same resolution [31,32]. Surprisingly, UHPLC has been quite scarcely employed in analysis of catechins. Eight naturally occurring catechins were successfully separated using UHPLC–UV in a run of only 1.7 min by Spáčil et al. [33]. In addition, even faster (0.5 min) UHPLC separation of seven catechin derivatives was reported by Guillarme et al. [34]. Nevertheless, none of these methods was fully validated for determination of catechins in real samples. Recently, fast UHPLC–UV method published by Naldi et al. [35] was fully validated for qualitative and quantitative analysis of 6 catechins and caffeine that were separated in a run of 3 min. In spite of higher selectivity and sensitivity of MS detection, it has been quite rarely used for this purpose. The development of UHPLC–MS/MS method that was partially validated was previously published by our group [36]. In this paper the focus was put on the optimization of MS conditions. To our knowledge a fast and selective UHPLC–MS/MS method that is fully validated for determination of 8 naturally occurring catechin derivatives in various tea samples catechins in various tea samples has not been published yet.

Sample preparation step of tea leaves prior to LC–MS analysis of catechins usually involves tea infusion preparation, filtration through a membrane filter and subsequent dilution. Despite all the advantages that LC–MS brings, it suffers from a major drawback called matrix effects. Is the sample treatment involving infusion preparation, filtration and dilution sufficient enough for reliable quantification of catechins in terms of overcoming matrix effects and providing acceptable validation results? Therefore, evaluation of this phenomena was included in the method validation. To our knowledge, no studies have been published evaluating matrix effects of catechins in tea samples. The aim of this work was to develop a complete procedure for routine quantification of catechins contained in various tea samples. Development of this procedure involved optimization of conditions for tea infusion preparation and development and validation of fast UHPLC–MS/MS method for quantification of all eight naturally occurring catechins in tea samples. Conditions for infusion preparation were optimized in terms of temperature, time and water-to-tea ratio. Validation of the method included also evaluation of matrix effects in both unflavored and flavored green, white and black teas. The resulting procedure was applied for quantification of catechins in tea samples commonly available for tea consumers in the Czech Republic to demonstrate its applicability to real sample analysis and to compare the total amount of catechins in flavored and unflavored green, white and black teas.

2. Experimental

2.1. Chemicals and reagents

The following standards of catechins: (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (±)-catechin hydrate (C), (+)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-gallocatechin gallate (GCG) and (−)-epigallocatechin gallate (EGCG) were purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile, methanol and mobile phase additives such as formic and acetic acid, all of them LC–MS grade, were obtained from Sigma–Aldrich (Steinheim, Germany). LC–MS grade water was prepared by Milli-Q reverse osmosis system (Millipore, Bedford, MA, USA) immediately prior to use. Green, white and black teas used for validation of UHPLC–MS/MS method and optimization of sample preparation step were obtained from Oxalis (Slušovice, Czech Republic). The other tea samples were purchased from local supermarkets or specialized tea shops in the Czech Republic and France.

2.2. Instrumentation and analytical conditions

ACQUITY Ultra Performance LC™ (UPLC) system (Waters, Milford, MA, USA) consisting of binary solvent manager and sample manager was coupled with Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters, Milford, MA, USA). All UHPLC analyses were performed on the analytical column CSH C_{18} (100 mm × 2.1 mm, 1.7 μm) (Waters, Milford, MA, USA) and the column was maintained at 40 °C. Samples were separated using a gradient elution with 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). The flow rate was set at 0.3 ml/min and the chromatographic run time was 4.0 min including equilibration of the system. The gradient started with 8.5% of solvent B, increased to 40.0% over 2.1 min and in 2.2 min the percentage of solvent B ramped to original conditions (8.5%). The injection volume was 5 μL. All the ion source and ion optic parameters were optimized and they were finally set as follows: capillary voltage 1.0 kV, extractor voltage 2.0 V, hexapole voltage 0.2 V, cone voltage 35 V (GC, EGC, C, EC), 30 V (EGCG, GCG, ECG, CG), cone gas flow rate 70 L h^{-1}, desolvation temperature 450 °C and desolvation gas flow rate 600 L h^{-1}. The resulting most intense SRM transitions with optimized collision energies and dwell times are shown in Table 1. Scheduled SRMs were used in order to maximize the dwell times for individual transitions. The data were acquired and processed using MassLynx™ software version 4.1 (Waters, Milford, MA, USA).

2.3. Standard solutions

Stock standard solutions of GC, EGC, C, EC, GCG, EGCG, CG and ECG were prepared by dissolving each compound in acidified methanol (0.1% formic acid) to give a solution with a concentration of 1.0 mg/mL. Formic acid was added due to the stability reasons. Stock standard solutions were stored at 4 °C until further dilution and they were prepared fresh every two weeks. Mixture consisting of 0.1% formic acid in water and 0.1% formic acid in methanol (50/50, v/v) was used for preparation of stock standard solutions as for dilution of natural samples.

2.4. Sample preparation

Both tea forms i.e. loose leaf tea and tea bag tea were homogenized before quantification of catechins. Loose leaf tea was taken from the bottom, middle and top of the pack and thoroughly mixed. Although smaller size of tea leaf particles would provide higher extraction efficiency, loose leaf tea samples were not crushed or rubbed to keep the conditions of tea infusion preparation close to the real procedure. Three tea bags of one and the same analyzed tea were cut open and their content was mixed. 1 g of resulting tea samples was weighed and subjected to sample preparation. Conditions for tea infusion preparation were optimized for green (Formosa gunpowder), white (Snow buds) and black (Keemun) teas. Both green and white tea infusions were prepared using 100 ml of water at 90 °C maintained for 20 min. During infusion preparation, tea samples were mildly stirred every 5 min. Black tea infusions were prepared according to the same procedure as used for green and white teas except temperature (100 °C). After infusion preparation, the resulting sample was filtered through hydrophilic 0.22 μm pore

Table 1
Parameters of SRM transitions for all 8 catechins.

Analyte	Precursor ion type	Precursor ion	Fragment ion	Dwell time (s)	Cone voltage (V)	Collision energy (V)	t_R (min)
GC	$[M-H]^-$	304.9	125.0	0.100	35	25	1.74
EGC	$[M-H]^-$	304.9	125.0	0.100	35	25	2.07
C	$[M-H]^-$	289.0	245.1	0.050	35	15	2.17
EC	$[M-H]^-$	289.0	245.1	0.050	35	15	2.51
EGCG	$[M-H]^-$	457.0	169.0	0.050	30	25	2.40
GCG	$[M-H]^-$	457.0	169.0	0.050	30	25	2.57
ECG	$[M-H]^-$	441.0	169.0	0.100	30	25	2.86
CG	$[M-H]^-$	441.0	169.0	0.100	30	25	3.03

size polytetrafluoroethylene (PTFE/L) syringe filter. Finally, all the tea samples had to be diluted in order to fit into the linear range of the UHPLC–MS/MS method using the same diluent mixture as used for dilution of standard stock solutions. Green and white tea infusions were diluted 50-fold for determination of C, GC, GCG, CG and 500-fold for determination of EGC, EC, EGCG and ECG. Whereas black tea samples were diluted 10-fold for determination of GC, EGC, C, EC, GCG and CG and 200-fold for EGCG and ECG.

2.5. Method validation

First, the repeatability of retention times and peak areas was evaluated at three concentration levels of 25 ng/ml, 250 ng/ml and 2500 ng/ml to perform system suitability test (SST) ($n = 10$). Both standard solutions and tea samples including green (Formosa gunpowder), white (Snow buds) and black (Keemun) tea infusions were used for validation of the UHPLC–MS/MS method. Validation was performed in terms of linearity, range, LOD, LOQ, intra-day and inter-day precision, accuracy and matrix effects. Linearity was evaluated using nine calibration levels for each compound. The limit of quantification (LOQ) was determined as the lowest concentration level with S/N ~ 10 and the limit of detection (LOD) as S/N ~ 3. Green, white and black tea samples spiked with standard solutions at three concentration levels (50 ng/ml, 250 ng/ml and 2500 ng/ml) were used for determination of method precision and method accuracy. Matrix effects were determined in unflavored and flavored teas. Regarding unflavored teas, the same tea samples as used for optimization of conditions for tea infusion preparation and validation was employed. Concerning flavored teas, green tea with strawberry pieces (1.6%) and lemon grass (10%), white tea with raspberries (min 2.4%), raisins and red currants and black tea with cocoa seeds (min 6.3%), cocoa husks (min 4.2%) and pomegranate seeds were subjected to the evaluation of matrix effects. They were evaluated by comparing the slopes of two calibration curves constructed in the diluent mixture and in tea infusion spiked with six concentration levels of standard solutions. The formed tea infusions were diluted as described in Section 2.4.

3. Results and discussion

3.1. UHPLC–MS/MS method development

All 8 analyzed catechins are structurally similar. They differ in the presence of hydroxyl groups and/or ester-bound gallic acid. In addition, some of them form a pair of epimers. Hence, the most critical issue is to separate these pairs because of the same m/z of their precursor and product ions. All four pairs of epimeric catechins were successfully separated using CSH C_{18} stationary phase as demonstrated in the full scan chromatogram (Fig. 1A) and in the SRM chromatograms (Fig. 1B). To fully exploit the potential of coupling UHPLC with MS, the purpose of this work was to develop a fast, efficient and robust UHPLC–MS/MS method. It allows to minimize the risk of analyte degradation, to analyze more samples and to reduce solvent consumption and analysis cost. UHPLC system using columns packed with 1.7 μm particles allows using higher flow rates of mobile phase and therefore to speed up the analysis. However, when using older MS platform, the limiting factor of the flow rate is the scan speed of the mass analyzer and its ability

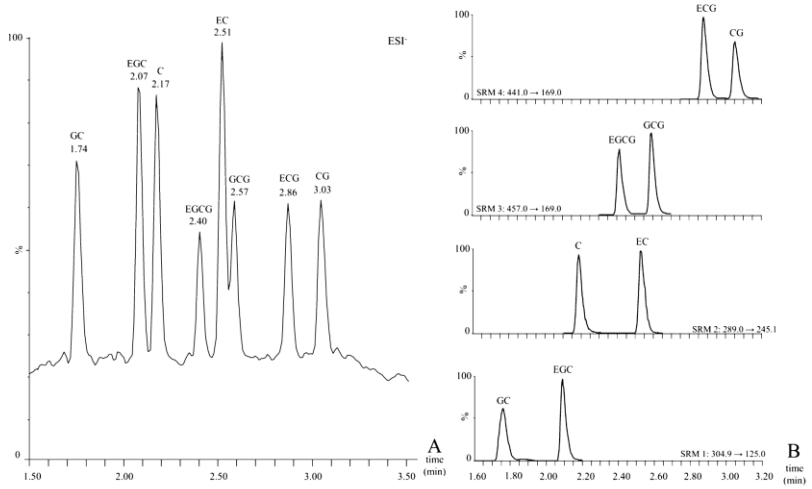

Fig. 1. Full-scan chromatogram of 8 catechins in negative mode of ionization (A) and SRM chromatograms of all four epimeric pairs of catechins (B).

to provide acceptable number of data points per peak. Sufficient number of data points (≥15) was obtained at LOQ levels for all 8 catechins when using flow rate 0.3 ml/min.

Another issue to be considered is a wide range of concentrations of 8 catechins in a single tea sample and also in various tea samples. Therefore, UHPLC–MS/MS method had to be linear in a wide range and infusions of tea samples had to be diluted to fit into this range. Preliminary quantification of catechins in some green, white and black tea samples revealed how much dilution of tea infusions was needed. The final dilution method for green, white and black tea infusions were employed as described in Section 2.4. In conclusion, these dilutions were suitable for quantitative analysis of all 70 different tea samples.

Initial shortcomings of poor linearity and narrow linear range were partially solved by adding formic acid (0.1%) in both components of the mobile phase. To further improve linearity and also peak shapes, composition of diluent mixture was taken into account. Thus, five different ratios 50:50, 60:40, 70:30, 80:20 and 90:10 (v/v) of diluent mixture consisting of 0.1% formic acid in water and 0.1% formic acid in methanol were tested. The ratio 50:50 (v/v) of acidified water (0.1% FA) and acidified methanol (0.1% FA) provided the best results in terms of both observed parameters. This diluent mixture was employed for preparation of both standard solutions and real samples.

In order to minimize matrix effects of catechins the highest possible dilution of tea infusions was needed. Level of dilution was also strongly associated with sensitivity of the method. Therefore, all the ion source and ion optic parameters as described in Section 2.2 were finely tuned to get the highest possible sensitivity. To further enhance sensitivity, the effect of concentration of mobile phase additive was evaluated using five concentrations (0.05%, 0.1%, 0.2%, 0.5% and 1.0%) of formic acid in both mobile phase components. The highest detector response was observed at the concentration 0.1%.

3.2. Optimization of conditions for tea infusion preparation

Conditions for preparation of tea infusions were optimized for all three analyzed types of tea. White tea (Snow Buds, Oxalis), green tea (Formosa Gunpowder, Oxalis) and black tea (Keemun, Oxalis) were subjected to the detailed examination of effect of water temperature, time of infusion preparation, and water-to-tea ratio on the extraction efficiency. All three tested teas were in the form of loose leaf tea. This tea form was chosen at the expense of tea bag form because this one needs more time for unfolding of tea leaves. The preparation of infusions was performed at the temperatures 70 °C, 80 °C, 90 °C and 100 °C. The samples were collected from tea infusions tempered at selected temperatures every 5 min for a 30-min period to evaluate the effect of time. The effect of water-to-tea ratio (ml/g) was tested using following ratios: 50:1, 75:1, 100:1, 125:1 and 150:1. The resulting conditions were subsequently applied for tea infusion preparation in real sample analysis.

3.2.1. Effect of water temperature on the total amount of catechins

An increasing temperature of used solvents makes the cell walls of tea leaves more permeable and increases both diffusion and solubility coefficients of tea catechins [37,38]. On the other hand, increasing temperature has a negative impact on the catechin stability. Catechins undergo epimerization, polymerization, degradation and oxidation [8]. The total amount of catechins (TAC) was increasing in the temperature range of 70–100 °C in the infusion of black tea (Fig. 2C), whereas in both green and white tea infusions only in the range of 70–90 °C (Fig. 2A and B). In both green and white teas at 100 °C degradation of catechins outweighed the benefit of high temperature on the extraction efficiency. Besides determining the total amount of catechins, the ratio between non-epi-forms and epi-forms of catechins was observed. The epimerization converting tea catechins to their corresponding isomers occurs at C-2

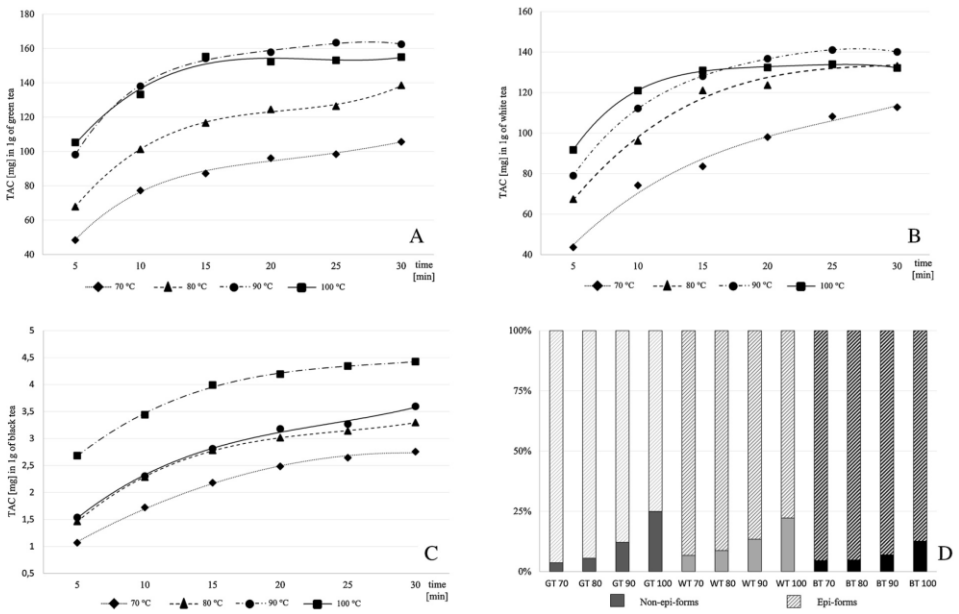

Fig. 2. Optimization of conditions for infusion preparation of green (A), white (B) and black (C) teas. Percentage of both epi- and non-epi-forms of all 8 catechins in the total amount of catechins in green (GT), white (WT) and black (BT) teas at 70, 80, 90 and 100 °C when sampling after 30 min (D).

position of dihydropyran heterocycle, which was previously confirmed by Seto et al. [39] using ^{1}H and ^{13}C NMR and optical rotation analyses, and also at C-3 position when oxidative degallation is taking place [40]. An increase of amount of non-epi-forms of catechins was observed in all three tea types when temperature rising. The percentage of non-epi-forms in the TAC obtained after 30 min of extraction was 3.7%, 5.6%, 12.2% and 25.0% at 70 °C, 80 °C, 90 °C and 100 °C in green tea infusion, 6.7%, 8.7%, 13.4% and 22.2% in white tea infusion and 4.6%, 4.8%, 6.9% and 12.6% in black tea infusion (Fig. 2D). These results confirmed that epi-forms of catechins are prone to epimerization and they also showed lower tendency for epimerization of catechins contained in black tea. Based on these results, temperature at 90 °C was chosen for preparation of green and white tea infusions whereas 100 °C for black tea infusions.

3.2.2. Effect of time of infusion preparation on the total amount of catechins

Besides temperature, time of infusion preparation is also an important factor that affects the TAC. Longer time of infusion preparation increased the extraction efficiency. However, at a certain point degradation of catechins prevailed. After 20 min of extraction performed at all temperatures any substantial increase (>10%) of TAC was not further observed as shown in Fig. 2A–C. In addition, after 20 min the increase of the TAC was less than 5% at temperatures (90 °C for green and white teas, 100 °C for black tea) that were chosen for final quantification of catechins. Based on these results, 20 min period was used for preparation of infusions of all three examined tea types. The 30-min interval of infusion preparation influenced the epimerization of catechins in a similar way as the 30-degree temperature range. As expected, the greatest effect was observed in the 30-min interval at 100 °C. The increase of non-epi-forms of catechins in the TAC between the first and the last sampling was more than 3-fold (7.6% → 25.0%) in green tea and 2-fold both in white (10.3% → 22.2%) and black (6.1% → 12.6%) teas.

3.2.3. Effect of water-to-tea ratio on the total amount of catechins

The impact of water-to-tea ratio was evaluated at 90 °C identically for all three types of tea. Samples were collected after 30 min. In the range of 50:1–150:1 (ml/g) the water-to-tea ratio did not affect the TAC as significantly as the temperature and time. The water-to-tea ratio 100:1 was finally set for all three types of tea. The most significant increase of the TAC was observed in green tea sample (gunpowder) that might be explained by a special form of these tea leaves that are rolled into pellets and need more space for unfolding of leaves.

3.3. Method validation

At first, the repeatability of retention times and peak areas both expressed as RSD% ($n = 10$) were evaluated at three concentration levels (25 ng/ml, 250 ng/ml and 2500 ng/ml). RSD for retention times was <0.3% and for peak areas <5.5%. The UHPLC–MS/MS method was fully validated in terms of intra-day and inter-day precision, accuracy, matrix effects, linearity, range, LOD and LOQ. The UHPLC–MS/MS method showed an excellent linearity expressed by correlation coefficient $r^2 = 0.9991–0.9996$ for all 8 catechins in the range of 25–5000 ng/ml. LOQ of the method was 5–25 ng/ml and LOD 1.5–7.5 ng/ml. Intra-day precision at concentration levels 50, 250 and 2500 ng/ml expressed as RSD ($n = 3$) was in the range of 0.9–5.9% in green tea, 0.9–4.9% in white tea and 1.1–8.2% in black tea. Inter-day precision was in the range of 1.8–11.6% in green tea, 1.6–8.7% in white tea and 3.1–15.7% in black tea. Accuracy of the UHPLC–MS/MS method expressed as recovery was 82.3–149.8% at concentration level 50 ng/ml, 83.1–126.4% at 250 ng/ml, 82.1–110.3% at 2500 ng/ml in green, white and black teas as shown in Tables 2–4. At first, the concentration level 25 ng/ml was included in the method validation. However, at this concentration level insufficient method accuracy was obtained. A higher bias observed at the lowest concentration level (50 ng/ml) for EGC and GCG does not represent a serious drawback of this method. The amount of catechins in tea infusions is high enough and the infusion has to be always diluted to fit into the linear range of UHPLC–MS/MS method. Therefore, a concentration level of catechins in the injected sample is adjusted as needed.

3.4. Matrix effects

Infusions of both flavored and unflavored green, white and black teas were subjected to evaluation of the matrix effects. They were evaluated by comparing the slopes of two calibration curves. The

Table 2
Validation of the UHPLC–MS/MS method in green tea samples.

Analyte	Green tea Intra-day precision RSD (%)			Green tea Inter-day precision RSD (%)			Green tea accuracy (%)			Matrix effects (%)	
	50 ng/ml	250 ng/ml	2500 ng/ml	50 ng/ml	250 ng/ml	2500 ng/ml	50 ng/ml	250 ng/ml	2500 ng/ml	Unflavored green tea	Flavored green tea
GC	3.79	2.10	1.15	3.61	2.30	3.77	91.9	83.1	94.8	+4.51	+7.32
EGC	3.52	2.02	2.50	2.45	2.70	2.25	82.3	97.2	100.5	−0.42	+2.51
C	3.49	3.35	1.11	5.88	3.28	1.76	108.2	95.1	100.8	+4.53	−1.41
EC	5.66	5.85	1.43	4.66	11.58	4.64	113.0	99.5	102.0	+4.64	−1.80
EGCG	5.70	3.29	1.16	3.45	4.45	4.69	94.4	89.7	106.9	+8.45	+4.90
GCG	2.29	3.75	1.11	8.58	7.65	5.03	92.0	98.2	98.4	+13.49	+10.39
ECG	5.07	3.77	0.93	3.49	6.40	5.68	93.9	99.3	98.6	−0.16	−2.17
CG	1.58	2.29	1.86	5.01	5.67	3.51	102.3	114.4	100.5	+8.97	−5.34

Table 3
Validation of the UHPLC–MS/MS method in white tea samples.

Analyte	White tea Intra-day precision RSD (%)			White tea Inter-day precision RSD (%)			White tea accuracy (%)			Matrix effects (%)	
	50 ng/ml	250 ng/ml	2500 ng/ml	50 ng/ml	250 ng/ml	2500 ng/ml	50 ng/ml	250 ng/ml	2500 ng/ml	Unflavored white tea	Flavored white tea
GC	1.59	1.35	1.93	3.32	1.83	2.12	95.4	87.1	82.1	−14.72	+11.21
EGC	3.49	1.15	0.89	3.67	2.68	1.63	146.7	86.1	98.3	−2.15	+8.05
C	2.55	2.38	2.32	2.23	3.81	2.68	100.2	100.8	98.1	+3.52	+5.05
EC	4.27	4.86	3.46	8.71	6.06	2.82	95.3	85.6	102.0	+1.35	+7.46
EGCG	3.40	2.26	2.05	2.72	4.15	2.27	108.6	100.7	110.3	+6.71	+10.50
GCG	3.16	3.30	3.06	4.65	3.81	3.89	98.8	109.1	100.1	+8.19	+12.74
ECG	2.78	2.70	3.04	2.64	4.93	2.18	120.3	105.1	99.1	−2.32	−0.83
CG	4.40	2.50	3.72	6.70	2.21	3.29	102.2	108.1	98.6	+4.36	+1.83

Table 4
Validation of the UHPLC–MS/MS method in black tea samples.

Analyte	Black tea intra-day precision RSD (%)			Black tea inter-day precision RSD (%)			Black tea accuracy (%)			Matrix effects (%)	
	50 ng/ml	250 ng/ml	2500 ng/ml	50 ng/ml	250 ng/ml	2500 ng/ml	50 ng/ml	250 ng/ml	2500 ng/ml	Unflavored black tea	Flavored black tea
GC	8.17	2.11	1.12	6.72	3.06	4.81	119.6	93.8	89.3	−4.40	+9.44
EGC	3.49	2.20	1.40	3.88	5.07	5.54	127.9	111.9	97.6	+7.38	+43.72
C	5.75	3.31	2.01	6.14	3.32	5.91	109.0	101.6	95.8	+1.90	+12.40
EC	5.58	1.59	2.48	15.73	6.37	7.26	107.3	99.3	106.7	−10.03	+34.84
EGCG	2.62	2.26	2.12	5.13	7.90	8.05	104.4	93.2	95.9	+0.83	+10.68
GCG	7.53	1.20	1.95	8.75	3.56	7.60	149.8	126.4	104.1	+14.49	+45.75
ECG	3.85	3.12	1.22	7.39	3.84	6.90	93.5	97.3	98.5	−2.01	+2.76
CG	4.84	1.23	2.74	4.82	4.63	7.86	121.8	110.7	97.7	+4.30	+13.27

first calibration curve was constructed using standards of catechins that were dissolved in the diluent mixture. The second one was constructed in tea infusion that was diluted and spiked with standards of catechins at six concentration levels in the range of 50–2500 ng/ml. In order to keep the same conditions as used for further quantification of catechins in real tea samples, each tea sample was prepared in the same way as described in Section 2.4 using two dilutions. The resulting matrix effects of each catechin expressed as percentage deviation of the slopes of calibration curves were lower than ±15% in all three unflavored teas. Therefore, only filtration through 0.22 μm PTFE/L filter and subsequent appropriate dilution of tea infusion was sufficient as a sample preparation step for quantification of catechins in unflavored teas. As shown in detail in Tables 2–4, matrix effects of all 8 catechins ranged from −0.4% to +13.5% in green tea, from −14.7% to +8.2% in white tea and from −10.0% to +14.5% in black tea. The matrix effects were not so significant in flavored green (−5.3% to +10.4%) and flavored white tea (−0.8% to +12.7%) as well. Whereas, catechins contained in flavored black tea were affected much more significantly. Indeed, the matrix effects ranged from +2.8% to +45.8%. Based on these results, 10-fold dilution of flavored black tea particularly for GCG (+45.8%), EGC (+43.7%) and EC (+34.8%) was not enough to avoid this phenomena. When evaluating matrix effects of these catechins in 200-fold diluted matrix, they were decreased below 8% for all of them. Thus, the high 200-fold dilution made possible to eliminate these effects. However, the amount of non-epi-forms of catechins, EGC and EC in black tea samples was too low to dilute the infusion more.

3.5. Quantification of catechins in tea samples

Eight catechin derivatives were quantified in 28 green tea, 22 white tea and 20 black tea samples that are commonly available for tea consumers in the Czech Republic, except 3 green tea samples purchased in France. Both forms of tea i.e. loose leaf teas and tea bag teas and also both unflavored and flavored teas were subjected to quantification of catechins. The selected tea samples should cover a representative range of both cheaper and more expensive and at the same time quite unknown and well-known tea brands. The total amount of catechins and the amount of individual catechins in green, white and black tea samples are shown in Tables 5–7. Overall, the highest TAC (133.3 mg/g dry weight) was determined in Chinese loose leaf white tea called Snow buds whereas the lowest TAC was detected in tea bag black tea produced by a supermarket. The TAC in 28 green tea samples was in the range of 34.5–125.4 mg/g, in 22 white tea samples 20.8–133.3 mg/g and in 20 black tea samples 8.8–85.7 mg/g. Overall, the average values of the TAC in green (76.7 mg/g) and white (66.1 mg/g) tea samples were similar. The average value of the TAC in black tea samples

Table 5
The amount of individual catechins (mg/g dry weight), the total amount of catechins (TAC) (mg/g dry weight), pH of formed infusions of green teas and price corresponding to 10 g of tea: € = 0.01–0.25€, €€ = 0.26–0.50€, €€€ = 0.51–0.75€, €€€€ = 0.76–1.00€ and €€€€€ = 1.01–1.25€. TBT indicates tea bag tea and LLT loose leaf tea.

Green tea	Tea form	GC (mg/g)	EGC (mg/g)	C (mg/g)	EC (mg/g)	EGCG (mg/g)	GCG (mg/g)	ECG (mg/g)	CG (mg/g)	TAC (mg/g)	pH	Price
Saga Green Tea	TBT	7.73	23.24	2.82	7.07	56.29	6.79	20.31	1.15	**125.40**	5.4	€€
Lipton Green Tea	TBT	7.25	25.73	2.99	8.71	53.36	4.02	18.77	0.63	**121.45**	5.5	€
Silver Prout Oxalis	LLT	4.43	26.04	1.33	5.28	45.81	6.91	10.90	0.69	**101.38**	6.0	€€
Teekanne Sencha	TBT	4.70	28.50	1.77	8.05	37.89	3.20	13.64	0.53	**98.29**	5.4	€€
Vietnam Ché ngon Oxalis	LLT	4.27	24.61	1.91	7.97	37.35	4.13	14.39	0.67	**95.31**	5.7	€€
Loyd Green Tea	TBT	4.67	22.42	1.14	5.04	44.05	6.55	10.22	0.70	**94.78**	5.5	€
Jemča Green Tea	TBT	3.79	24.30	1.04	5.06	45.16	4.58	10.11	0.52	**94.55**	5.5	€
Jasmin Green Tea	LLT	3.36	17.22	1.45	6.40	42.39	6.74	16.07	0.87	**94.49**	5.7	€€€
Jeoncha Oxalis	LLT	4.96	31.09	1.20	6.54	35.10	4.24	8.39	0.44	**91.95**	5.8	€€€€
Ahmad Greean Tea	TBT	3.46	14.31	3.69	8.31	32.62	4.77	23.23	1.25	**91.63**	5.6	€€
Dukát Green Tea	TBT	4.29	20.48	1.00	4.24	42.52	7.00	9.16	0.72	**89.40**	5.7	€
Pickwick Green Tea	TBT	4.79	21.42	1.24	6.20	34.86	4.04	11.19	0.56	**84.29**	5.5	€€
Zlatý šálek Green Tea	TBT	4.41	19.54	1.34	5.83	33.83	3.44	11.64	0.52	**80.56**	5.4	€
Albert Bio Green Tea	TBT	3.88	20.66	1.22	6.38	33.07	2.71	8.77	0.31	**77.01**	5.2	€€
Jemča Gunpowder	LLT	3.08	14.52	0.95	3.55	33.29	5.64	10.14	0.60	**71.77**	5.7	€
Coconut and Lemon Oxalis	LLT	3.68	18.98	1.02	6.15	27.80	2.69	10.34	0.39	**71.04**	5.5	€€
Tesco Green Tea	TBT	4.23	16.72	1.90	6.25	25.30	3.32	10.94	0.66	**69.33**	4.9	€€
Pickwick Green Tea Mango	TBT	3.29	15.58	1.08	4.30	26.07	3.33	8.42	0.42	**62.47**	5.5	€€
Teekanne Zen Chai	TBT	2.44	12.48	1.23	4.57	28.07	3.19	9.92	0.55	**62.45**	5.5	€€
Pickwick Green Tea Cranberry	TBT	2.51	17.58	0.76	4.58	25.20	1.40	8.20	0.20	**60.43**	4.5	€€
Théophile Palais des Tés	LLT	3.37	19.65	1.01	5.22	21.56	1.55	7.58	0.19	**60.12**	5.4	€€€€
Thé du Hammam Palais des Tés	LLT	3.34	17.95	0.91	4.66	22.90	2.13	7.39	0.25	**59.54**	5.4	€€€€
Pickwick Green Tea Lemon	TBT	3.16	15.15	1.00	4.40	23.96	2.97	8.14	0.42	**59.20**	5.4	€€€
Dilmah Green Tea	TBT	2.70	13.70	1.34	5.39	21.83	2.49	8.21	0.36	**56.01**	5.4	€€€
Raspberry and Mint Oxalis	LLT	3.01	14.42	0.93	4.33	20.58	1.93	7.80	0.31	**53.30**	5.3	€€
Thé des Vahinés Palais des Tés	LLT	2.58	14.20	0.62	3.55	18.21	1.73	5.63	0.21	**46.72**	5.3	€€€€
Sea Buckthorn Oxalis	LLT	1.68	10.79	0.57	3.85	15.36	1.01	6.20	0.14	**39.58**	4.9	€€
Pickwick Green Tea Strawberry	TBT	1.84	9.03	0.56	2.93	13.70	1.33	4.91	0.20	**34.49**	5.3	€€

Table 6
The amount of individual catechins (mg/g dry weight), the total amount of catechins (TAC) (mg/g dry weight), pH of formed infusions of white teas and price corresponding to 10 g of tea: € = 0.01–0.25€, €€ = 0.26–0.50€, €€€ = 0.51–0.75€, €€€€ = 0.76–1.00€ and €€€€€ = 1.01–1.25€. TBT indicates tea bag tea and LLT loose leaf tea.

White tea	Tea form	GC (mg/g)	EGC (mg/g)	C (mg/g)	EC (mg/g)	EGCG (mg/g)	GCG (mg/g)	ECG (mg/g)	CG (mg/g)	TAC (mg/g)	pH	Price
Snow Buds Oxalis	LLT	2.42	10.05	3.63	6.89	69.27	5.94	33.83	1.26	**133.30**	5.8	€€€€
White Yun Cui Oxalis	LLT	3.07	8.46	7.22	9.83	40.06	3.75	38.62	1.48	**112.49**	5.5	€€€
Lipton Asian White	TBT	5.28	17.10	2.59	6.47	54.81	5.12	18.97	0.78	**111.11**	5.5	€€€
White Monkey Oxalis	LLT	2.82	11.27	2.04	5.82	50.79	8.73	17.63	1.27	**100.36**	5.8	€€€€
Teekanne White Tea Citrus	TBT	3.04	9.98	3.85	7.13	39.66	5.49	24.87	1.66	**95.69**	5.5	€€€€
London FaH Pure White Tea	TBT	2.71	11.21	3.30	7.50	35.90	3.09	23.28	0.74	**87.72**	5.2	€€€
Lipton White Tea Raspberry	TBT	4.04	14.34	2.16	6.34	38.86	2.97	15.57	0.44	**84.72**	5.3	€€
London FaH Pomegranate	TBT	2.81	13.23	0.96	4.54	40.18	4.99	10.70	0.57	**77.97**	5.5	€€€
Teekanne White Tea	TBT	3.17	16.01	1.03	4.40	37.91	4.45	8.73	0.52	**76.21**	5.5	€€€
Teekanne White Tea Red berries	TBT	2.68	15.10	0.92	4.03	35.13	3.83	7.86	0.44	**69.99**	5.4	€€€
Tesco White Tea	TBT	4.12	14.93	2.07	5.78	28.10	2.69	10.40	0.41	**68.48**	5.5	€€
Mistral White Tea and Cherry	TBT	2.39	12.84	1.00	4.51	33.56	3.35	9.81	0.44	**67.90**	5.4	€€€€
Mistral Mango White Tea	TBT	2.77	13.58	1.05	4.60	28.33	2.40	8.89	0.34	**61.95**	5.4	€€€€
Pai Mu Tan Oxalis	LLT	0.92	4.93	0.88	2.71	23.12	2.15	9.54	0.40	**44.65**	5.6	€€€
Peach and Nectarine Oxalis	LLT	1.27	7.53	0.79	3.56	19.72	1.57	7.17	0.28	**41.89**	5.0	€€
Sweet Grape Oxalis	LLT	1.26	5.20	0.74	2.92	21.05	1.94	8.36	0.36	**41.82**	5.3	€€
Shou mei Oxalis	LLT	1.16	5.37	1.29	3.82	15.98	1.42	9.31	0.38	**38.71**	5.5	€€
Juicy Raspberry Oxalis	LLT	1.26	5.67	1.05	3.24	17.35	0.86	8.11	0.17	**37.72**	4.1	€€
Clipper White Tea with Orange	TBT	1.21	6.20	0.53	3.00	15.84	1.49	5.48	0.24	**33.98**	5.4	€€€
Bonthé Shou Mei	TBT	0.52	2.90	0.28	1.95	13.55	1.50	4.24	0.24	**25.17**	5.6	€€€
Bonthé Strawberry and Aloe	TBT	0.48	3.43	0.39	2.25	11.13	0.58	3.92	0.12	**22.31**	5.0	€€€
Herbal White Tea	TBT	0.57	2.90	0.30	1.80	10.65	1.14	3.23	0.19	**20.78**	5.6	€€€€€

Table 7
The amount of individual catechins [mg/g dry weight], the total amount of catechins (TAC) (mg/g dry weight), pH of formed infusions of black teas and price corresponding to 10 g of tea: € = 0.01–0.25€, €€ = 0.26–0.50€, €€€ = 0.51–0.75€, €€€€ = 0.76–1.00€ and €€€€€ = 1.01–1.25€. TBT indicates tea bag tea and LLT indicates loose leaf tea.

Black tea	Tea form	GC (mg/g)	EGC (mg/g)	C (mg/g)	EC (mg/g)	EGCG (mg/g)	GCG (mg/g)	ECG (mg/g)	CG (mg/g)	TAC (mg/g)	pH	Price
Darjeeling rishehat Oxalis	LLT	2.64	8.98	1.75	3.73	42.30	10.18	14.26	1.81	**85.66**	4.9	€€€€
Ahmad Darjeeling	LLT	2.10	6.92	1.67	3.55	42.36	5.23	14.43	1.22	**77.47**	5.5	€€
Loyd Gold	LLT	0.89	2.75	0.99	2.97	15.79	1.99	10.28	0.58	**36.24**	5.5	€€
Cocoa Pods Oxalis	LLT	1.44	6.52	1.14	3.98	9.47	1.40	6.41	0.33	**30.71**	5.4	€€
Ceylon Oxalis	LLT	0.44	1.83	0.68	2.18	10.41	1.37	10.88	0.53	**28.33**	5.5	€€
Assam Nahorhabi Oxalis	LLT	0.33	1.33	0.84	1.73	7.38	1.23	7.90	0.54	**21.28**	4.5	€€
Irish cream Oxalis	LLT	0.42	1.57	0.55	1.81	6.45	0.97	5.98	0.38	**18.14**	5.4	€€
Lipton Earl Grey	TBT	0.67	1.73	0.75	1.80	6.20	0.80	5.36	0.31	**17.62**	5.8	€€
Earl Grey Tesco	LLT	0.55	1.56	0.60	1.66	6.37	0.72	5.78	0.23	**17.45**	5.5	€
Lipton Yellow Label	TBT	0.58	1.53	0.83	1.54	4.75	0.63	4.28	0.24	**14.37**	5.7	€
Ahmad Earl Grey	TBT	0.54	1.60	0.67	1.51	4.04	0.49	4.09	0.21	**13.16**	5.5	€€
Dukát Ceylon	TBT	0.43	1.31	0.52	1.21	4.22	0.49	3.75	0.20	**12.12**	5.5	€
Dilmah Ceylon	TBT	0.40	1.46	0.43	1.15	3.86	0.47	3.80	0.21	**11.80**	5.4	€€
Teekanne Gold	TBT	0.49	1.51	0.34	0.96	4.14	0.55	2.55	0.15	**10.68**	5.6	€€
Pickwick Earl Grey	TBT	0.40	1.35	0.38	1.05	3.68	0.49	3.06	0.17	**10.58**	5.5	€€
Saga	TBT	0.41	1.31	0.38	1.02	3.77	0.52	2.88	0.18	**10.45**	5.7	€€
Caramel Oxalis	LLT	0.23	0.81	0.28	0.75	4.41	0.46	3.33	0.18	**10.45**	5.4	€€
Jemča Ceylon	TBT	0.27	0.96	0.30	0.87	3.43	0.44	3.30	0.20	**9.77**	5.4	€
Dukát Assam	TBT	0.33	1.03	0.36	0.80	3.16	0.37	2.78	0.16	**8.98**	6.0	€
Earl Grey Tesco	TBT	0.38	1.23	0.35	0.86	3.20	0.42	2.25	0.14	**8.84**	5.4	€€

was significantly lower (22.7 mg/g) compared to green and white tea samples, as expected. Indeed, these results correspond to the differences in post-harvest processing of tea leaves, when non-fermented white and green teas contain higher amount of catechins in comparison with fully-fermented black tea. However, the TAC in two Darjeeling black tea samples was much higher (85.7, 77.5 mg/g) than in the other black tea samples (8.8–36.2 mg/g). Due to the high TAC, some catechins contained in these Darjeeling black tea samples and in Cocoa Pods black tea had to be quantified in the 200-fold diluted fraction instead of 10-fold diluted one. The difference between Darjeeling that is often called "the Champagne of teas", and another black tea samples was obvious at the first sight. Both Darjeeling teas were visually heterogeneous due to the presence of both black tea leaves and green tea leaves. The reason of much higher TAC and different appearance may lie in a different way of post-harvest processing of tea leaves. Due to an incomplete oxidation of tea leaves Darjeeling black tea is closer to oolong tea with respect to catechin amount and tea leaves appearance [41]. The results shown in Tables 5–7 suggest that the manufacturer and the price of the tested tea samples do not play a significant role in the total amount of catechins. Apart from individual catechins and the TAC, pH of all prepared infusions was determined because of its effect on catechin stability. Nevertheless, the average pH of all three tea types was practically the same, indeed 5.4 in green tea, 5.4 in white tea and 5.5 in black tea infusions. All the tea samples were in the narrow range of pH between 4.1 and 6.0. In conclusion, pH did not play an important role in the catechin quantification. Regarding the price of the tested tea samples, no obvious trend between the price and the total amount of catechins was observed.

4. Conclusions

A complete procedure involving sample preparation and fast UHPLC–MS/MS method was developed for quantitative

determination of 8 catechins in infusions of green, white and black teas. In terms of the total amount of catechins, 100 ml of water at 90 °C used for 20 min was the most suitable way to prepare infusions of green and white teas. The conditions were the same for infusion preparation of black tea except the higher temperature (100 °C). The UHPLC–MS/MS was fully validated in terms of intra-day and inter-day precision, accuracy, matrix effects, linearity, range, LOD and LOQ. Matrix effects affecting quantification of catechins were evaluated in both flavored and unflavored green, white and black tea infusions. Matrix effects were in an acceptable range from −14.7% to +14.5% observed in all three unflavored tea types as in flavored green and white teas. Nevertheless, catechins in black tea were affected by matrix effects more significantly (range from +2.8 to +45.8%). However higher dilution allowed to decrease this signal enhancement. On the other hand, due to the lower catechin content in black tea infusion, it cannot be diluted more. Therefore, filtration through 0.22 μm PTFE/L filter followed by appropriate dilution was sufficient as a sample pretreatment step for unflavored teas. All 8 catechins were quantified in 28 green, 22 white and 20 black tea samples. Average of the total amount of catechins in green, white and black teas was 76.7, 66.1 and 22.7 mg/g dry weight, respectively. These results correspond to the effect of post-harvest processing of tea leaves on the total amount of catechins and also indicate that a tea brand and the cost of tea do not play a significant role in the total amount of catechins.

Acknowledgment

The authors gratefully acknowledge the financial support of the Charles University, project SVV 260 184. The publication is co-financed by the European Social Fund and the state budget of the Czech Republic. Project no. CZ.1.07/2.3.00/20.0235, the title of the project: TEAB.

References

[1] S. Ahmed, J.R. Stepp, Green Tea: The Plants, Processing, Manufacturing and Production, Tea in Health and Disease Prevention, 2013, pp. 19–31, http://dx.doi.org/10.1016/B978-0-12-384937-3.00002-1
[2] R.S.S. Kumar, S. Murugesan, G. Kottur, D. Gyamfi, Black Tea: The Plants, Processing/Manufacturing and Production, Tea in Health and Disease Prevention, 2013, pp. 41–57, http://dx.doi.org/10.1016/B978-0-12-384937-3.00004-5
[3] Y. Wang, Ch.T. Ho, Polyphenolic chemistry of tea and coffee: a century of progress, J. Agric. Food Chem. 57 (2009) 8109–8114, http://dx.doi.org/10.1021/jf804025c
[4] N. Khan, H. Mukhtar, Tea polyphenols for health promotion, Life Sci. 81 (2007) 519–533, http://dx.doi.org/10.1016/j.lfs.2007.06.011
[5] C. Cabrera, R. Gimenéz, M.C. López, Determination of tea components with antioxidant activity, J. Agric. Food Chem. 51 (2003) 4427–4435, http://dx.doi.org/10.1021/jf0300801
[6] M.G. Sajilata, P.R. Bajaj, R.S. Singhal, Tea polyphenols as nutraceuticals, Compr. Rev. Food Sci. Food Saf. 7 (2008) 229–254, http://dx.doi.org/10.1111/j.1541-4337.2008.00043.x
[7] H. Wang, G.J. Provan, K. Helliwell, Tea flavonoids: their functions, utilisation and analysis, Trends Food Sci. Technol. 11 (2000) 152–160, http://dx.doi.org/10.1016/S0924-2244(00)00061-3
[8] V.K. Ananingsih, A. Sharma, W. Zhou, Green tea catechins during food processing and storage: a review on stability and detection, Food Res. Int. 50 (2013) 469–479, http://dx.doi.org/10.1016/j.foodres.2011.03.004
[9] S. Sang, S. Tian, H. Wang, R.E. Stark, R.T. Rosen, Ch.S. Yang, Ch.T. Ho, Chemical studies of the antioxidant mechanism of tea catechins: radical reaction products of epicatechin with peroxyl radicals, Bioorg. Med. Chem. 11 (2003) 3371–3378, http://dx.doi.org/10.1016/S0968-0896(03)00367-5
[10] Y. Dobashi, T. Hirano, M. Hirano, Y. Ohkatsu, Antioxidant and photo-antioxidant abilities of catechins, J. Photochem. Photobiol. A 197 (2008) 141–148, http://dx.doi.org/10.1016/j.jphotochem.2007.12.019
[11] S.V. Jovanovic, Y. Hara, S. Steenken, G. Simic, Antioxidant potential of theaflavins. A pulse radiolysis study, J. Am. Chem. Soc. 119 (1997) 5337–5343, http://dx.doi.org/10.1021/ja970120f
[12] L.K. Leung, Y. Su, R. Chen, Z. Zhang, Y. Huang, Z.H. Chen, Theaflavins in black tea and catechins in green tea are equally effective antioxidants, J. Nutr. 131 (2001) 2248–2251.
[13] M.P. Almajano, R. Carbó, J.A.L. Jiménez, M.H. Gordon, Antioxidant and antimicrobial activities of tea infusions, Food Chem. 108 (2008) 55–63, http://dx.doi.org/10.1016/j.foodchem.2007.10.040
[14] C.C. Chou, L.L. Lin, K.T. Chung, Antimicrobial activity of tea as affected by the degree of fermentation and manufacturing season, Int. J. Food Microbiol. 48 (1999) 125–130, http://dx.doi.org/10.1016/S0168-1605(99)00034-3
[15] M. Friedman, Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas, Mol. Nutr. Food Res. 51 (2007) 116–134, http://dx.doi.org/10.1002/mnfr.200600173
[16] J.M. Song, K.H. Lee, B.L. Seong, Antiviral effect of catechins in green tea on influenza virus, Antivir. Res. 68 (2005) 66–74, http://dx.doi.org/10.1016/j.antiviral.2005.06.010
[17] M. Hirasawa, K.J. Takada, Multiple effects of green tea catechin on the antifungal activity of antimycotics against *Candida albicans*, J. Antimicrob. Chemother. 53 (2004) 225–229, http://dx.doi.org/10.1093/jac/dkh046
[18] B.J. Park, J.Ch. Park, H. Taguchi, K. Fukushima, S.H. Hyon, K. Takatori, Antifungal susceptibility of epigallocatechin 3-O-gallate (EGCg) on clinical isolates of pathogenic yeasts, Biochem. Biophys. Res. Commun. 347 (2006) 401–405, http://dx.doi.org/10.1016/j.bbrc.2006.06.037
[19] P. Bhardwaj, D. Khanna, Green tea catechins: defensive role in cardiovascular disorders, Chin. J. Nat. Med. 11 (2013) 345–353, http://dx.doi.org/10.3724/SP.J.1009.2013.00345
[20] Y. Zuo, H. Chen, Y. Deng, Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with photodiode array detector, Talanta 570 (2002) 307–316, http://dx.doi.org/10.1016/S0039-9140(02)00030-9
[21] X.R. Yang, Ch.X. Ye, J.K. Xu, Y.M. Jiang, Simultaneous analysis of purine alkaloids and catechins in *Camellia sinensis*, *Camellia ptilophylla* and *Camellia assamica* var. *kucha* by HPLC, Food Chem. 100 (2007) 1132–1136, http://dx.doi.org/10.1016/j.foodchem.2005.11.021
[22] P.L. Fernández, F. Pablos, M.J. Martín, A.G. González, Study of catechin and xanthine tea profiles as geographical tracers, J. Agric. Food Chem. 50 (2002) 1833–1839, http://dx.doi.org/10.1021/jf0114435
[23] L. Yao, Y. Jiang, N. Datta, R. Singanusong, X. Liu, J. Duan, K. Raymont, A. Lisle, Y. Xu, HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia, Food Chem. 84 (2004) 253–263, http://dx.doi.org/10.1016/S0308-8146(03)00209-7
[24] Y. Masukawa, Y. Matsui, N. Shimizu, N. Kondou, H. Endou, M. Kuzukawa, T. Hase, Determination of green tea catechins in human plasma using liquid chromatography-electrospray ionization mass spectrometry, J. Chromatogr. B. 834 (2006) 26–34, http://dx.doi.org/10.1016/j.jchromb.2006.02.008
[25] L. Peng, X. Song, X. Shi, J. Li, Ch. Ye, An improved HPLC method for simultaneous determination of phenolic compounds, purine alkaloids and theanine in Camellia species, J. Food Comp. Anal. 21 (2008) 559–563, http://dx.doi.org/10.1016/j.jfca.2008.05.002
[26] J.-Q. Jin, J.-Q. Ma, Ch.-L. Ma, M.-Z. Yao, L. Chen, Determination of catechin content in representative Chinese tea germplasms, J. Agric. Food Chem. 62 (2014) 9436–9441, http://dx.doi.org/10.1021/jf5024559
[27] S. Sang, M.-J. Lee, Z. Hou, Ch.-T. Ho, Ch.S. Yang, Stability of tea polyphenol (−)-epigallocatechin-3-gallate and formation of dimers and epimers under common experimental conditions, J. Agric. Food Chem. 53 (2005) 9478–9484, http://dx.doi.org/10.1021/jf0519055
[28] M. Araya-Farias, E. Alain Gaudreau, L. Rozoy, Bazinet, Rapid HPLC–MS method for the simultaneous determination of tea catechins and folates, J. Agric. Food Chem. 62 (2014) 4241–4250, http://dx.doi.org/10.1021/jf4053258
[29] J.G. Napolitano, T. Gödecke, D.C. Lankin, B.U. Jaki, J.B. McAlpine, S.-N. Chen, G.F. Pauli, Orthogonal analytical methods for botanical standardization: determination of green tea catechins by qNMR and LC–MS/MS, J. Pharmaceut. Biomed. Anal. 93 (2014) 59–67, http://dx.doi.org/10.1016/j.jpba.2013.06.017
[30] A.A. Rahim, S. Nofrizal, B. Saad, Rapid tea catechins and caffeine determination by HPLC using microwave-assisted extraction and silica monolithic column, Food Chem. 147 (2014) 262–268, http://dx.doi.org/10.1016/j.foodchem.2013.09.131
[31] D.T.-T. Nguyen, D. Guillarme, S. Rudaz, J.-L. Veuthey, Chromatographic behavior and comparison of column packed with sub-2 μm stationary phases in liquid chromatography, J. Chromatogr. A. 1128 (2006) 105–113, http://dx.doi.org/10.1016/j.chroma.2006.06.069
[32] D. Guillarme, D.T.-T. Nguyen, S. Rudaz, J.-L. Veuthey, Recent developments in liquid chromatography-impact on qualitative and quantitative performance, J. Chromatogr. A. 1149 (2007) 20–29, http://dx.doi.org/10.1016/j.chroma.2006.11.014
[33] Z. Späčil, L. Nováková, P. Solich, Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography, Talanta 76 (2008) 189–199, http://dx.doi.org/10.1016/j.talanta.2008.02.021
[34] D. Guillarme, C. Casetta, C. Bicchi, J.-L. Veuthey, High throughput qualitative analysis of polyphenols in tea samples by ultra-high pressure liquid chromatography coupled to UV and mass spectrometry detectors, J. Chromatogr. A 1217 (2010) 6882–6890, http://dx.doi.org/10.1016/j.chroma.2010.08.060
[35] M. Naldi, J. Fiori, R. Gotti, A. Périat, J.-L. Veuthey, D. Guillarme, V. Andrisano, UHPLC determination of catechins for the quality control of green tea, J. Pharmaceut. Biomed. Anal. 88 (2014) 307–314, http://dx.doi.org/10.1016/j.jpba.2013.08.054
[36] Z. Späčil, L. Nováková, P. Solich, Comparison of positive and negative ion detection of tea catechins using tandem mass spectrometry and ultra high performance liquid chromatography, Food Chem. 123 (2010) 535–541, http://dx.doi.org/10.1016/j.foodchem.2010.04.048

[37] Q.V. Vuong, J.B. Golding, C.E. Stathopoulos, M.H. Nguyen, P.D. Roach, Optimizing conditions for the extraction of catechins from green tea using hot water, J. Sep. Sci. 34 (2011) 3099–3106, http://dx.doi.org/10.1002/jssc.201000863
[38] Q.V. Vuong, J.B. Golding, M.H. Nguyen, P.D. Roach, Extraction and isolation of catechins from tea, J. Sep. Sci. 33 (2010) 3415–3428, http://dx.doi.org/10.1002/jssc.201000438
[39] R. Seto, H. Nakamura, F. Nanjo, Y. Hara, Preparation of epimers of tea catechins by heat treatment, Biosci. Biotech. Biochem. 61 (1997) 1434–1439, http://dx.doi.org/10.1271/bbb.61.1434
[40] P. Coggon, G.A. Moss, H.N. Graham, G.W. Sanderson, The biochemistry of tea fermentation: oxidative degallation and epimerization of the tea flavanol gallates, J. Agric. Food Chem. 21 (1973) 727–733.
[41] S. Dubeau, G. Samson, H.-A. Tajmir-Riahi, Dual effect of milk on the antioxidant capacity of green, Darjeeling, and English breakfast teas, Food Chem. 122 (2010) 539–545, http://dx.doi.org/10.1016/j.foodchem.2010.03.005.

8.2 Synthesis of a molecularly imprinted sorbent for selective solid-phase extraction of β-*N*-methylamino-L-alanine

Talanta 144 (2015) 1021–1029

Contents lists available at ScienceDirect

Talanta

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

Synthesis of a molecularly imprinted sorbent for selective solid-phase extraction of β-N-methylamino-L-alanine

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ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form
10 July 2015
Accepted 17 July 2015
Available online 17 July 2015

Keywords:
Molecularly imprinted silica
Sol–gel
Solid phase extraction
β-N-methylamino-l-alanine

ABSTRACT

The aim of the work was to synthesize a molecularly imprinted material for the selective solid-phase extraction (SPE) of β-N-methylamino-L-alanine (L-2-amino-3-methylpropionic acid; BMAA) from cyanobacterial extracts. BMAA and its structural analogs that can be used as template are small, polar and hydrophilic molecules. These molecules are poorly soluble in organic solvents that are commonly used for the synthesis of acrylic-based polymers. Therefore, a sol gel approach was chosen to carry out the synthesis and the resulting sorbents were evaluated with different extraction procedures in order to determine their ability to selectively retain BMAA. The presence of imprinted cavities in the sorbent was demonstrated by comparing elution profiles obtained by using molecularly imprinted silica (MIS) and non-imprinted silica (NIS) as a control. The molecularly imprinted solid-phase extraction (MISPE) procedure was first developed in a pure medium (acetonitrile) and further optimized for the treatment of cyanobacterial samples. It was characterized by high elution recoveries (89% and 77% respectively in pure and in real media).The repeatability of the extraction procedure in pure medium, in real medium and the reproducibility of MIS synthesis all expressed as RSD values of extraction recovery of BMAA were equal to 3%, 12% and 5%, respectively. A MIS capacity of 0.34 μmol/g was measured. The matrix effects, which affected the quantification of BMAA when employing a mixed mode sorbent, were completely removed by adding a clean-up step of the mixed-mode sorbent extract on the MIS.

1. Introduction

β-N-methylamino-L-alanine (BMAA) is a neurotoxic, non-proteinogenic amino acid which was first isolated from seeds of *Cycas circinalis* L. by Vega and Bell in 1967 [1]. This compound is supposed to be produced by nitrogen-fixing cyanobacteria of the genus Nostoc living in symbiosis with the coralloid roots of cycad trees as well as by various taxa of free living cyanobacteria [2]. BMAA is hypothesized to be linked to amyotrophic lateral sclerosis [3] and it is also assumed to have caused amyotrophic lateral sclerosis/Parkinson dementia complex (ALS/PDC) in the indigenous Chamorro people living on the island of Guam [4]. Although BMAA is a polar and hydrophilic compound, some studies hypothesized the possibility of BMAA biomagnification in the environment and in the food chain which would lead to a protein-bound form [5]. The traditional diet of Guam's people contains a source of BMAA in the form of cycad seed flour and flying foxes (*Pteropus mariannus mariannus*) feed on sarcotesta of cycad seeds [6]. This unusual amino acid may be a threat outside the Guam Island. It is due to the fact that BMAA producing cyanobacteria are assumed to be widespread all over the world and BMAA was detected in brain tissues of patients who suffered from neurodegenerative diseases whereas it was not generally detected in parallel control experiments on healthy subjects [7,8].

BMAA is commonly analyzed in complex matrices such as cyanobacteria, plant, animal and human tissue samples [2,7–21]. One of the most frequently used analytical method for quantification of BMAA involves the derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and the analysis of derivatized BMAA by liquid chromatography coupled with fluorescence detector (LC-FLD) and more recently by tandem mass

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http://dx.doi.org/10.1016/j.talanta.2015.07.052
0039-9140/

spectrometry [12–17]. As the fluorescent derivative agent, AQC, reacts with both primary and secondary amines, it may react with all the primary and secondary amines occurring in the sample [22]. As cyanobacterial matrix contains a lot of interfering compounds such as other amino acids and isobaric compounds, this may consequently lead to inaccurate quantification and even to false-positive results. The quantification of BMAA contained in cyanobacterial samples shows contradictory results. Some studies [12,13] failed to detect and quantify BMAA whereas other studies [2,9–11] found a considerable amount of this neurotoxin in various taxa of cyanobacteria. The results of a study comparing three analytical methods commonly used for BMAA quantification showed that the LC-FLD method using AQC may lead to false positive response [16]. Sample preparation using mixed mode sorbent involving reversed-phase and ion-exchange interactions was previously reported as an effective clean-up procedure before LC–MS/MS analysis of underivatized BMAA contained in cyanobacterial samples [23,24]. However, significant matrix effects were observed by Combès et al. using this type of sorbent for sample preparation [15].

The selectivity of an extraction procedure can be improved by molecularly imprinted polymers (MIPs) that are synthetic materials possessing specific recognition sites (cavities) for a target analyte. Cheap, easy and rapid synthesis and cartridge preparation are also among the advantages of this technique [25]. The complexation of a template molecule by a functional monomer starts the process of synthesis. It is followed by polymerization in the presence of a cross-linker initiated by acidic or basic compound in sol–gel approach or by radical initiator for the synthesis of acrylates. The template molecule is subsequently removed by a washing step that disrupts the interactions between template and monomer. Formed cavities are complementary to the template in terms of size, shape and functional groups. The control of the imprinting process is mostly realized by an extraction procedure performed in parallel on a non-imprinted polymer (NIP). NIP is synthesized according to the same procedure as MIP except that no template is used for polymerization [26]. BMAA and other structural analogs that can be used as template are polar and hydrophilic compounds poorly soluble in the organic solvents commonly used as porogen for the synthesis of acrylic-based imprinted polymers. Therefore, an alternative sol–gel method using aminosilane monomer and alkoxysilane cross-linker was employed for the synthesis of a sorbent with specific recognition sites. During the formation of a rigid, porous and interconnected network, a liquid sol is converted into a solid gel that is finally fully dried to get a xerogel [27]. Compared to the acrylic based imprinted polymers, silica based ones have been used much less frequently. However some powerful imprinted silica sorbents applied as SPE sorbent have been already described such as for the extraction of triazine herbicides from sugar cane juice [28], nitroaromatic explosives from post-blast samples [29] or ibuprofen from human urine [30]. This work describes for the first time the synthesis of a molecular imprinted silica (MIS) for the extraction of BMAA. Different syntheses of MISs were performed to get a sorbent that retains BMAA selectively. 2,4-diaminobutyric acid (DAB), a natural hepatotoxic and neurotoxic isomer of BMAA, was found to occur with BMAA in cyanobacteria [13]. Therefore, in this work, the study of extraction of DAB was taken into account. After development and optimization of a molecularly imprinted solid-phase extraction (MISPE) method in a pure medium, the repeatability of the extraction procedure and the reproducibility of MIS synthesis were evaluated and the capacity was determined. Finally, the potential of this MIS to extract BMAA and DAB from cyanobacterial extracts was evaluated.

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile, MS grade acetonitrile, HPLC grade methanol and formic acid (FA) were purchased from Carlo Erba (Val de Reuil, France). Milli-Q water was produced by a Millipore water purification system (Molsheim, France). Trichloroacetic acid solution, ammonia hydroxide solution, tetraethyl orthosilicate, L-histidine, 3-aminopropyltriethoxysilane, β-N-methylamino-L-alanine hydrochloride (BMAA) and 2,4-diaminobutyric acid dihydrochloride (DAB) were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). 2,4-diaminobutyric acid-2,4,4-D_3 dihydrochloride (D_3-DAB) was provided by CDN isotopes (Pointe Claire, Canada). Hydrochloric acid solution and L-alanine were purchased from Merck (Darmstadt, Germany). N-methyl-β-alanine was synthesized by Synchem OHG (Felsberg, Germany).

Standard stock solutions (100 μg/mL) of BMAA and DAB were prepared by dissolution in water and they were stored at 4 °C until further dilution.

2.2. Apparatus and analytical conditions

The LC/MS–MS analysis was performed by a high-performance liquid chromatography system Agilent 1200 series (Agilent Technology, Massy, France) which was coupled to a triple quadrupole mass spectrometer Agilent 6410 (Agilent Technology, Massy, France). The chromatographic separation was performed on a ZIC-HILIC column (3.5 μm, 150 × 2.1 mm; Merck Sequant AB, Umeå, Sweden). A previously described LC/MS–MS procedure was used [15]. Briefly, samples were analyzed using linear gradient elution with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient started with 65% of solvent B, decreased to 55% over 25 min, held for 2 min, returned to initial composition within 3 min and kept 15 min to equilibrate the system. The flow rate was set at 0.2 mL/min and the sample injection volume was 5 μL. The mass spectrometer was operated in positive mode of ionization. The ion transitions were set as follows: BMAA 119.1 → 102.1; DAB 119.1 → 101.1 and D_3-DAB 122.1 → 104.2. The fragmentor voltage and the collision energy were set for all three transitions at 71 V and at 4 V, respectively. Moreover, for BMAA, this ion transition was also monitored using the second fragmentor voltage: 46 V. The ion source parameters were as follows: capillary voltage: 4 kV; desolvation gas temperature: 350 °C and desolvation gas flow rate: 6 L/min.

2.3. Synthesis of molecularly imprinted silica sorbents and preparation of cartridges

Templates (N-methyl-β-alanine, L-alanine and L-histidine), porogens (ethanol, water or a mixture EtOH/H_2O, 70/30, v/v), monomer (3-aminopropyltriethoxysilane), cross-linker (tetraethyl orthosilicate) and catalysts (0.01 M hydrochloric acid or 0.01 M ammonium hydroxide) were used for the synthesis of six different molecularly imprinted silica (MIS) sorbents. MISs were synthesized according to the ratio of reagents (template/monomer/cross-linker) either 1/4/30 or 1/4/20 (*n*/*n*/*n*). Conditions for the synthesis of all these sorbents are summarized in Table 1. The detailed procedure is described for the synthesis of MIS 6 that was used for all further experiments. First, the template molecule (0.25 mmol) weighed into a 7 mL glass vial was dissolved in the porogen (3.9 mL). Next, the monomer (1 mmol), the cross-linker (5 mmol), a magnetic stir bar and 50 μL of the catalyst were added successively to the glass vial containing the solution of the template molecule. The resulting solution (5.3 mL) was stirred thoroughly after addition of each reagent. Immediately after catalyst addition

Table 1
Reagents used for the syntheses of six MIS sorbents.

Sorbent	Template	Monomer	Cross-linker	Molar ratio	Catalyst	Solvent
MIS 1	N-methyl-β-alanine	APTES	TEOS	1/4/30	NH_3(Aq)	EtOH
MIS 2	N-methyl-β-alanine	APTES	TEOS	1/4/30	NH_3(Aq)	H_2O
MIS 3	L-alanine	APTES	TEOS	1/4/30	HCl	EtOH/H_2O, 70/30
MIS 4	L-histidine	APTES	TEOS	1/4/30	HCl	EtOH/H_2O, 70/30
MIS 5	L-histidine	APTES	TEOS	1/4/20	HCl	EtOH/H_2O, 70/30
MIS 6	N-methyl-β-alanine	APTES	TEOS	1/4/20	HCl	EtOH/H_2O, 70/30

and stirring the mixture, the screw capped glass vial was immersed in a silicone oil bath heated to 40 °C, stirred and kept there for 24 hours. Then, the product of synthesis was kept at room temperature for 2 h. Thereafter it was placed in an oven heated to 120 °C and kept there for 18 h in order to evaporate the solvent and dry the sorbent. After cooling, the block of polymer was crushed and manually sieved. The fraction with particle sizes between 25 μm and 36 μm was collected and subjected to sedimentation in order to remove fine particles. The sedimentation process was performed three times in a methanol/water (80/20, v/v) mixture and, the polymer was dried at room temperature for at least 24 h. Non-imprinted silica (NIS) sorbents lacking specific recognition sites were synthesized according to the same procedure as used for the synthesis of MIS, except that no template was added to the mixture of reagents.

25 mg of MIS or NIS sorbents were packed into 1 mL propylene cartridges (Interchim, Montluçon, France) between two polyethylene frits (Supelco, Saint-Germain-en-Laye, France). Afterwards, both cartridges were rinsed 7 times with 25 mL of methanol in order to eliminate the template from the MIS. The process of template elimination was checked by the LC–MS/MS analysis of each washing fraction. The reproducibility of MIS sorbent synthesis was evaluated by performing two extraction procedures on five MIS 6 sorbents resulting from five synthesis processes. Three synthesis processes were started on three different days (MIS 6a, MIS 6b and MIS 6c) and another two of them on the same day (MIS 6d and MIS 6e).

2.4. Characterization of MIS sorbent in pure medium

Three extraction procedures ($n=3$) were performed on MIS 6a on three different days to evaluate the repeatability of the MISPE procedure. In both cases, 100 ng of BMAA and 100 ng of DAB dissolved in 1 mL of acidified acetonitrile (0.1% FA) were percolated through the MIS and NIS cartridges. 1 mL of acetonitrile and 2 mL of acetonitrile/methanol/water mixture (80/18/2, v/v/v) were assayed for the washing step and the elution step was performed by 3 mL of methanol. The recovery of each SPE fraction was calculated by comparing its concentration with the concentration of a quality control sample. This control sample contained the same amount of BMAA and DAB as well as the sample loaded onto MIS and NIS and it was subjected to evaporation at the same time as the MISPE fractions. The "witness" sample was used to neglect the influence of ambient conditions on evaporation. After each extraction procedure, the MIS and NIS sorbents were dried under a stream of nitrogen for 30 min to eliminate the residue of elution solvent. After that both MIS and NIS were filled with 1 mL of acetonitrile and stored in the fridge at 4 °C until further use.

Finally, to complete the characterization of the MIS 6 sorbent in pure medium, 1 mL of solution at eight different concentration levels of BMAA (0.1; 0.15; 0.25; 0.5; 0.75; 1.0; 1.5; 2.0 ng/mL) were percolated through the cartridge to determine the maximum capacity of the MIS sorbent.

2.5. Treatment of cyanobacterial samples

The strain *Oscillatoria* sp. PCC 8946 was obtained from the Pasteur Culture Collection of Cyanobacteria (PCC). It was grown according to Cadel-Six et al. [31] in BG 11 medium and with constant illumination. 200 mL of *Oscillatoria* sp. PCC 8946 culture were centrifuged at 4000*g*, for 30 min at 4 °C. The cells pellet was recovered and lyophilized, and 10 mg of dried cyanobacterial cell pellets were weighed into a 15 mL plastic centrifuge tube and 3 mL of 0.1 M trichloroacetic acid were added. The mixture was vortexed for 1 min, sonicated for 2 min at 4 °C using a probe tip sonicator (Vibra Cell Ultrasonic Processor, Sonics & Materials Inc., Danbury, CT, USA) and centrifuged at 3500*g* for 10 min at room temperature. Finally, the supernatant was collected and subjected to further experiments.

2.6. Automated SPE procedure on a mixed mode sorbent

An automated SPE method based on a mixed mode sorbent, developed and fully validated for environmental samples containing BMAA by Combes et al. [15], was applied as the initial sample pretreatment step of the cyanobacterial samples. The extraction procedure using a mixed mode sorbent (Oasis-MCX, 3 cc, 60 mg, Waters, Milford, USA) was fully automated (GX271 liquid handler system, Gilson, Villiers le Bel, France). Briefly, the SPE sorbent was equilibrated with 2 mL of methanol and with 1 mL of water acidified with formic acid (pH=3). After loading of 3 mL of cyanobacterial extract (PCC 8946), the sorbent was washed with 1 mL of 0.1 M HCl and with 2 mL of methanol. The elution step was carried out using 3 mL of methanol containing 5% of NH_3(Aq).

2.7. Clean-up of cyanobacterial extract on MIS

Before the sample loading, MIS 6 and NIS 6 sorbents were equilibrated with 3 mL of acetonitrile and with 1 mL of 0.1% formic acid in acetonitrile. The cyanobacterial extract resulting from the mixed mode SPE procedure was evaporated under a stream of nitrogen, spiked with 100 ng of BMAA and DAB dissolved in acidified acetonitrile (0.1% formic acid) and then percolated through both MIS and NIS cartridges. The washing step was performed by 1 mL of acetonitrile and by 3 mL of mixture consisting of acetonitrile, methanol and water (80/18/2, v/v/v). The elution step was carried out by 3 mL of methanol. Each fraction was evaporated under a stream of nitrogen and then reconstituted by adding 200 μL of mobile phase mixture containing 0.1% formic acid in acetonitrile and 0.1% formic acid in water (65/35, v/v).

2.8. Matrix effects evaluation

Matrix effects were evaluated by comparing the slopes of three calibration curves constructed in the mobile phase, in the elution fraction resulting from the SPE on mixed mode sorbent applied to cyanobacterial samples and in the elution fraction obtained by applying first the mixed mode SPE procedure followed by the MISPE procedure. For this, 40 mg of lyophilized cyanobacteria (PCC

8946) were suspended in 12 mL of 0.1 M trichloroacetic acid, vortexed, sonicated, centrifuged (described in Section 2.5.) and the supernatant was percolated through the Oasis MCX cartridge (described in Section 2.6). The resulting elution fraction (12 mL) was equally divided into two parts. Regarding the first part (6 mL), 1 mL of elution fraction was transferred to each of five Eppendorf test tubes. The entire volume was evaporated and the residue was reconstituted in 66.7 μL of mobile phase containing 50, 125, 250, 500 and 1000 ng of BMAA and DAB. The second part (6 mL) was divided into two fractions, each of them was evaporated, reconstituted in 1 mL of acetonitrile containing formic acid (0.1%) and percolated through the MIS. Both elution fractions obtained by MIS extraction were combined (6 mL), vortexed and 1 mL of the elution fraction was transferred to each of five Eppendorf test tubes, the entire volume was evaporated and reconstituted in the same way as previously. The whole procedure involving both sample preparation and calibration curve construction was performed in two replicates. Each calibration point was injected three times during the analysis of the first procedure and twice during the second one.

3. Results and discussion

3.1. Optimization of MIS synthesis and extraction procedure

In order to avoid template bleeding when applying the imprinted sorbent to real samples, BMAA ($\log P = -3.77$) was not employed as a template for the synthesis. Three other polar amino acids, with similar structures, were used as structural analogs to create cavities: (a) N-methyl-β-alanine ($\log P = -2.95$) that differs from BMAA only by the absence of an amino group at C2-position, (b) L-alanine ($\log P = -2.84$) that lacks the methylamino group at C3-position and (c) L-histidine ($\log P = -3.29$) that differs most significantly (structure are shown in Fig. 1). Due to the poor solubility of these molecules in organic solvents, it was not possible to synthesize imprinted sorbents by radical polymerization using acrylic-based monomers. Six different imprinted sorbents were therefore synthesized by sol–gel method based on the use of organosilanes. According to the functional groups present in the target analyte (amino and carboxyl group), both 3-aminopropyltriethoxysilane as monomer and tetraethyl orthosilicate as cross-linker were used for the synthesis of all six MISs. All synthesis conditions are summarized in Table 1.

First, the synthesis of the MIS 1 failed because of the poor solubility of the template in ethanol. All other syntheses of MIS using water or water/ethanol mixture as a solvent were successful. Concerning the development of the SPE procedure, acetonitrile and acidified acetonitrile (0.1% FA) were used as percolating solvents to facilitate polar interactions between the MIS and BMAA during the sample loading. This means that it was performed in "hydrophilic interaction liquid chromatography (HILIC) like mode". In HILIC mode, elution strength of solvents increases as follows: acetonitrile < methanol < water [32]. In this context, the percolation step was performed in acidified acetonitrile (0.1% AF) and the washing steps were tested on the MIS/NIS 2 to 6 with the mixtures containing decreasing amount of acetonitrile (100, 95, 90, 80, 75, 70 and 60) and increasing amounts of methanol (0, 5, 10, 20, 25, 30 and 40). BMAA was retained completely during percolation step on MIS 2. However a poor selectivity was observed on MIS 2, BMAA also being strongly retained on NIS 2 even when the washing step was optimized.

In return, some selectivity between MIS and NIS was observed for all the other MISs. However, the ability of MIS 3, 4 and 5 to retain BMAA was lower than for the MIS6. A loss of about 20% of BMAA during the washing step with 2.5 mL of ACN/MeOH (80/20, v/v) occurred on the MIS 3, 4 and 5 while no loss of BMAA was observed under these conditions on the MIS 6. In addition, cavities in the MIS 6 were created with N-methyl-β-alanine as template whereas MIS 3 and MIS 4, 5 were developed using L-alanine and L-histidine as template. The purpose of this work being to develop a sorbent for the clean-up of cyanobacterial samples that naturally contain high amounts of these proteinogenic amino acids, a risk of competition may occur particularly if using these MISs during the extraction of BMAA. Therefore, even if the MISs synthesized using histidine (MIS 4 and 5) or alanine (MIS 3) as a template showed a higher selectivity, they would not be applied in the following assays and MIS 6 was preferred.

Therefore, further studies were conducted to improve the selectivity of the sorbent. A significant increase of selectivity between the MIS and the NIS was obtained by the addition of a small amount of water to the solvent used for the washing step. Mixtures consisting of acetonitrile, methanol and water (80/18/2), (80/15/5, v/v/v) and (80/10/10, v/v/v) were tested for the washing step. Ratios of water higher than 2% lead to an important loss of BMAA from the MIS. With the mixture consisting of acetonitrile, methanol and water (80/18/2, v/v/v), a significant increase of selectivity between the MIS and the NIS was observed. Indeed, addition of 2% of water in the washing step caused a loss of retention during this washing step from the NIS 6 ($37 \pm 3\%$ to $47 \pm 7\%$) without affecting the performance of the procedure on the corresponding MIS 6. Indeed recoveries of $90 \pm 6\%$ and $89 \pm 3\%$ for elution fraction were obtained for the extraction procedures respectively for the extraction procedure without and with 2% of water during the washing step.

The final extraction procedure consisted then of conditioning steps using 3 mL of acetonitrile and 1 mL of acidified acetonitrile (0.1% FA). The sample loading step was carried out by acidified acetonitrile (0.1% FA) spiked with BMAA and DAB, the washing step by 1 mL of acetonitrile and by 2 mL of mixture consisting of acetonitrile, methanol and water (80/18/2, v/v/v) and the elution step by 3 mL of methanol.

3.2. Repeatability of the extraction procedure on MIS and reproducibility of the MIS synthesis

To evaluate the repeatability of the MISPE procedure, three extraction procedures were performed on the MIS 6a and on the corresponding NIS 6a within three days ($n = 3$). The results showed good extraction recoveries obtained on MIS for both BMAA (89%) and DAB (76%) as seen on Fig. 2. The RSD values describing repeatability were 3% and 7% for BMAA and DAB, respectively. The

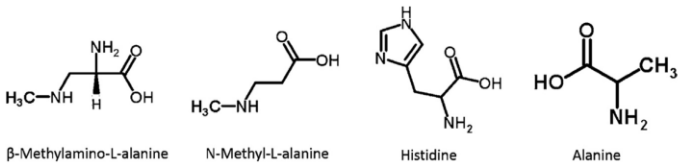

Fig. 1. Chemical structures of BMAA and templates used for the MIS synthesis.

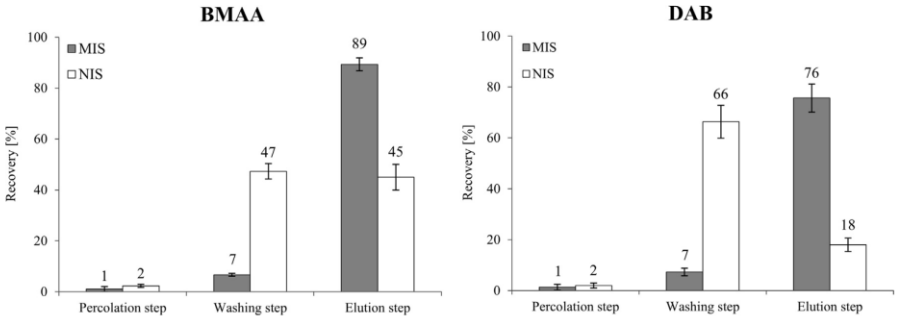

Fig. 2. Repeatability of the extraction procedure. Elution profiles of BMAA and DAB obtained on the MIS 6a/NIS 6a in pure medium. Percolation step: 1 mL of 0.1% formic acid in ACN spiked with 100 ng of BMAA and DAB; washing step: 1 mL of ACN and 2 mL of ACN/MeOH/H_2O 80/18/2 (v/v/v); elution step: 3 mL of MeOH. The average recoveries ± SD (n=3) are reported on the figure.

extraction recoveries obtained on NIS were 45% (RSD=11%) for BMAA and 18% (RSD=14%) for DAB. Thus, the selectivity of the MIS 6 was higher for the DAB molecule. The total recovery yield of BMAA was almost 100% and in the case of DAB it was lower than 85%. That fact was also observed in all further experiments. The evaporation test compared with standard spiked solvents revealed that the loss of DAB was caused by evaporation. The evaporation step was inevitable due to the nature of the LC separation, the HILIC mode being highly sensitive to the composition of the injected solution that has to be very close to the composition of the mobile phase.

To verify the reproducibility of the synthesis, five syntheses of MIS 6 and NIS 6 were performed. To show the global reproducibility of the MISPE method, i.e. sorbent synthesis and SPE extraction procedure, the optimized extraction procedure described at the end of Section 3.1 was performed in pure medium twice on each of five MIS 6a–6e (n=10) (Fig. 3). RSD values of 5% for BMAA and of 8% for DAB of extraction recoveries obtained on MISs 6 demonstrated a very good global reproducibility of the MISPE method in pure medium and a good reproducibility of the MIS synthesis.

3.3. Capacity of the MIS

The capacity of the MIS was evaluated in order to complete its characterization in pure medium. The capacity represents the maximum amount of analyte that can be retained on the sorbent under given conditions without a decrease of extraction recovery. Increasing amounts of BMAA were percolated through the cartridges filled with 25 mg of MIS 6b and NIS 6b sorbents and the quantity of BMAA in the elution fraction was determined. The amount of water contained in samples that are percolated through the MIS plays an important role in the retention mechanism. Because water acts as an elution agent on this sorbent. Therefore, to carry out this experiment the amount of water was controlled and lower than 0.2% in all the spiked samples. The results are shown on Fig. 4. The curve obtained on MIS 6b presents a linear part up to 1 μg of percolated BMAA. This value obtained for 25 mg of MIS corresponds to a capacity of 40 μg/g, i.e. 0.34 μmol/g, and represents the saturation of the specific cavities. It is lower than the range (1 to 40 μmol/g) of capacity of MIP reviewed by Pichon et al. for MIPs resulting from radical polymerization of organic monomers but still sufficient for the application of this MIS to real samples [25]. The result obtained for NIS 6 corresponds to the retention of BMAA by non-specific interactions. The comportment of the NIS sorbent is close to the comportment of the MIS when percolating quantity of BMAA larger than the capacity.

3.4. Optimization and repeatability of the extraction procedure on MIS 6 for cyanobacteria samples

The extraction procedure developed in pure medium (acetonitrile) was applied to real cyanobacterial samples in order to demonstrate the potential of MIS for the treatment of complex matrices. Cyanobacterial strain PCC 8946 was chosen as the blank matrix because of its low content of both BMAA (0.06 pg/mg dry

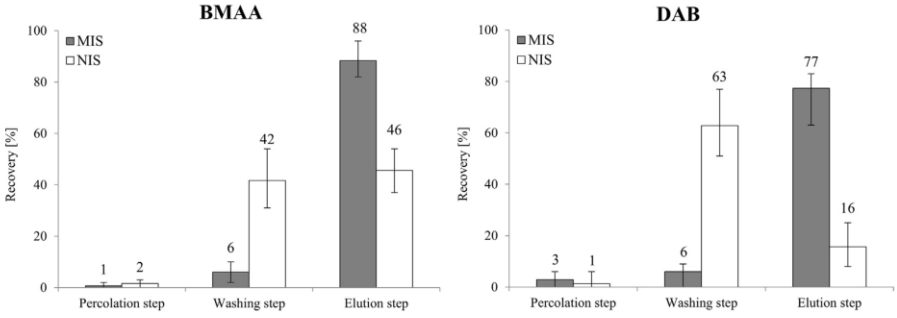

Fig. 3. Reproducibility of the synthesis. Elution profiles of BMAA and DAB obtained on MIS 6a-e/NIS 6a-e in pure medium. Percolation step: 1 mL of 0.1% formic acid in ACN spiked with 100 ng of BMAA and DAB; washing step: 1 mL of ACN and: 2 mL of ACN/MeOH/H_2O 80/18/2 (v/v/v); elution step: 3 mL of MeOH. The average recoveries ± SD (n=10, 2 assays on each MIS/NIS) are reported on the figure.

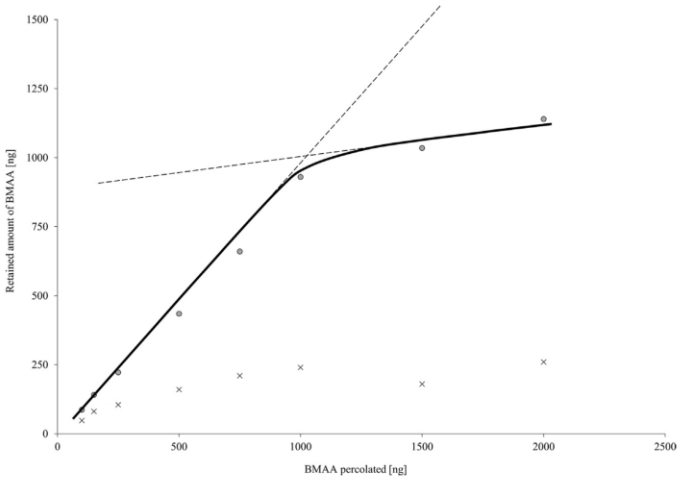

Fig. 4. Capacity of the MIS 6: Amount of BMAA (ng) quantified in the elution fraction obtained on the MIS 6b and on the corresponding NIS 6b as a function of the amount of BMAA percolated. Circles correspond to the MIS and crosses to the NIS.

weight) and DAB (0.1 pg/mg dry weight) (Combès, Méjean et al. unpublished data). After the treatment of dried cyanobacterial cell pellets (described in Section 2.5.), the evaporation of the cyanobacterial extract and spiking with 100 ng of BMAA and DAB dissolved in acidified acetonitrile (0.1% FA), the MIS was not able to retain either BMAA or DAB. Both analytes were eluted during the sample loading step and washing step. It was due to the high complexity of the cyanobacterial matrix that contains a lot of interfering compounds such as other amino acids and polar low-molecular-weight compounds. Therefore the cyanobacterial samples were first treated by a percolation through a mixed-mode sorbent and the resulting extract was cleaned-up through the MIS. In these conditions, both compounds were completely retained on the MIS. The average ($n=3$, $\pm$SD) extraction recoveries obtained on MIS were 85% $\pm$ 7.2 for BMAA and 55% $\pm$ 11.3 for DAB. The extraction recoveries equal to 21% $\pm$ 6.1 (BMAA) and 8% $\pm$ 4.7 (DAB) were obtained on the corresponding NIS. Therefore, the MIS allows the selective extraction of BMAA and DAB from real sample extracts with a recovery close to those in pure medium. Moreover the selectivity between the MIS and the NIS for BMAA is even higher in real medium (21% on the NIS) than in pure medium (46% on the NIS) by employing the same extraction procedure. In order to further improve the clean-up of the cyanobacterial sample, by eliminating more molecules that can interfere with subsequent LC/MS–MS analysis, the volume used in the washing step was increased from 2 to 3 mL. Under these conditions the efficiency of the clean-up was maintained. Indeed, similar reductions of extraction recoveries of BMAA both on MIS (77% $\pm$ 9.1) and NIS (15% $\pm$ 3.8) were observed, while the extraction recoveries of DAB were less affected (51% $\pm$ 9.8 and 7% $\pm$ 2.6) as seen on Fig. 5.

Considering the global analytical procedure, taking into account the recovery of 72% for BMAA on the Oasis MCX on cyanobacteria extract [15] and to the recovery of 77% for BMAA on the MIS during the second step of purification (Fig. 5), to the absence of matrix effect during LC/MS–MS analysis, to the volume percolated on the Oasis MCX cartridge (3 ml of extract issued from 10 mg of lyophilisate cyanobacteria) and to the volume of solvent used for suspension of the elution fraction on the MIS before injection on LC/MS–MS system (200 µl), the LOQ (define for $S/N=10$) of the global procedure was 0.22 ng/mg dry weight of cyanobacteria.

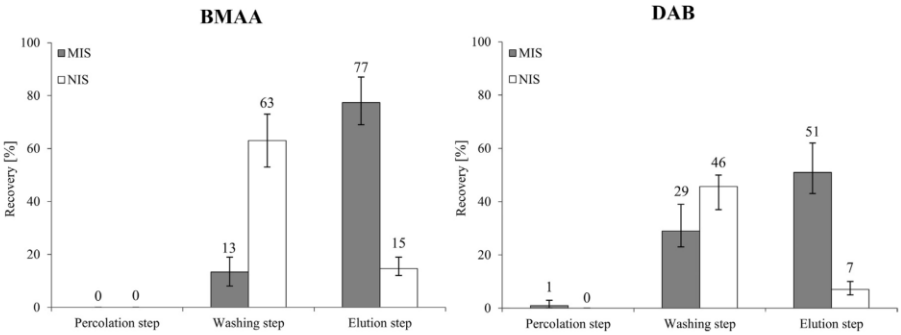

Fig. 5. Elution profiles in a cyanobacterial extract of BMAA and DAB obtained on the MIS 6c/NIS 6c. Percolation step: 1 mL of 0.1% formic acid in ACN spiked with 100 ng of BMAA and DAB; washing step: 1 mL of ACN and 3 mL of ACN/MeOH/H_2O 80/18/2 (v/v/v); elution step: 3 mL of MeOH. The average recoveries $\pm$ SD ($n=3$) are reported on the figure.

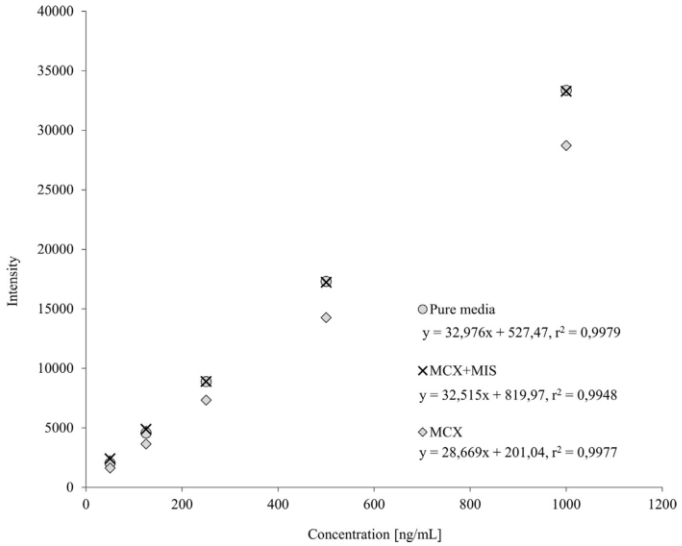

Fig. 6. Matrix effects evaluation. Calibration curves for BMAA constructed in pure medium, in the elution fraction of cyanobacterial sample obtained by MCX SPE and in the elution fraction of a cyanobacterial sample obtained by MCX SPE followed by MIS 6d.

3.5. Matrix effects and efficiency of the clean-up of the cyanobacterial matrix

Matrix effects were evaluated by comparing the slopes of the different calibration curves. The first calibration curve was constructed by injecting solutions corresponding to the composition of the mobile phase spiked with BMAA. The second one was constructed by injecting the elution fraction that was obtained by applying the mixed-mode sorbent to a cyanobacterial extract spiked with BMAA and the third one by injecting the elution fraction obtained by combination of the mixed mode sorbent and the MIS sorbent applied to a cyanobacterial extract spiked with BMAA. The calibration curves (Fig. 6) were drawn using the average values of response ($n=5$) whereas both correlation coefficients and the slopes of the curves were calculated from five single values. The selectivity brought by the MIS sorbent to the sample pretreatment step made the clean-up of the matrix more effective. The matrix effects observed for the cyanobacterial extract that was purified using only the mixed mode sorbent were of about −13% for BMAA. It was lower than previously observed by Combès et al. (−41%) [15]. However, this matrix effect remains troublesome for the reliable and reproducible quantification of BMAA in complex matrices. Regarding DAB, the observed matrix effects were negligible. It may be due to the fact that another Oscillatoria strain (PCC 8946 instead of PCC 6506) was analyzed and that another mass spectrometer was used for the evaluation of matrix effects. If low matrix effects were observed using only the mixed mode sorbent, no matrix effects (Fig. 6) were observed while quantifying BMAA in the extract that was purified by extraction on MCX and subsequently on MIS sorbents. Indeed this calibration curve overlapped the one of standard solutions. The efficiency of the MIS to clean-up the sample is also illustrated in chromatograms acquired in full-scan mode as seen on Fig. 7. Indeed when using only the mixed mode sorbent a high background of compounds appears on the full scan chromatogram between 0 and 15 min. This background is significantly reduced by adding a clean-up step on MIS. This removal of matrix components also allowed us to increase the life time of the analytical column and to limit the clogging of the source of the mass spectrometer.

3.6. Regeneration and reusability of the MIS

Besides the selectivity, the reusability is another indisputable advantage of the MISPE sorbents compared to the common, commercial SPE. The most important issue is to find out how to store and regenerate the sorbent. The MIS was purged with nitrogen after each SPE procedure to remove the residue of the elution solvent, then were filled with acetonitrile and finally stored at 4 °C until further use. The MIS 6 sorbent was synthesized and prepared in five replicates. Two SPE procedures were performed on each of them to evaluate the reproducibility of the synthesis and global procedure. Furthermore, MIS 6a was employed to optimize the SPE procedure and to evaluate its repeatability; altogether ten SPE procedures were performed on it. MIS 6b was used to determine the binding capacity (used 12 times). MIS 6c was applied to optimize the SPE procedure in real medium (used 11 times) and MIS 6d to evaluate matrix effects (used 4 times). Neither a decrease in selectivity nor a decrease in extraction recovery was observed by repeating the same extraction procedure on the same MIS thus proving the reusability of these sorbents.

4. Conclusions

This study has shown the feasibility of the synthesis of a molecularly imprinted material for the selective SPE extraction of a small, polar and highly hydrophilic molecule. The MIS was successfully synthesized by the sol–gel method using N-methyl-β-alanine as a template, 3-aminopropyltriethoxysilane as a monomer and tetraethylorthosilicate as a cross-linker with a ratio of reagents 1/4/20, *n*/*n*/*n*. The polymerization was catalyzed by hydrochloric acid and took place in ethanol/water (70/30, v/v) mixture. The MIS extraction procedure operate in "HILIC-like mode". The sample was loaded in acetonitrile acidified with formic acid

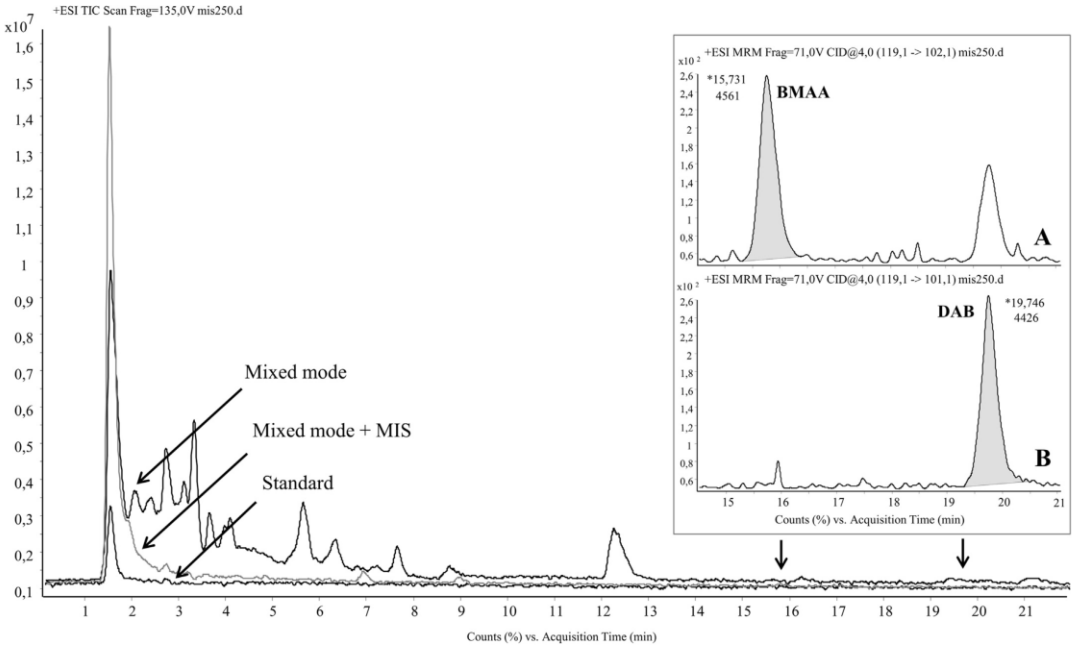

Fig. 7. LC/MS chromatogram (TIC from $m/z=100$ to $m/z=1000$) corresponding to a concentration of 250 ng/mL of both BMAA and DAB dissolved in the mobile phase or in the elution fraction that was obtained by the mixed mode sorbent with or without purification of the resulting extract on MIS 6d. Arrows under the insert figure indicate the retention time of BMAA and DAB. In the insert figure, the SRM chromatograms for specific transitions of A –BMAA (119.1→102.1) and B-DAB (119.1→101.1) in the elution fraction that was obtained by combining the mixed mode sorbent and the MIS are shown.

(0.1%), the MIS was further washed by a mixture consisting of acetonitrile, methanol and water (80/18/2, v/v/v) and the analyte of interest was eluted by methanol. The MIS sorbent showed a good selectivity as compared to the non-imprinted control material and the selectivity between the MIS and the NIS was even higher in a real medium represented by a cyanobacterial extract than in a pure medium. The extraction procedure was characterized by a high elution recovery (89% and 77% respectively in pure and in real media) with a good repeatability (RSD=3% and 9.1% in pure and in real media, respectively). The reproducibility of the MIS synthesis was also demonstrated (RSD=5%). The capacity of MIS was determined and reached a value of 40 μg/g. The MIS sorbent applied as an additional step of sample pretreatment successfully eliminated matrix effects that affected the mixed-mode SPE procedure developed and validated by Combès et al. [15] for the analysis of BMAA in cyanobacterial extracts, the mixed-mode sorbent being not efficient enough to clean up the cyanobacterial sample.

Acknowledgments

We would like to thank Annick Mejean and Olivier Ploux for the gift of the lyophilized strain of cyanobacteria. This work was supported by the French National Research Agency (ANR Program CESA 2011-BMAALS), and by the Charles University In Prague (project SVV 260 184). It is also co-financed by the European Social Fund and the state budget of the Czech Republic (project no. CZ.1.07/2.2.00/28.0194, FAFIS). We would like to thanks the BMAALS publishing group issues of this ANR (Philippe Couratier, Benoit Marin, Pierre-Marie Preux, Michel Druet Cabanac, Marie Nicol, François Paraf, Aurelie Delzor, Annick Mejean, Olivier Ploux, Audrey Combes, Farid Boumédiene, Jean Philippe Leleu, Edwige Garnier, Luc Brient, Marion Lengronne, Valérie Pichon, Saïda El Abdellaoui, Vincent Bonneterre, Dominique Bicout, Emmeline Lagrange, Gérard Besson, William Camu, Nicolas Pageot, Raoul Morales, Véronique Rigaud, Estelle Masseret, Béatrice Bec, Eric Abadie).

References

[1] A. Vega, E.A. Bell, Phytochemistry 6 (1967) 759.
[2] P.A. Cox, S.A. Banack, S.J. Murch, U. Rasmussen, G. Tien, R.R. Bidigare, J. S. Metcalf, L.F. Morrison, G.A. Codd, B. Bergman, Proc. Natl. Acad. Sci. USA 102 (2005) 5074.
[3] T.A. Caller, N.C. Field, J.W. Chipman, X. Shi, B.T. Harris, E.W. Stommel, Amyotroph. Lateral Scler. Off. Publ. World Fed. Neurol. Res. Group Mot. Neuron Dis. 13 (2012) 25.
[4] P.S. Spencer, J. Hugon, A. Ludolph, P.B. Nunn, S.M. Ross, D.N. Roy, H. H. Schaumburg, Ciba Found. Symp. 126 (1987) 221.
[5] S.J. Murch, P.A. Cox, S.A. Banack, Proc. Natl. Acad. Sci. USA 101 (2004) 12228.
[6] S.A. Banack, P.A. Cox, Neurology 61 (2003) 387.
[7] J. Pablo, S.A. Banack, P.A. Cox, T.E. Johnson, S. Papapetropoulos, W.G. Bradley, A. Buck, D.C. Mash, Acta Neurol. Scand. 120 (2009) 216.
[8] S.J. Murch, P.A. Cox, S.A. Banack, J.C. Steele, O.W. Sacks, Acta Neurol. Scand. 110 (2004) 267.
[9] H.E. Johnson, S.R. King, S.A. Banack, C. Webster, W.J. Callanaupa, P.A. Cox, J. Ethnopharmacol. 118 (2008) 159.
[10] M. Esterhuizen, T.G. Downing, Ecotoxicol. Environ. Saf. 71 (2008) 309.
[11] J.S. Metcalf, S.A. Banack, J. Lindsay, L.F. Morrison, P.A. Cox, G.A. Codd, Environ. Microbiol. 10 (2008) 702.
[12] T. Krüger, B. Christian, B. Luckas, Toxicon 54 (2009) 302.
[13] J. Rosén, K.-E. Hellenäs, Analyst 133 (2008) 1785.
[14] A. Combes, S.E. Abdellaoui, J. Vial, E. Lagrange, V. Pichon, Anal. Bioanal. Chem.

406 (2009) 4627.
[15] A. Combes, S.E. Abdellaoui, J. Vial, E. Lagrange, V. Pichon, Anal. Bioanal. Chem. (2014) 1.
[16] E.J. Faassen, F. Gillissen, M. Lürling, PLoS ONE 7 (2012) e36667.
[17] L. Jiang, B. Aigret, W.M. De Borggraeve, Z. Spacil, L.L. Ilag, Anal. Bioanal. Chem. (2012).
[18] S.A. Banack, P.A. Cox, Bot. J. Linn. Soc. 143 (2003) 165.
[19] M.A. Al-Sammak, K.D. Hoagland, D. Cassada, D.D. Snow, Toxins 6 (2014) 488.
[20] S. Downing, V. Contardo-Jara, S. Pflugmacher, T.G. Downing, Ecotoxicol. Environ. Saf. 101 (2014) 51.
[21] E.L. Purdie, S. Samsudin, F.B. Eddy, G.A. Codd, Aquat. Toxicol. Amst. Neth 95 (2009) 279.
[22] S.A. Cohen, Analyst 137 (2012) 1991.
[23] A. Li, H. Fan, F. Ma, P. McCarron, K. Thomas, X. Tang, M.A. Quilliam, The Analyst 137 (2012) 1210.
[24] T. Kubo, N. Kato, K. Hosoya, K. Kaya, Toxicon: Off. J. Int. Soc. Toxinol. 51 (2008) 1264.
[25] V. Pichon, K. Haupt, J. Liq. Chromatogr. Relat. Technol. 29 (2006) 989.
[26] V. Pichon, J. Chromatogr. A 1152 (2007) 41.
[27] M.E. Díaz-García, R.B. Laínño, Microchim. Acta 149 (2005) 19.
[28] R. Gomes Costa Silva, C. Rosa Morais Vigna, C.B.G. Bottoli, C.H. Collins, F. Augusto, J. Sep. Sci. 33 (2010) 1319.
[29] S. Lordel, F. Chapuis-Hugon, V. Eudes, V. Pichon, Anal. Bioanal. Chem. 399 (2011) 449.
[30] K. Farrington, F. Regan, Talanta 78 (2009) 653.
[31] S. Cadel-Six, C. Peyraud-Thomas, L. Brient, N.T. de Marsac, R. Rippka, A. Méjean, Appl. Environ. Microbiol. 73 (2007) 7605.
[32] B. Buszewski, S. Noga, Anal. Bioanal. Chem. 402 (2012) 231.

8.3 Development of MEPS-UHPLC-MS/MS multistatin methods for clinical analysis

Research Article

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Bioanalysis

Development of MEPS-UHPLC–MS/MS multistatin methods for clinical analysis

Background: Statins are the microsomal 3-hydroxy-3methylglutaryl-coenzyme A reductase inhibitors used for the treatment of hypercholesterolemia. Some recent studies revealed also the extra-lipid effects and anticancer activities. Due to the wide incidence of cancer diseases, the number of studies dealing with anticancer statin activities has grown in recent years. Development of one universal multistatin method will be a very convenient way of providing practical and economical multiple statin analysis. **Results/methodology:** Fast and sensitive methods for determination of seven clinically relevant statins, their interconversion products and metabolites (17 analytes in total) in biological samples using microextraction by packed sorbent for sample preparation and UHPLC–MS/MS for subsequent analysis were developed and validated. Three MS platforms with different ion sources, transfer optics, collision cell technologies and scan speed parameters were compared. **Conclusion:** Significant differences among the methods were observed in terms of selectivity and sensitivity. Microextraction by packed sorbent was successful in the extraction of all 17 analytes from biological matrix.

First draft submitted: 14 September 2015; Accepted for publication: 18 November 2015; Published online: 9 February 2016

Keywords: bioanalysis • dwell time • interconversion • MEPS • multianalyte • protein precipitation • SPE • statins • triple quadrupole systems • UHPLC–MS/MS

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Statins are competitive liver-specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme of cholesterol biosynthesis. They represent the most effective drugs for the treatment of hypercholesterolemia and reduce the mortality and morbidity associated with cardiovascular diseases [1–5]. Several recent studies also revealed the extralipid effects: improving or restoring endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, inhibiting platelet aggregation, immunomodulation, stimulation of bone formation and inhibition of the growth of tumor cells [1,6–10]. Due to the huge expansion of cancer diseases, the number of studies dealing with anticancer statin activities has grown in recent years. Therefore a suitable bioanalytical method for the determination of individual statins is highly required [4].

Statins can be divided into three groups: natural (lovastatin, mevastatin), semisynthetic (simvastatin, pravastatin) and synthetic compounds (fluvastatin, atorvastatin, cerivastatin, rosuvastatin and pitavastatin) [1,2]. However, only seven of them are clinically relevant. Mevastatin as the first identified statin has never been used in clinical practice. Cerivastatin was withdrawn from the market in 2001 due to the serious adverse effects. Pitavastatin is a relatively new, highly effective statin available for the treatment only in Japan, the USA and in a part of Europe, so far [1].

Statin molecules exist in lactone (prodrug) and open-ring hydroxy-acid (active) form.

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Some statins are administrated in the active form and some of them in the prodrug form (structures shown in Supplementary Figure 1). The interconversion between lactone and acid form occurs both *in vivo* and *in vitro*. The elimination of the interconversion during the sample preparation and LC–MS analysis is achieved by maintaining pH between 4 and 5. Increasing pH above 6 facilitates the conversion of the lactone to the acid while decreasing pH facilitates the conversion of acid to the lactone [1,11–14]. Therefore, the analysis of both statin forms and their chromatographic separation is one of the key factors for their accurate quantification.

The complex matrices, such as biological materials, contain a lot of endogenous sample components (proteins, salts and various organic compounds), which mostly induce the significant matrix effects (ME) in LC–MS analysis. Therefore, the sample preparation step is necessary initial step of most bioanalytical methods [15–17]. Conventional sample preparation techniques including protein precipitation (PP) and SPE are still the most often used in bioanalysis. However, miniaturized forms of SPE, which bring some advantages such as speed, automation and low sample and solvent consumption, have recently become a trend in the field of sample preparation techniques [16,18–19]. Microextraction by packed sorbent (MEPS) is one of these miniaturized SPE techniques. Recently, the number of commercially available MEPS sorbents has been significantly extended (C18, C8, C2, C8-SCX, silica, polystyrene-divinylbenzene [PS-DVB], R-AX, R-CX and porous graphitic carbon) which provides further flexibility in method development. MEPS provides a rapid and cost-effective alternative sample preparation method, especially in bioanalysis [20,21].

UHPLC–MS/MS system with triple quadrupole (QqQ) detection is the gold standard in modern bioanalytical methods due to the high sensitivity, selectivity and capability to acquire hundreds of SRM (selected reaction monitoring) transitions in one analysis. To improve the compatibility of QqQ with UHPLC system producing very narrow peaks, the dwell time (time spent with acquiring the specific SRM transition) and interchannel/inter-scan delay times of QqQ devices have been decreased over the last years while maintaining or increasing sensitivity. The dwell time is selected according to the peak width and the number of compounds that are monitored simultaneously in order to obtain at least 15 points across the chromatographic peak. Therefore, older MS devices (with high dwell times) often did not provide sufficient number of data points per peak and accurate quantitative analysis when they were used with UHPLC and high flow rates [16,22–23].

So far no method analyzing simultaneously a large number of statins in biological samples has been presented. Only one method determining atorvastatin, lovastatin, pravastatin and simvastatin in water samples was published. However, the lactone forms of statins were not evaluated, which is a considerable drawback of this method [5]. Several UHPLC–MS/MS methods for determination of statins were published, but only few of them analyzed both forms (lactone and hydroxy-acid) in one method [1,24–30]. Another disadvantage of most LC–MS methods was the use of only one internal standard for a group of statins which does not provide guaranteed compensation of ME (different retention times of internal standards and individual analytes). According to literature, the most widely used sample preparation methods for biological samples in statins analysis were PP, SPE and liquid–liquid extraction [1]. Only few methods used modern microextraction techniques [19,31]. Employment of MEPS technique in the analysis of a large number of analytes is challenging and such a method has not been published in scientific literature yet.

The aim of this study was to develop and validate a fast and sensitive UHPLC–MS/MS method for the determination of clinically relevant statins, their interconversion products and metabolites (17 analytes in total) in human serum using PP and MEPS as sample preparation techniques. The MEPS-UHPLC–MS/MS method was developed and validated on three different QqQ systems with different technical specifications (Supplementary Table 1). The results, advantages and drawbacks of individual methods were compared with the regard to MS spectrum (amount of sodium adducts), sensitivity, selectivity, other validation parameters and possibility of dwell time setting to use the second qualifier SRM transition. Such data are important to help the bioanalyst make a wise selection of method set-up. In spite of the fact that statins are not used simultaneously during the treatment, such a procedure will be useful in routine analysis of many samples from still increasing number of studies dealing with biological activity of various statins, especially anticancer effect. Such a universal multistatin method will be definitely much simpler, practical and economical to develop, validate and use in multiple statin analysis compared with an approach using the multiple different procedures individually repeated for each statin. The method applicability was verified on several samples of patients treated by atorvastatin and rosuvastatin, which are the most common agents in clinical practice.

Experimental

Chemicals & reagents

Working standards of atorvastatin calcium salt (AT), atorvastatin lactone (ATL), *p*-hydroxy atorvastatin disodium salt (*p*-OH AT), *o*-hydroxy atorvastatin monosodium salt (*o*-OH AT), rosuvastatin calcium salt (RV),

rosuvastatin lactone (RVL), *N*-desmethyl rosuvastatin disodium salt monohydrate (*N*D-RV), pravastatin sodium salt (PV), pravastatin lactone (PVL), pitavastatin calcium salt (PTV), pitavastatin lactone (PTVL), fluvastatin sodium salt (FV), fluvastatin lactone (FVL), simvastatin (SV), simvastatin acid ammonium salt (SVA), lovastatin (LV) and lovastatin acid sodium salt (LVA) were purchased from Toronto Research Chemicals (Ontario, Canada). All deuterium labeled internal standard (atorvastatin-d5 sodium salt, atorvastatin lactone-d5, *p*-hydroxy atorvastatin-d5 disodium salt, *o*-hydroxy atorvastatin-d5 disodium salt, rosuvastatin-d6 sodium salt, rosuvastatin lactone-d6, *N*-desmethyl rosuvastatin-d6 disodium salt, pravastatin-d3 sodium salt, pravastatin lactone-d3, pitavastatin-d5 sodium salt, pitavastatin lactone-d5, fluvastatin lactone-d5, simvastatin-d6, simvastatin acid-d6 ammonium salt, lovastatin-d3 and lovastatin acid-d3 sodium salt) were purchased from Toronto Research Chemicals (Ontario, Canada), as well.

Acetic acid, reagent grade, ammonia, reagent grade and acetonitrile, LC–MS grade, were purchased from Sigma Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (MA, USA) and it meets European Pharmacopoeia requirements.

Chromatography & MS

UHPLC system Acquity UPLC® (Waters, MA, USA) was used for the purpose of this study. It consisted of ACQ-binary solvent manager and ACQ-sample manager. This UHPLC system was coupled with three different triple quadrupole systems, namely Quattro *micro* API™–device I, Xevo® TQD–device II and Xevo® TQ-S–device III (Waters, Manchester, UK). The technical specifications of the individual devices are shown in Supplementary Table 1.

In early experiments the systematic method development was used for the choice of analytical column and mobile phase. Four analytical UHPLC columns: Acquity UPLC BEH C18, BEH Shield RP 18, BEH Phenyl, all of them (50 × 2.1 mm; 1.7 µm), and HSS T3 (50 × 2.1 mm; 1.8 µm), two pH of mobile phase (pH 3 and 9) and two organic modifiers (acetonitrile [ACN] and methanol [MeOH]) were tested. Finally, chromatographic separation was performed on Acquity UPLC BEH C18 analytical column based on Bridged Ethyl Hybrid (BEH) particles. Mobile phase was composed of ACN (A) and 0.5 mM ammonium acetate pH 4.0 (B) using gradient elution with initial ratio (30:70, A:B). Mobile phase flow rate was different for the device I and devices II/III, respectively. Mobile phase flow rate was 0.30 ml/min for the device I and initial composition of mobile phase was changed within 7 min to 65% of ACN. Subsequently the equilibration step took 3.5 min. Mobile phase flow rate for the devices II and III was 0.5 ml/min and linear gradient profile was recalculated for this higher flow rate using procedure described in [32,33] as follows: 0 min, 30% of ACN; 4.2 min, 65% of ACN; 4.5–6.3 min, 30% of ACN. The analytical columns were kept at 30°C by column oven. The sample solutions were stored in the autosampler at 4°C.

All three MS systems were equipped with a Multi-Mode Ionization Source (ESCI). The MS conditions were tuned in both positive and negative ESI modes individually on all three MS systems. Nitrogen was used as the desolvation and cone gas and argon as the collision gas for all three MS devices. The settings of MS device I were as follows: capillary voltage: 2.5 kV and -2.5 kV, source temperature: 130°C, extractor: 1.0 V, RF lens: 0.5 V, desolvation gas flow 650 l/h, desolvation temperature 450°C, cone gas flow 70 l/h and collision gas pressure 5×10^{-3} mbar. The settings of MS device II were: capillary voltage: 2.25 kV and -2.25 kV, source temperature: 130°C, extractor: 3.0 V, desolvation gas flow 600 l/h, desolvation temperature 550°C, cone gas flow 70 l/h and collision gas flow 0.1 ml/min. The settings of MS device III were: capillary voltage 1.5 kV and -1.5 kV, source temperature: 130°C, source offset: 50 V, desolvation gas flow 600 l/h, desolvation temperature 550°C, cone gas flow 130 l/h, nebulizer gas 7.0 bar and collision gas flow 0.1 ml/min.

Cone voltages (CV) and collision energies (CE) were set-up individually for each analyte. Quantification of all analytes was performed using SRM experiment. Two specific transitions were optimized and used for each analyte on the devices II and III. The first most intense transition was used for the quantification and the second transition for the confirmation of analytes. On the device I only one transition was optimized and used for each molecule. The individual SRM transitions, optimal CV and CE are shown in Table 1. The MassLynx 4.1 Data System was used for MS control and data acquisition. QuanLynx and TargetLynx softwares were used for data processing and quantification – regression analysis of standard, matrix calibration curves and calculation of concentrations.

Preparation of standard solutions & internal standard solutions

The stock standard solutions were prepared by dissolving 1.0 mg of each compound in 1.0 ml of dissolution media which were chosen according to the solubility properties. The stock solutions of pravastatin lactone, rosuvastatin lactone, atorvastatin lactone, atorvastatin, pitavastatin lactone, fluvastatin lactone, lovastatin and simvastatin were prepared in pure acetonitrile. The stock solutions of *N*-desmethyl rosuvastatin, rosuvastatin, pravastatin, fluvastatin, *p*-hydroxy and *o*-hydroxy atorvastatin, pitavastatin, lovastatin acid and simvas-

Table 1. Individual selected reaction monitoring transitions, ionization mode, optimal cone voltages, collision energies and used SIL-ISs for the device I (A) and for the devices II and III (B).

Compounds	t_R		Mode		Precursor		Fragment		CV (V)		CE (eV)		ISs
	A	B	A	B	A	B	A	B	A	B	A	B	
N-desmethyl rosuvastatin	0.58	1.02	ESI⁻		466		404	404 378	30		15	15 20	*N*-desmethyl rosuvastatin-d6
Pravastatin	0.72	1.26	ESI⁻		423		321	321 303	30		15	15 20	Pravastatin-d3
Pravastatin lactone†	1.27	2.14	ESI⁺		407		183	209 183	20		15	20 15	Pravastatin lactone-d3
p-hydroxy atorvastatin	1.34	2.35	ESI⁻		573		413	413 278	35	40	30	35 40	Pravastatin lactone-d3
Rosuvastatin	1.34	2.37	ESI⁻	ESI⁺	480	482	418	258 272	30	50	15	40 40	Rosuvastatin-d6
Pitavastatin	1.97	3.39	ESI⁻	ESI⁺	420	422	358	290 318	25	45	15	30 35	Pitavastatin-d5
Rosuvastatin lactone	2.18	3.72	ESI⁺		464		270	270 282	50		35	40 40	Rosuvastatin lactone-d6
o-hydroxy atorvastatin	2.30	4.00	ESI⁻		573		278	278 134	40	45	40	45 40	*o*-hydroxy atorvastatin-d5
Fluvastatin	2.52	4.29	ESI⁻		410		348	348 322	25	30	15	15 20	Atorvastatin-d5
Atorvastatin	2.52	4.37	ESI⁻		557		278	278 397	40		40	45 25	Atorvastatin-d5
Lovastatin acid	2.83	4.93	ESI⁻		421		319	319 101	35	25	15	20 30	Lovastatin acid-d3
Pitavastatin lactone	3.09	5.25	ESI⁺		404		290	290 274	45		30	30 45	Pitavastatin lactone-d5
Simvastatin acid	3.21	5.65	ESI⁻		435		319	319 115	30	35	15	20 30	Simvastatin acid-d6
Atorvastatin lactone	3.21	5.44	ESI⁺		541		278	448 422	30		40	20 25	Atorvastatin lactone-d5
Fluvastatin lactone	3.50	5.95	ESI⁺		394		376	376 290	25	35	5	5 15	Simvastatin-d6
Lovastatin	3.85	6.51	ESI⁺		405		285	199 285	20		10	15 10	Lovastatin-d6
Simvastatin	4.33	7.32	ESI⁺		419		285	199 285	25		10	15 10	Simvastatin-d6

Individual values in column B are written only if the value is different from the value in the column A. In other cases the values are identical.
†Pravastatin lactone had to be quantified as an adduct $[M+Na]^+$ on the device III. Selected reaction monitoring transitions were 429 → 309 (quantifier) and 429 → 292 (qualifier), CV and CE were similar.
CE: Collision energies; CV: Cone voltages.

tatin acid were prepared in the mixture corresponding to the initial composition of mobile phase ACN: 0.5 mM ammonium acetate pH 4.0, 30:70. Stock standard solutions were further diluted with the mixture corresponding to the initial composition of the mobile phase to obtain the required concentration levels.

The stock internal standard solutions were prepared at the concentration of 1 mg/ml. The mixture of all

internal standards, mentioned in the 'Chemicals and reagents' section, was made at the concentration of 50 µg/ml and further diluted with the mixture corresponding to the initial composition of the mobile phase to obtain the required concentration levels. The concentration levels of SIL-ISs in the sample were 500 ng/ml for the device and 100 ng/ml for the device II and device III.

All stock standard and internal standard solutions were prepared fresh every week.

Sample preparation: protein precipitation & microextraction by packed sorbent

Serum samples were kept at -80°C and after the thaw cycle they were processed immediately by PP and MEPS procedure and analyzed by UHPLC–MS/MS. Briefly, the initial step of sample preparation procedure included PP with ACN as a deproteinization agent. 100 µl of ACN was added to 50 µl of serum sample containing mixture of internal standards (SIL-ISs, listed in Table 1). This mixture was shaken and subsequently incubated for 10 min at laboratory temperature. The precipitated sample was centrifuged at 4200 RPM for 10 min and then the supernatant was collected and diluted with 1900 µl of 0.01 M ammonium acetate pH 4.5. The mixture was loaded on a MEPS C8 sorbent previously activated three-times with 100 µl of ACN and conditioned three-times with 100 µl of 0.01 M ammonium acetate pH 4.5. The sorbent was then washed two times with 100 µl of 0.01 M ammonium acetate pH 4.5 and with 100 µl of the mixture of ACN and 0.01 M ammonium acetate pH 4.5 in ratio 5/95. Analytes were eluted with 100 µl of the mixture of ACN and 0.01 M ammonium acetate pH 4.5 in ratio 95/5. The eluate was evaporated to dryness and reconstituted in 100 µl of the mixture corresponding to the initial composition of mobile phase. Afterwards it was filtered via PTFE microfilter (0.22 µm, Membrane Solutions) and the sample (5 µl) was injected onto UHPLC system.

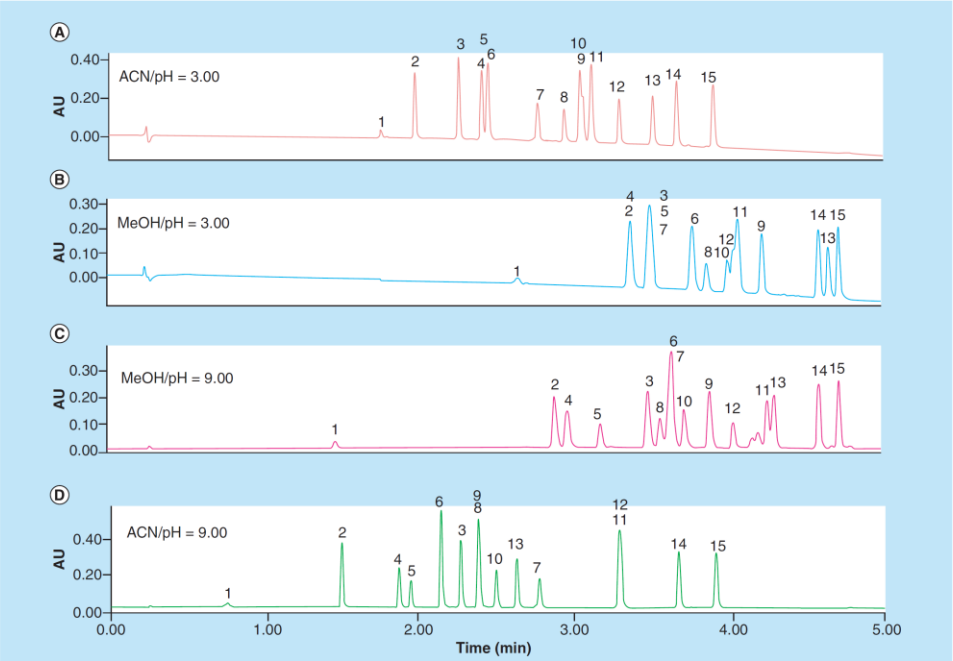

Figure 1. Chromatograms of standard solution obtained during the method development on the Acquity BEH C18 columns.
Mobile phase was composed from ACN/buffer pH 3 **(A)**, MeOH/buffer pH 3 **(B)**, MeOH/buffer pH 9 and ACN/buffer pH 9 **(C)**. Standard solution contains 15 compounds: 1-*N*-Desmethylrosuvastatin, 2-Pravastatin, 3-Pravastatin lactone, 4-Rosuvastatin, 5-*p*-Hydroxyatorvastatin, 6-Pitavastatin, 7-Rosuvastatin lactone, 8-*o*-Hydroxyatorvastatin, 9-Fluvastatin, 10-Atorvastatin, 11-Pitavastatin lactone, 12-Atorvastatin lactone, 13-Simvastatin acid, 14-Lovastatin, 15-Simvastatin.

Method validation

Newly developed methods employing the coupling of PP, MEPS and UHPLC–MS/MS were validated in terms of linearity, accuracy, precision, selectivity, sensitivity (limit of detection and LLOQ) and matrix effects according to the requirements of International Conference on Harmonization (ICH) [34] and EMA guidelines [35]. For the determination of linearity, two calibration curves of all analytes were prepared: matrix calibration curve using blank serum sample, which was spiked and then treated by MEPS procedure, and standard calibration curve where the stock standard solutions were diluted by the mixture corresponding to the initial composition of mobile phase. Method linearity was evaluated in different ranges for individual devices, in other words, 5–1000 ng/ml for the device I, 1–500 ng/ml for the device II and 0.1–100 ng/ml for the device III. Limits of detection and LLOQs were determined based on the S/N ratio approach. Limit of detection was expressed as S/N = 3, limit of quantitation was expressed as S/N = 10.

For evaluation of accuracy, precision and matrix effects three concentration levels and LLOQ concentration were selected for individual devices according to the linear range. Individual concentration levels are shown in section concerning the results of method validation. The method accuracy was evaluated as an average of recovery experiments measured in three replicates at three above mentioned concentration levels and LLOQs. Recovery was determined via a comparison of the response of blank matrix samples spiked before the sample preparation step with the response of blank matrix samples that were first treated with the sample preparation procedure and then spiked with the mixture of analytes and SIL-ISs [36]. For method precision, spiked blank serum treated by PP and MEPS at three above mentioned concentration levels and LLOQs were measured in three replicates in order to calculate the % of RSD, which describes the closeness of agreement between the series of measurements. Matrix effects were evaluated using blank serum samples, which were first treated by sample preparation procedure and then spiked with standard solution at three above mentioned concentration levels and LLOQs. The results were compared with the measurement of standard solution and matrix effects were calculated. Based on the previous extensive studies of statin stability [37–42] only short-term measurement of stability of the samples in standard stock and mobile phase solution at laboratory temperature (20°C) and autosampler temperature (4°C) were evaluated.

Results & discussion

Development of UHPLC–MS/MS method

UHPLC was used as separation technique for the analysis of statins, their interconversion products and metabolites. In early experiments the systematic method development was used for the choice of suitable analytical column and mobile phase. During the method development, four UHPLC columns: BEH C18, BEH Shield RP 18, BEH Phenyl and HSS T3, two pH of mobile phase (pH 3 and 9) and two organic solvents (ACN and MeOH) were employed. Only 15 analytes were available (without fluvastatin lactone and lovastatin acid) for this initial screening.

The systematic method development demonstrated that MeOH is inconvenient for the separation of statins at all four tested columns under UHPLC conditions used (Figure 1). Its elution strength was substantially lower than that of ACN. Most analytes were eluted in the final part of gradient window and separation efficiency was completely insufficient (Figure 1B,C). The pH 9 provided better chromatographic separation than pH 3 especially for the lately eluted analytes (Figure 1A,D). The most suitable separation was obtained on the BEH C18 column when employing the mobile phase, acetonitrile/ammonium acetate pH 9. Unfortunately, at pH 9 visible degradation of statins occurred, which is in agreement with the inconvenience of this pH due to the occurrence of interconversion between acid and lactone form of statins [11,13]. In spite of less favorable results, more detailed optimization of gradient elution had to be further performed at lower pH due to the stability reasons. The separation was finely tuned at the pH 4 and 4.5 [11,13] using ammonium formate and ammonium acetate in the concentration range 0.1–5 mM. No major effect on chromatographic separations was observed, with the exception of the lowest concentration (0.1 mM) toward less reproducible results in terms of peak area repeatability.

However, the MS sensitivity was influenced by the type and concentration of additives (Supplementary Figure 2). In both negative and positive ionization mode, ammonium formate at pH 4.5 provided lower sensitivity than ammonium acetate at pH 4.0 except for lovastatin and simvastatin, which provided higher sensitivity at pH 4.5. As shown in Supplementary Figure 2, increase in additive concentrations led to decrease of MS sensitivities in both ionization modes. Finally with the regard to MS sensitivity, the mobile phase containing 0.5 mM ammonium acetate pH 4.0 and ACN was chosen using gradient elution described in the 'Chromatography and MS' section. LC conditions were the same for all three devices, only the flow rates of mobile phase for the devices II and III were higher (0.5 ml/min) than for the device I (0.3 ml/min) and the gradient profile was recalculated. All of these changes were enabled by the improved construction of the ionization sources and scan speed parameters of the two latter devices.

The device I was limited by acquisition speed and sensitivity.

The optimization of individual MS parameters, especially ESI settings, ionization mode and SRM transitions, was optimized individually for each MS device due to the different construction of the ionization sources, different transfer optics, scan speed parameters of the devices investigated and different flow-rates applied in chromatographic separation. First, the ESI parameters and SRM transitions were optimized and subsequently more suitable ionization mode was selected for each analyte (Table 1). For most analytes, the negative ionization mode provided clearer spectra with the most intense peak of deprotonated molecule $[M-H]^-$ except for lactone forms weakly ionized as $[M+Cl]^-$, $[M+HCOO]^-$ and $[M+CH_3COO]^-$ adducts. Identically for all the three devices, all lactone forms were generally analyzed in positive ionization mode and all hydroxy acid forms were analyzed in negative ionization mode. Only rosuvastatin and pitavastatin were quantified in negative ionization mode on the device I and in positive ionization mode on the devices II and III (Table 1). On the other hand, a huge difference of adduct intensity was observed on all three MS systems in positive ionization mode. On the devices I and II, the protonated molecules $[M+H]^+$were the most intensive peaks in the spectra while $[M+Na]^+$ and $[M+K]^+$ adducts showed very low abundances. The presence of both adducts was significantly higher on the device III. In several cases, $[M+Na]^+$ and $[M+K]^+$ adducts were dominant peaks in the spectra or even the protonated molecules were not present at all, which significantly affected the sensitivity of the method for several analytes. Particularly, the assessment of pravastatin lactone was challenging on the device III as this molecule was not ionized in negative mode and in addition, in positive mode it provided only $[M+Na]^+$ and $[M+K]^+$ adducts. Therefore, pravastatin lactone had to be quantified as an adduct $[M+Na]^+$. The exact explanation of the significantly higher presence of adducts on the device III is not perfectly obvious. However, a different construction of an ionization source could play an important role. A sample cone is used as transfer optics in the ionization sources of devices I and II, while the device III contains a new type of ionization source, using step wave as transfer optics. The results of this study showed that this kind of ion source was more prone to the formation of adducts.

Individual spectra of all analytes obtained on the devices II and III are shown in Supplementary Figure 3.

The scheduled SRMs and two SRM transitions (quantifier and qualifier) for each analyte were used to improve method selectivity on the devices II and III, which substantially increases method selectivity. The occurrence of any interferences would be revealed by the change of the ratio of the two SRM transitions, which is a further parameter to enhance method selectivity. However, only one SRM transition could be employed on the device I due to a low scan speed and lower SRM acquisition rates of the MS instrument (minimal dwell time of device I–10 ms, device II–3 ms and device III–1 ms; see Supplementary Table 1). Most of the SRM transitions were identical for all the three devices. Nevertheless, several analytes provided different intensities of quantifier and qualifier transitions for individual devices. SRM transitions, cone voltages and collision energies of all analytes and all three devices are shown in Table 1.

Development of MEPS procedure

Generally, sample preparation is an indispensable part of bioanalytical LC–MS methods. Currently, the main criteria for the choice of the suitable sample preparation technique in clinical analysis is not only respecting the regulatory approval but also to the possibility of automation and miniaturization in order to limit solvent and sample consumption. This is especially limiting factor in case of laboratory animals. MEPS perfectly meets this criteria using only 50 µl of sample. Combination with PP prevents from cartridge clogging, which is often case also in conventional SPE. Moreover, SPE cartridges are made for a single use, while MEPS sorbents can be used repeatedly. Finally, MEPS extraction is less time-consuming compared with SPE. Therefore, it brings many advantages into sample preparation step [16] and was selected in this study. According to previously published results [19,31] silica C8 and C18 were used as the suitable commercially available MEPS sorbents for the initial testing. First, the influence of the % of organic solvent in the elution agent using the mixture of ACN and 0.01 M ammonium acetate pH 4.5 in the concentration range 80–95% of ACN (Supplementary Figure 4) was verified. The pH effect and pure ACN were not possible to test in the large extent due to the instability of statins. To our knowledge, the pH 4.5 is appropriate for the elimination of acid-lactone interconversion of statins [11,13]. The suitability of the elution solvent was evaluated based on the method recovery. Overall, the results show that the decrease in concentration of ACN in the elution solvent decreased the recoveries of the analytes (Supplementary Figure 4). Therefore, 95% of ACN was selected as the optimal elution solvent for the extraction of all seventeen analytes. Second, the recoveries of the statin standards on different sorbents (C8 and C18) were evaluated. Both sorbents exhibited similar results, indicating that statins could be effec-

tively enriched by commercially available MEPS. Only more hydrophilic analytes (pravastatin and rosuvastatin) provided slightly higher recovery on C8 sorbent. Therefore, the C8 was selected as an optimal sorbent. The influence of the composition of the elution solvent on the recoveries of the all analytes is shown in Supplementary Figure 4.

In the next step, elution volumes (50 and 100 µl) were optimized. The volume of 50 µl 95% of ACN and 0.01 M ammonium acetate pH 4.5 was not sufficient for the elution of analytes from C8 sorbent, as the recovery was only about 15%. Therefore, higher elution volume of 100 µl was employed for sufficient recovery of all investigated analytes (Supplementary Figure 4). All above mentioned steps were optimized using the standard solutions.

As the optimal elution solvent (95% of ACN and 0.01 M ammonium acetate pH 4.5) was not compatible with initial LC gradient composition, the evaporation and reconstitution step had to be included. To minimize the distorted peak shapes in the first part of gradient, different types of reconstitution solvents were tested: the mixture of ACN and 0.5 mM ammonium acetate pH 4.0 in the ratios 50/50, 40/60 and 30/70 (corresponding to the initial gradient composition). The evaporated samples were reconstituted in 100 µl of dissolution media, which represented the minimal acceptable volume still enabling subsequent filtration of the sample. All the three reconstitution solvents provided acceptable peak shapes. The mixture corresponding to the initial gradient composition was therefore chosen as the optimal dissolution medium providing overall acceptable peak shapes and good solubility of analytes.

Finally, using the spiked serum samples, other steps including the protein precipitation, the composition of the loading and washing solvent were also optimized. To remove the sorbent clogging during the sample loading step, the protein precipitation had to be implemented before the MEPS extraction using ACN in the ratio 2:1 (ACN: sample). Due to the high content of ACN in the precipitate, which would prevent from the retention of analytes, the supernatant had to be diluted 20× with 0.01 M ammonium acetate pH 4.5. Furthermore, the composition and volume of the washing solvents were determined with the regard to the selectivity, cleaning efficiency and recovery of the analytes in the spiked serum samples [16]. During the optimization of washing step, 0.01 M ammonium acetate pH 4.5 was chosen as the first washing solvent to remove a large content of salt from the sample. In following washing step aqueous ACN was employed to obtain clearer extract. The composition and volume of the washing solvent was determined with the regard to the selectivity, cleaning efficiency and recovery of the analytes in the spiked serum samples. The optimization of its concentration had to be performed in the concentration range 1–10% of ACN. A concentration of 5% of ACN was finally used to ensure negligible loss of analytes. The whole developed MEPS procedure, including the loading, washing and elution steps, was further verified and validated using the spiked serum samples. The samples were finally prepared according to the protocol described in the 'Sample preparation: protein precipitation & microextraction by packed sorbent' section.

Validation of MEPS-UHPLC–MS/MS methods

The MEPS-UHPLC–MS/MS methods for the determination of seven statins, their seven interconversion products and three metabolites were developed and validated on three different MS devices: an old platform (device I), a newer one (device II) and the state-of-the-art (device III) triple quadrupole systems (Supplementary Table 1). Method validation including determination of linearity, method accuracy, precision, matrix effects, limits of detection and LLOQs was performed for all three methods. The corresponding results are shown in Tables 2–4. SRM chromatograms of blank serum sample spiked with all tested analytes are shown in Figure 2 and SRM chromatograms of blank serum sample spiked with LLOQ concentration of all tested analytes are shown in Supplementary Figure 5. The evaluation of statin stability was performed based on the previous extensive studies [37–42]. Anyway, a short-term stability was confirmed with stock standard solution and mobile phase solutions at laboratory (20°C) and autosampler (4°C) temperature. All analyzed statins were stable at laboratory temperature at least for 6 h, which is sufficient for the sample preparation and sample manipulation. At lower temperature (in the autosampler) the statins were stable for at least 72 h, which is substantially longer than the stability at laboratory temperature and sufficient for routine clinical analysis. These results are in agreement with previous findings.

Device I (Quattro *micro* API)

Method linearity was evaluated in the calibration range 1–1000 ng/ml for standard calibration curves and 5–1000 ng/ml for matrix calibration curves with linear response for all analytes ($r^2 > 0.997$). The results of both standard and matrix calibrations are shown in Table 2. Method accuracy, precision and matrix effects were assessed at three concentration levels of the calibration curve – at high (1000 ng/ml), medium (100 ng/ml) and low (10 ng/ml). Method accuracy expressed as recovery ranged from 84 to 119%. Method precision provided RSD <13% for all analytes. Matrix

effects ranged from -19 to +19% for most analytes except for pravastatin lactone (+47%), rosuvastatin lactone (32%), atorvastatin lactone (25%) and lovastatin (39%) at the concentration level 10 ng/ml. Individual values of method accuracy, precision and matrix effect are shown in Tables 3 & 4. However, the LLOQs of most analytes for matrix samples were 10 ng/ml, which is completely insufficient for the quantitative analysis of real biological samples (see Table 2).

Device II (Xevo TQD)

Method linearity was measured in the concentration range 0.1–500 ng/ml for standard calibration curve and in 0.5–500 ng/ml for matrix calibration curve. Linearity of both calibration curves was higher than 0.995 except for pravastatin lactone in matrix solution (r^2 = 0.990). LLOQs of individual analytes in matrix solution were in the range 1–10 ng/ml with 5 or 1 ng/ml for most of the analytes. Fluvastatin showed the lowest sensitivity with LLOQ of 10 ng/ml. Except for *N*-desmethyl-rosuvastatin and fluvastatin, the sensitivity was two- to 20-times higher compared with the sensitivity of the device I. For some analytes measured in biological samples (*N*-desmethyl rosuvastatin, pravastatin, *p*-hydroxy atorvastatin, fluvastatin, lovastatin hydroxy acid, simvastatin hydroxy acid, fluvastatin lactone, lovastatin and simvastatin) the sensitivity was marginal, as the usual concentration range of statins is 1–100 ng/ml [24,43–45]. Method accuracy, precision and matrix effects were measured at three concentration levels: at high (500 ng/ml), medium (50 ng/ml), low (5 ng/ml) and LLOQs. The individual values of these parameters are shown in Tables 3 & 4. Method accuracy was in the range 79.0–117.1% except for pitavastatin, fluvastatin lactone and lovastatin hydroxy acid. Method precision provided RSD <10% for all analytes at all concentration levels, except for LLOQ level for fluvastatin lactone (<20%). The matrix effects were in the range from -19 to +14%. Only for pravastatin lactone, pitavastatin, *o*-hydroxy atorvastatin, atorvastatin lactone and lovastatin the higher positive matrix effects (between +20 and +40%) were observed especially at higher concentration levels.

Device III (Xevo TQ-S)

Method linearity was measured in the concentration range 0.01–100 ng/ml for standard calibration curve and 0.1–100 ng/ml for matrix calibration curve. Linearity of both calibration curves was higher than 0.993 for all measured analytes except for lovastatin (r^2 = 0.990). Sensitivity was significantly higher compared with the sensitivity of the devices I and II for most analytes. However, LLOQs of fluvastatin, lovastatin acid and simvastatin acid were only two-times lower than those on the device I (Table 2). This could be caused by the negative matrix effects. Due to the higher sensitivity compared with the other devices the much lower concentration levels were selected for the measurement of method accuracy, precision and matrix effects: high (100 ng/ml), medium (10 ng/ml), low (1 ng/ml) and LLOQs, as shown in Tables 3 & 4. Method accuracy was in the range of 84–118% except for *p*-hydroxy atorvastatin and simvastatin. Method precision provided RSD <15% for all analytes and concentration levels including LLOQs. Only fluvastatin lactone provided worse precision (19%) at LLOQ level, similarly to the device II. The matrix effects were in the range from -15 to +17% for all analytes at all concentration levels except for *p*-hydroxy atorvastatin and fluvastatin showing high negative matrix effects between up to -56% (Table 4).

Based on this observation, the matrix effects were evaluated also without SIL-ISs correction. Subsequently, the values of these matrix effects were compared with the matrix effects with SIL-IS correction on the device III (Table 4). In general the matrix effects of analytes eluted until 2.2 min were negligible except for *p*-OH AT (50–70%). In contrast, very significant negative matrix effects were shown for the analytes eluted in the second part of the gradient. To eliminate the influence of complex biological matrices, higher concentration of ACN in washing solvent might potentially help. However, higher ACN concentration caused the loss of more hydrophilic analytes during MEPS procedure, thus it could not be applied in this generic approach. Fluvastatin and simvastatin hydroxy acid were affected by the most intensive negative matrix effects (-87%), which probably caused the lower method sensitivity in the real matrix. Importantly, our results confirmed the indispensability of SIL-ISs for the analysis of complex matrices such as biological sample even when using thorough sample preparation step (Table 4). Overall, these validation experiments and the calculated accuracy and precision of the new methods demonstrate their reliability and usefulness for the routine determination of statins.

Application to real samples

Although the newly developed UHPLC–MS/MS method with PP and MEPS as the sample preparation for the determination of seven statins, their metabolites and interconversion products (17 analytes in total) were optimized and validated on three MS devices, only two methods were applicable for the serum samples of patients treated by atorvastatin or rosuvastatin. As described above, the low sensitivity of the device I prohibited the analysis and determination of statins in biological samples, because the concentrations of all tested statins in serum samples are usually units to

Table 2. Method validation data showing standard and matrix calibration curve linearity and LLOQ for all three methods measured on the devices I, II and III.

	ND-RV	PV	PVL	*p*-OH AT	RV	PTV	RVL	*o*-OH AT	FV	AT	LVA	PTVL	ATL	SVA	FLVL	LV	SV
LINEARITY–STD																	
Device I	0.9996	0.9993	0.9986	0.9994	0.9996	0.9994	0.9995	0.9997	0.9996	0.9997	0.9974	0.9997	0.9991	0.9996	0.9995	0.9991	0.9994
Device II	0.9982	0.9984	0.9982	0.9977	0.9993	0.9990	0.9955	0.9968	0.9952	0.9992	0.9985	0.9975	0.9980	0.9981	0.9990	0.9982	0.9998
Device III	0.9996	0.9996	0.9992	0.9983	0.9995	0.9996	0.9980	0.9990	0.9943	0.9988	0.9928	0.9995	0.9988	0.9989	0.9986	0.9980	0.9984
LINEARITY–MATRIX																	
Device I	0.9993	0.9994	0.9991	0.9990	0.9996	0.9990	0.9994	0.9990	0.9989	0.9990	0.9991	0.9999	0.9988	0.9989	0.9995	0.9992	0.9986
Device II	0.9988	0.9976	0.9984	0.9986	0.9987	0.9977	0.9983	0.9987	0.9987	0.9984	0.9932	0.9995	0.9956	0.9976	0.9974	0.9972	0.9970
Device III	0.9996	0.9995	0.9992	0.9990	0.9995	0.9995	0.9992	0.9948	0.9940	0.9993	0.9951	0.9994	0.9962	0.9939	0.9293	0.9900	0.9970
LOQ–STD (ng/ml)																	
Device I	1	2.5	10	2.5	2.5	1	2.5	2.5	2.5	2.5	10	2.5	2.5	1	2.5	2.5	2.5
Device II	0.5	1	0.5	0.5	0.5	0.1	0.5	0.5	0.5	0.5	1	0.1	0.5	1	1	0.1	0.1
Device III	0.05	0.05	0.01	0.05	0.05	0.01	0.05	0.05	0.1	0.1	0.5	0.01	0.01	1	0.1	0.05	0.1
LOQ–MATRIX (ng/ml)																	
Device I	5	10	10	10	10	5	10	10	10	10	10	10	5	10	10	10	10
Device II	5	5	0.5	5	0.5	1	1	1	10	1	5	1	0.5	5	5	5	5
Device III	0.5	0.5	0.1	1	0.1	0.1	0.5	1	5	1	5	5	1	5	1	1	1

AT: Atorvastatin; ATL: Atorvastatin lactone; FLVL: Fluvastatin lactone; FV: Fluvastatin; LV: Lovastatin; LVA: Lovastatin acid; ND-RV: *N*-desmethyl rosuvastatin; *o*-OH AT: *o*-hydroxy atorvastatin; *p*-OH AT: *p*-hydroxy atorvastatin; PTV: Pitavastatin; PTVL: Pitavastatin lactone; PV: Pravastatin; PVL: Pravastatin lactone; RV: Rosuvastatin; RVL: Rosuvastatin lactone; SV: Simvastatin; SVA: Simvastatin acid.

Table 3. Method validation data showing accuracy and precision for all three methods measured on the devices I, II and III.

		ND-RV	PV	PVL	*p*-OH AT	RV	PTV	RVL	*o*-OH AT	FV	AT	LVA	PTVL	ATL	SVA	FLVL	LV	SV
ACCURACY - 1 (%)	1000	101.4	97.9	102.49	87.7	90.6	95.8	103.8	99.3	91.7	93.2	109.2	105.0	98.9	98.9	84.4	107.8	102.2
	100	105.6	100.2	100.24	100.5	97.9	100.8	105.2	102.3	89.9	100.5	101.3	112.5	96.1	98.1	119.9	96.7	100.6
	10	110.2	114.7	118.98	111.6	107.2	102.6	104.3	110.7	105.6	117.5	95.0	109.7	90.6	104.4	118.5	81.5	113.2
ACCURACY - 2 (%)	500	100.4	117.1	100.7	86.1	103.9	76.9	106.8	99.4	100.3	96.8	130.9	113.1	89.2	99.5	106.5	81.5	102.1
	50	100.5	95.2	80.4	93.7	112.7	75.5	104.5	81.1	99.1	95.8	116.1	97.6	102.4	96.9	163.2	83.5	111.4
	5	98.3	112.2	93.62	93.8	98.7	108.6	114.7	108.7	–	107.2	127.2	91.8	99.3	109.3	98.7	102.5	114.3
	LOQ	–	–	114.1	–	85.9	113.5	80.2	87.9	–	111.8	–	79.0	116.0	–	–	–	–
ACCURACY - 3 (%)	100	100.9	116.9	86.3	100.3	106.5	113.0	93.7	102.1	114.6	91.3	113.8	105.9	92.3	96.1	86.6	87.0	74.8
	10	107.7	113.1	103.6	98.6	112.4	106.9	95.8	99.7	110.6	97.4	97.0	117.9	106.1	121.9	85.7	106.0	86.6
	1	104.9	108.2	95.9	69.2	117.8	112.3	83.9	106.0	–	93.9	–	–	118.6	–	104.0	115.3	132.7
	LOQ	109.2	116.8	128.9	–	110.2	104.9	109.6	–	–	–	–	98.4	–	–	–	–	–
PRECISION - 1 (%)	1000	2.2	1.8	1.8	0.8	1.5	0.3	0.8	0.4	2.4	0.4	0.3	0.9	2.9	1.3	1.5	3.1	0.7
	100	3.1	1.2	5.2	2.3	1.4	1.4	2.4	2.1	4.0	3.3	4.8	0.3	5.2	2.0	8.3	1.5	1.4
	10	9.4	2.7	10.4	0.3	4.5	4.8	10.2	2.8	6.8	0.6	8.9	8.2	8.9	12.5	9.4	3.7	1.2
PRECISION - 2 (%)	500	6.9	2.0	3.6	7.5	3.5	6.4	1.1	1.3	3.6	5.8	8.6	3.7	3.3	3.7	2.3	0.8	0.8
	50	7.0	2.8	8.5	1.5	1.3	2.6	5.3	1.4	5.0	2.8	9.9	3.4	1.9	6.2	6.5	2.1	2.0
	5	13.5	19.1	5.7	8.9	1.4	8.2	4.4	8.8	–	7.6	13.0	5.7	5.7	8.2	19.1	3.1	0.8
	LOQ	–	–	5.5	–	4.9	11.8	13.9	16.7	–	4.3	–	10.0	6.7	–	–	–	
PRECISION - 3 (%)	100	2.4	2.9	9.0	6.6	10.4	12.9	6.8	7.7	4.3	1.3	1.3	1.6	1.8	10.1	4.3	5.7	4.5
	10	2.8	2.9	4.2	5.4	6.2	1.5	4.4	2.3	10.3	6.6	5.4	5.1	5.0	12.3	10.6	1.1	11.5
	1	10.0	9.2	8.4	4.4	1.4	6.6	1.2	7.9	–	1.5	–	–	6.0	–	19.2	4.0	3.5
	LOQ	13.1	14.1	6.5	–	6.7	4.3	3.7	–	–	–	–	13.3	–	–	–	–	–

AT: Atorvastatin; ATL: Atorvastatin lactone; FLVL: Fluvastatin lactone; FV: Fluvastatin; LV: Lovastatin; LVA: Lovastatin acid; ND-RV: *N*-desmethyl rosuvastatin; *o*-OH AT: *o*-hydroxy atorvastatin; *p*-OH AT: *p*-hydroxy atorvastatin; PTV: Pitavastatin; PTVL: Pitavastatin lactone; PV: Pravastatin; PVL: Pravastatin lactone; RV: Rosuvastatin; RVL: Rosuvastatin lactone; SV: Simvastatin; SVA: Simvastatin acid.

Table 4. Method validation data showing matrix effects with SIL-ISs compensation for all three methods measured on the devices I, II and III and matrix effects without SIL-ISs compensation for the method on device III.

		ND-RV	PV	PVL	*p*-OH AT	RV	PTV	RVL	*o*-OH AT	FV	AT	LVA	PTVL	ATL	SVA	FLVL	LV	SV
ME - 1 (%)	1000	-1.2	+1.0	+4.3	-3.1	-2.0	+1.5	-4.1	-0.7	+4.3	-5.6	+5.8	-1.7	-6.2	-1.4	-33.1	+11.2	+1.15
	100	0.0	+0.4	+5.0	-4.1	-0.1	+1.1	+13.1	-2.7	-1.5	+0.5	+10.8	+2.1	-1.1	+4.9	-18.4	+19.7	+1.80
	10	+5.3	+5.2	+46.8	-7.2	+4.4	+3.1	+31.6	-3.7	+8.6	-8.7	+6.1	+19.3	+25.0	+2.5	+1.4	+38.9	+3.74
ME - 2 (%)	500	+3.9	-9.9	-0.1	-1.2	-1.4	+26.3	-3.5	+0.5	+3.8	0.0	-3.8	-13.0	+7.1	-4.7	-20.8	+20.7	-0.4
	50	+12.5	-3.5	+40.7	-7.5	-11.2	+32.6	-3.6	+25.7	+3.6	-2.8	-3.5	-16.9	+22.9	-3.7	-14.7	+14.2	-2.9
	5	+2.8	+0.8	+6.9	+1.9	-7.3	+0.3	-3.2	+0.1	–	+3.0	-5.6	-15.8	+34.1	+1.5	-2.8	-4.3	+7.50
	LOQ	–	–	-18.7	–	+3.0	-6.3	+13.7	+5.6	–	-14.2	–	+11.6	+14.5	–	–	–	
ME - 3 (%)	100	-2.7	-3.0	+4.2	-28.5	+4.7	-0.4	+5.1	+12.1	+8.1	+10.2	+14.4	-5.8	+13.7	-5.0	+11.7	+23.4	-5.0
	10	+2.5	+3.8	-2.1	-45.1	-0.2	-1.3	+3.4	-1.4	-56.2	-1.7	+11.8	-14.2	+3.4	-12.2	-0.3	+4.0	-20.0
	1	+11.2	+8.4	-3.4	-40.1	+1.5	+2.2	+5.0	-13.0	–	+7.6	–	–	+17.1	–	+17.9	+2.0	+14.83
	LOQ	-3.3	+12.2	+6.2	–	+16.1	+10.6	+5.2	–	–	–	–	-11.6	–	–	–	–	
ME - 3 (%) without SIL-IS correction	100	-7.7	-6.0	+3.8	-30.0	+2.4	-6.0	+3.1	-32.7	-34.4	-33.3	-17.2	-12.1	-8.8	-67.8	-21.0	-5.5	-32.5
	10	+1.4	+4.9	+6.2	-41.2	+6.4	-6.2	+4.9	-37.1	-75.3	-37.8	-11.4	-13.4	-19.9	-86.1	-29.2	-18.6	-52.8
	1	+9.1	+12.3	+1.2	-37.5	+6.4	-2.9	+4.2	-40.7	–	-21.0	–	–	-10.5	–	-27.4	-28.1	-53.0
	LOQ	-13.0	+2.0	+9.0	–	+8.0	-10.0	-3.0	–	–	–	–	-21.6	–	–	–	–	–

AT: Atorvastatin; ATL: Atorvastatin lactone; FV: Fluvastatin; FVL: Fluvastatin lactone; LV: Lovastatin; LVA: Lovastatin acid; ND-RV: *N*-desmethyl rosuvastatin; *o*-OH AT: *o*-hydroxy atorvastatin; *p*-OH AT: *p*-hydroxy atorvastatin; PTV: Pitavastatin; PTVL: Pitavastatin lactone; PV: Pravastatin; PVL: Pravastatin lactone; RV: Rosuvastatin; RVL: Rosuvastatin lactone; SV: Simvastatin; SVA: Simvastatin acid.

tens ng/ml and only in some cases reach up to hundreds ng/ml [24,43–46]. Validation experiments showed the sufficient method selectivity and sensitivity of the devices II and III.

The efficiency of both methods was demonstrated by the analysis of the same real samples of serum. The results of the measurements were compared by the t-statistic test. As expected, the differences of the concentrations of measured analytes obtained by both methods were insignificant. Moreover, *N*-desmethyl rosuvastatin was not detected in any patient samples. Overall, box plots and analysis of variability by the t-statistic test describing the seven statins in the real samples of patients measured by device II and device III and analysis of variability are shown in Figure 3 and Supplementary Table 2. These results confirm that the new methods for the MS-based target profiling of statins are powerful tools with high levels of specificity and the number of analytes used in our assay is no way limiting factor neither compromising speed nor sensitivity. However, due to the higher method sensitivity of the device III, this method is more suitable for the analysis of real samples and potentially applicable for the trace analysis of statins, for example, in waste water or in other matrices.

Conclusion

Rapid methods for the determination of seven statins, interconversion products and metabolites in human serum using UHPLC–MS/MS coupled to the combination of PP and MEPS as sample treatment were developed. The MEPS method and the LC conditions were the same, only the flow rates of mobile phase for the devices II and III were higher (0.5 ml/min) and gradient profile was recalculated. MS conditions were optimized individually for all three MS systems and were found to be different. The optimal SRM transitions were identical in most cases. However, the device III provided a significantly higher intensity of sodium and potassium adducts in positive ionization mode compared with other devices. This fact caused challenging quantification of PVL on the device III, which did not provide any signal for protonated molecule and quantification had to be performed using sodium adduct as precursor ion. Selectivity of the method on the devices II and III was increased using two SRM transitions (quantifier and qualifier) and secondary isotopic ratio for all analytes, which was not possible for the device I due to a low scan speed and higher dwell times. Precise and accurate quantification was ensured by individual deuterium labeled internal standards for most compounds. All three MEPS-UHPLC–MS/MS methods were validated, but sensitivity of the methods was significantly different. As expected the oldest device I showed the lowest sensitivity

(LLOQ for matrix samples generally 10 ng/ml), device II provided slightly higher sensitivity for most compounds and the newest device III provided significantly higher sensitivity (LLOQ for matrix samples 0.1–1 except for simvastatin hydroxy acid, lovastatin hydroxy acid, pitavastatin lactone and fluvastatin). Finally, the meth-

Figure 2. SRM chromatograms of blank serum sample spiked with the all tested analytes (100 ng/ml).

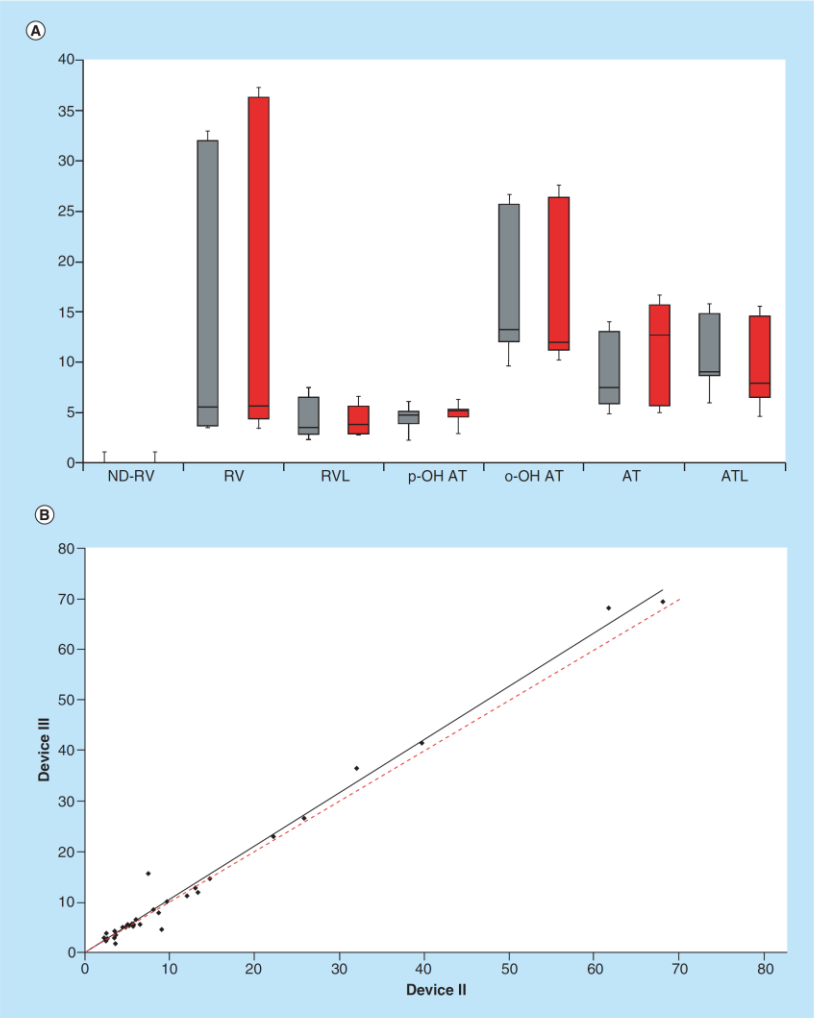

Figure 3. Statistical evaluation of measurements of patient samples. **(A)** Box plots describing the seven statins in the real samples of patient measured on device II (gray) and device III (red). Each boxplot characterizes the variability of data sets using average values of median, first and third quartiles, minimum and maximum. **(B)** Analysis of variability describing the seven statins in the real samples of patient measured device II and device III by the t-statistic. The red line described the ideal results with the variability of 0.

ods II and III were used for the analysis of real samples of patients treated by atorvastatin and rosuvastatin, which are the most common in clinical practice. The results of this study clearly demonstrated the limitations of older platforms in multi-analyte determination and also shown some drawback of newer platforms, which is very

useful for practical method development. In the future perspective our findings might be practically applicable for further development of the methods for the trace analysis of statins in other application fields.

Future perspective

Several recent studies revealed the extralipid effects of statins, such as improving or restoring endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, inhibiting platelet aggregation, immunomodulation, stimulation of bone formation and inhibition of the growth of tumor cells. These effects are now further studied using laboratory animal models. For this reason, new methods for the determination of statins and their metabolites, and even those less widely used in current clinical practice, are needed.

Therefore, newly developed multistatin method allowing the analysis of seven statins and their interconversion products and metabolites provides a high potential for such studies. Multistatin method allows a comparison of the effects among individual statins, which are never administered simultaneously. However, the studies using animal models include simultaneous testing of more different statins in one study. Therefore, it was definitely much simpler, practical and economical to develop, validate and use one universal method with high efficiency, sensitivity and selectivity, than to do this repeatedly once for each statin. For the moment, the method was applied to the analysis of serum sample from atorvastatin and rosuvastatin treated patients because these are the most widely used statins. In future, multistatin methods are likely to be used for the analysis of human as well as laboratory animal samples.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.15.245

Financial & competing interests disclosure

The project is co-financed by the European Social Fund and the state budget of the Czech Republic, project no. CZ.1.07/2.3.00/20.0235, the title of the project: TEAB and research project SVV/2015/260184. This work was also funded by the Ministry of Education, Youth and Sports of the Czech Republic – grant from the National Program for Sustainability I (LO1204). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The UHPLC column BEH C18 offered the most effective separation of individual statins (especially acid and lactone forms).
- Microextraction by packed sorbent together with protein precipitation were shown as the suitable combination of sample preparation techniques for the multi-analyte determination.
- Purity of spectrum and amount of adducts depended on the construction of ionization source.
- Newer platforms provided the higher selectivity and sensitivity.
- The limitation of older platforms in multi-analyte determination were demonstrated.

References

Papers of special note have been highlighted as:
• of interest

1 Nirogi R, Mudigonda K, Kandikere V. Chromatography–mass spectrometry methods for the quantitation of statins in biological samples. *J. Pharm. Biomed. Anal.* 44, 379–387 (2007).

2 Shitara Y, Sugiyama Y. Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug–drug interactions and interindividual differences in transporter and metabolic enzyme functions. *Pharmacol. Ther.* 112, 71–105 (2006).

3 Sacks FM. The relative role of low-density lipoprotein cholesterol and highdensity lipoprotein cholesterol in coronary artery disease: evidence from largescale statin and fibrate trials. *Am. J. Cardiol.* 88, N14–N18 (2001).

4 Pisanti S, Picardi P, Ciaglia E, D'Alessandro A, Bifulco M. Novel prospects of statins as therapeutic agents in cancer. *Pharmacol. Res.* 88, 84–98 (2014).

5 Miao XS, Metcalfe CD. Determination of cholesterol-lowering statin drugs in aqueous samples using liquid chromatography–electrospray ionization tandem mass spektrometry. *J. Chromatogr. A* 998, 133–141 (2003).

6 Farmer JA. Pleiotropic effects of statins. *Curr. Atheroscler. Rep.* 2, 208–217 (2000).

7 Bellosta S, Ferri N, Bernini F, Paoletti R, Corsini A. Non-lipid-related effects of statins. *Ann. Med.* 32, 164–176 (2000).

8 Kendall MJ, Toescu V. Non-lipid properties of statins. *J. Clin. Pharm. Ther.* 24, 3–5 (1999).

9 Wheeler DC. Are there potential non-lipid-lowering uses of statins. *Drugs* 56, 517–522 (1998).

10 Mundy G, Garret R, Harris S *et al.* Stimulation of bone formation *in vitro* and in rodents by statins. *Science* 296, 1946–1949 (1999).

11 Yang DJ, Hwang LS. Study on the conversion of three natural statins from lactone forms to their corresponding hydroxy acid forms and their determination in Pu-Erh tea. *J. Chromatogr. A* 30, 277–284 (2006).

12 Jacobsen W, Kuhn B, Soldner A *et al.* Lactonization os the critical first step in the disposition of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin. *Drug Metab. Dispos.* 28, 1369–1378 (2000).

13 Nováková L, Šatínský D, Solich P. HPLC methods for the determination of simvastatin and atorvastatin. *Trends Anal. Chem.* 27, 352–367 (2008).

• **A review addressing analysis of several statins.**

14 Erturk S, Onal A, Cetin SM. A nalytical methods for the quantitative determination of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in biological samples. *J. Chromatogr. B* 793, 193–205 (2003).

15 Nováková L. Challenges in the development of bioanalytical liquid chromatography-mass spectrometry method with emphasis on fast analysis. *J. Chromatogr. A* 1292, 25–37 (2013).

• **A review addressing general challenges in method development in bioanalysis.**

16 Nováková L, VlČková H. A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation. *Anal. Chim. Acta* 656, 8–35 (2009).

• **A review addressing current trends in bioanalysis.**

17 Saraji M, Khaje N. Recent advances in liquid microextraction techniques coupled with MS for determination of small-molecule drugs in biological samples. *Bioanalysis* 4, 725–739 (2012).

18 Alves G, Rodrigues M, Fortuna A, Falcao A, Queiroz J. A critical review of microextraction by packed sorbent as a sample preparation approach in drug bioanalysis. *Bioanalysis* 5(11), 1409–1442 (2013).

19 VlČková H, Solichová D, Bláha M, Solich P, Nováková L. Microextraction by packed sorbent as sample preparation step for atorvastatin and its metabolites in biological samples–critical evaluation. *J. Pharm. Biomed. Anal.* 55, 301–308 (2011).

20 Abdel-Rehim M. Microextraction by packed sorbent (MEPS): a tutorial. *Anal. Chim. Acta* 701, 119–128 (2011).

• **A tutorial on microextraction by packed sorbent sample preparation technique.**

21 Abdel-Rehim M. Recent advances in microextraction by packed sorbent for bioanalysis. *J. Chromatogr. A* 1217, 2569–2580 (2010).

22 Nováková L, Havlíková L, Vlčková H. HILIC UHPLC–MS/MS for fast and sensitive bioanalysis: accounting for matrix effects in method development. *Bioanalysis* 5(19), 2345–2357 (2013).

23 Rodriguez-Aller M, Gurny R, Veuthey J-L, Guillarme D. Coupling ultra high-pressure liquid chromatography with mass spectrometry: constraints and possible applications. *J. Chromatogr. A* 1292, 2–18 (2013).

24 Novakova L, Vlčkova H, Šatinský D *et al.* Ultra high performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin. *J. Chromatogr. B* 877, 2093–2103 (2009).

25 Deng JW, Kim KB, Song IS, Shon JH, Zhou HH, Liu KH. Determination of two HMG-CoA reductase inhibitors, pravastatin and pitavastatin, in plasma samples using liquid chromatography-tandem mass spektrometry for pharmaceutical study. *Biomed. Chromatogr.* 22, 131–135 (2008).

26 Pilli NR, Mullangi R, Inamadugu JK, Nallapati IK, Seshagiri Rao JVLN. Simultaneous determination of simvastatin, lovastatin and niacin in human plasma by LC–MS/MS and its application to a human pharmacokinetic study. *Biomed. Chromatogr.* 22, 511–518 (2008).

27 Qi X, Ding L, Wen A, Zhou N, Du X, Shakya S. Simple LC–MS/MS methods for simultaneous determination of pitavastatin and its lactone metabolite in human plasma and urine involving a procedure for inhibiting the conversion of pitavastatin lactone to pitavastatin in plasma and its application to a pharmacokinetic study. *J. Pharm. Biomed. Anal.* 72, 8–15 (2013).

28 Palagani SR, Pilli NR, Gandu V. High performance liquid chromatography mass spectrometric method for the simultaneous quantification of pravastatin and aspirin in human plasma: pharmacokinetic application. *J. Pharm. Anal.* 2(3), 206–213 (2012).

29 Nirogi RVS, Kandikere VN, Shrivastava W, Mudigonda K, Datla PV. Liquid chromatography/negative ion electrospray tandem mass spectrometry method for the quantification of fluvastatin in human plasma: validation and its application to pharmacokinetic studies. *Rapid Commun. Mass Spectrom.* 20, 1225–1230 (2006).

30 Yuan H., Wang F, Tu J, Peng W, Li H. Determination of lovastatin in human by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry and its application in a pharmacokinetic study. *J. Pharma. Biomed. Anal.* 46, 808–813 (2008).

31 VlČková H, Rabatinová M, Mikšová A, Kolouchová G, MiČuda S. Determination of pravastatin and pravastatin lactone in rat plasma and urine using UHPLC–MS/MS and microextraction by packed sorbent. *Talanta* 90, 22–29 (2012).

32 Chimie analytique pharamaceurique: telechargement. www.unige.ch/sciences/pharm/fanal/lcap/telechargement

33 Guillarme D, Nguyen D, Rudaz S, Veuthey JL. Method transfer for fast liquid chromatography in pharmaceutical analysis: application to short columns packed with small particle. Part II: gradient experiments Eur. *J. Pharm. Biopharm.* 68, 430–440 (2008).

34 International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH). Q2 (R1): Text on Validation of Analytical Procedures. US FDA Federal Register (2005). www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm065005.htm

35 Committee for medicinal products for human use. Guideline on validation of bioanalytical methods. EMA, London, UK (2011). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf

36 Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* 75, 3019–3030 (2003).

37 Partani P, Verma SM, Gurule S, Khuroo A, Monif T. Simultaneous quantitation of atorvastatin and its two active metabolites in human plasma by liquid chromatography/(–) electrospray tandem mass spectrometry. *J. Pharm. Anal.* 4, 26–36 (2014).

38 Siddartha B, Babu IS. Estimation and validation for determination of rosuvastatin in human plasma by LC/MS/MS method. *J. Glob. Trends Pharm. Sci.* 5(3), 1979–1988 (2014).

39 Ramesh B, Manjula N, Bijargi SR, Sarma VUM, Devi PS. Comparison of conventional and supported liquid extraction methods for the determination of sitagliptin and simvastatin in rat plasma by LC–ESI–MS/MS. *J. Pharm. Anal.* 5(3), 161–168 (2015).

40 Saha A, Jangala H, Vats P, Thakur R, Khuroo A, Monif T. Stability indicating LC-MS/MS method for estimation of lovastatin in human plasma: application to a bioequivalence study. *J. Anal. Sci Technol.* 6, 19–30 (2015).

41 Polagani SR, Pilli NR, Gandu V. High performance liquid chromatography mass spectrometric method for the simultaneous quantificationof pravastatin and aspirin in human plasma: pharmacokinetic application. *J. Pharm. Anal.* 2(3), 206–213 (2012).

42 Nakashima A, Saxer C, Niina M, Masuda N, Iwasaki K, Furukawa K. Determination of fluvastatin and its five metabolites in human plasma using simple gradient reversed-phase high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. B* 760, 17–25 (2001).

43 Qi X, Ding L, Wen A, Zhou N, Du X, Shakya S. Simple LC–MS/MS methods for simultaneous determination of pitavastatin and its lactone metabolite in human plasma and urine involving a procedure for inhibiting the conversion of pitavastatin lactone to pitavastatin in plasma and its application to a pharmacokinetic study. *J. Pharm. Biomed. Anal.* 72, 8–15 (2013).

44 Lv H, Sun JG, Wang GJ *et al.* Determination of pitavastatin in human plasma via HPLC-ESI-MS/MS and subsequent application to a clinical study in healthy Chinese volunteers. *Clin. Chim. Acta* 386, 25–30 (2007).

45 Siekmeier R, Lattke P, Mix C, Park JW, Jaross W. Dose dependency of fluvastatin pharmacokinetics in serum determined by reversed phase HPLC. *J. Cardiovasc. Pharmacol. Ther.* 6(2), 137–145 (2001).

46 Gazzerro P, Proto MC, Gangemi G *et al.* Pharmacological actions of statins: a critical appraisal in the management of cancer. *Pharmacol. Rev.* 64, 102–146 (2012).

8.4 Development and optimization of ultra-high performance supercritical fluid chromatography mass spectrometry method for high-throughput determination of tocopherols and tocotrienols in human serum

Analytica Chimica Acta 934 (2016) 252–265

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Development and optimization of ultra-high performance supercritical fluid chromatography mass spectrometry method for high-throughput determination of tocopherols and tocotrienols in human serum

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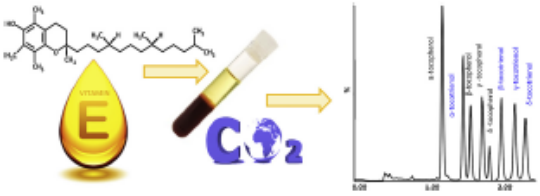
HIGHLIGHTS

- Two fast, selective and sensitive UHPSFC-MS methods were developed and validated.
- Eight isomeric forms of vitamin E were fully separated in less than 4.5 min.
- The influence of individual SFC parameters was evaluated in detail.
- High-throughput LLE was used for sample preparation with sufficient selectivity and sensitivity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form
2 June 2016
Accepted 4 June 2016
Available online 9 June 2016

Keywords:
Ultra-high performance supercritical fluid chromatography
Mass spectrometry
Liquid liquid extraction
Tocopherols
Tocotrienols
Human serum

ABSTRACT

The goal of this study was to develop an effective supercritical fluid chromatography method using single quadrupole MS for analysis of all isomeric forms of vitamin E. Finally, two fast and effective methods, the high resolution one and the high speed one, for the determination of 8 vitamin E isomers in human serum were developed.

Rapid high-throughput liquid-liquid extraction was selected as a sample preparation step. Sample pretreatment of 100 µL human serum was consisted of protein precipitation with 200 µL ethanol and liquid-liquid extraction by 400 µL hexane/dichloromethane (80/20, v/v). The separation was performed on BEH 2-EP (3.0 × 100 mm, 1.7 µm) stationary phase, using isocratic elution with carbon dioxide and 10 mM ammonium formate in methanol in the ratio 98:2 for high resolution method with run time 4.5 min and in the ratio 95:5 for high speed method, where the run time was 2.5 min. The method development included optimization of key parameters: the choice of the suitable stationary phase and the composition of mobile phase, where an influence of various modifiers, their ratio and additives were tested, and optimization of fine tunning parameters including BPR pressure, flow-rate and column temperature. Quantification of all isomeric forms was performed using SIM (single ion monitoring) experiments in ESI positive ion mode. Both high speed and high resolution chromatographic methods were validated in terms of precision, accuracy, range, linearity, LOD, LOQ and matrix effects using the same LLE procedure. The high resolution method provided more sensitive results (LOD: 0.017

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http://dx.doi.org/10.1016/j.aca.2016.06.008

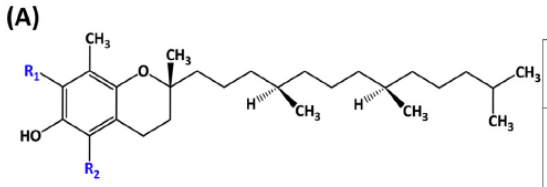
−0.083 μg mL^{-1}) and better linearity (r^2 > 0.9930) than the high speed one (LOD: 0.083−0.25 μg mL^{-1}, r^2 > 0.9877) at the cost of double time of analysis.

1. Introduction

Vitamin E belongs among fat-soluble vitamins associated with important biological activity that is essential for human health. Vitamin E exists in eight different stereoisomeric forms: α-; β-; γ- and δ-tocopherols and α-; β-; γ- and δ-tocotrienols. All tocochromanols are amphiphilic molecules characterized by the same chemical structure based on a 2-methyl-6-chromanol ring and a lipophilic saturated isoprenoid C_{16} side chain (tocopherols) or a farnesyl side chain (tocotrienols). Derivatives of both tocotrienols and tocopherols differ in the methylation of the chromanol ring (Fig. 1) [1–3]. Vitamin E is considered as one of the most powerful antioxidants. The most biologically active homologue is α-tocopherol with phenolic group on chromanol ring donating a hydrogen atom to reduce free radicals activity. Lipophilic side chain allows penetration of vitamin E into biological membranes and protects them from oxidation caused by free radicals [4]. Tocochromanols are hypothesized to induce apoptosis of various tumour cells (skin, lung, prostate, pancreas, breast). They also exhibit cardioprotective, immunomodulatory, neuroprotective activity and blood cholesterol lowering effect [5–8]. Vegetable oils, nuts, whole grains and green leafy vegetables represent the important plant sources of vitamin E. The vitamin E level in animal tissues and products is not so significant in comparison with plant tissues containing tocochromanols [9–12]. After the oral administration vitamin E is absorbed in the gastrointestinal tract and transported by plasma lipoproteins to other tissues. Distribution and concentration of vitamin E in body compartments are influenced by lipids in various tissues. Plasma level of vitamin E is regulated by specific proteins binding tocopherols and tocotrienols in cell membranes. In general, the major amount (up to 90%) of vitamin E is redistributed in adipose tissues in normal subjects [13,14]. A physiological range of vitamin E in human plasma belongs typically to confidence interval 4–12 μg mL^{-1}. Lower levels of tocochromanols are commonly associated with malabsorption syndromes, including biliary atresia, cystic fibrosis, abetalipoproteinemia; and with preterm born neonates [15]. The human deficiency syndrome of vitamin E is manifested as a peripheral neuropathy caused by dying back of sensory axons, which signalizes the sensitivity of the central nervous system to oxidative stress. The vitamin E intake is an essential dietary factor for reproductive health [16,17]. Unlike other fat-soluble vitamins, vitamin E is not accumulated in the liver or extrahepatic tissues with a high intake. Only the increase of bleeding tendency was observed as its toxic effect. In fact, this effect was found to be advantageous as a prevention of venous thrombosis [17,18].

Tocopherols and tocotrienols were mostly analysed by high performance liquid chromatography (HPLC) in various matrices as shown in Table 1.

These separations were performed on both reversed (C18, PFP) and normal (silica) stationary phases using isocratic elution and UV or fluorescent detection. Because of very similar structures and physico-chemical properties, the separation of eight vitamin E forms is very challenging, especially the separation of critical pairs of β- and γ-isomeric forms. Therefore, the most of the previously developed methods for vitamin E analysis enable the determination of only a few isomers of tocopherols and tocotrienols, most commonly α-tocotrienol, α- and γ-tocopherol [19]. As it is shown in Table 1, only few methods (NP-HPLC, RP-HPLC, and also supercritical fluid chromatography - SFC) have been developed for the simultaneous determination of all isomeric forms of tocopherol and tocotrienol [23,26–28,32]. Despite this fact, the determination often took typically tens of minutes (20–60). Even the fastest developed LC method using PFP column took 15 min, so the analysis time was quite long for routine high throughput applications. Modern SFC methods with packed column took less than 10 min, but only one of these methods allowed to determine all isomeric forms of vitamin E [23] without application on any real matrix, such as biological or food samples to show the potential when the real samples are analysed. Sample pretreatment step of complex samples (oils, food, serum, blood or tissues) typically involves liquid-liquid extraction (LLE), LLE based techniques, protein precipitation (PP), solid phase extraction (SPE) followed by evaporation to dryness and reconstitution in solvents compatible with the mobile phase, which is the main disadvantage of the approaches combined with RP-HPLC. Only one method developed in 1999 where supercritical fluid extraction (SFE) coupled with capillary SFC were used



Compound	Molecular formula	Molecular weight [g/ mol]	logP	pKa	R_1 substituent	R_2 substituent
α-tocopherol	$C_{29}H_{50}O_2$	430.71	10.51	10.80	$-CH_3$	$-CH_3$
β-tocopherol	$C_{28}H_{48}O_2$	416.67	9.99	10.47	$-CH_3$	-H
γ-tocopherol	$C_{28}H_{48}O_2$	416.67	9.99	10.47	-H	$-CH_3$
δ-tocopherol	$C_{27}H_{46}O_2$	402.65	9.48	10.14	-H	-H
α-tocotrienol	$C_{29}H_{44}O_2$	424.66	9.23	10.80	$-CH_3$	$-CH_3$
β- tocotrienol	$C_{28}H_{42}O_2$	410.63	8.78	10.47	$-CH_3$	-H
γ- tocotrienol	$C_{28}H_{42}O_2$	410.63	8.78	10.47	-H	$-CH_3$
δ- tocotrienol	$C_{27}H_{40}O_2$	396.60	8.26	10.14	-H	-H

Fig. 1. Chemical structures of tocopherols (A) and tocotrienols (B) and physico-chemical properties of their isomeric forms [45].

Table 1
Methods for determination of tocopherols and tocotrienols in various matrices.

Analyte	Sample	Sample pretreatment	Separation technique	Analytical column	Mobile phase flow rate [mL min^{-1}]	Analysis time [min]	Detection	Sensitivity [LOQ]	Year of publication ref.
α-T, β-T, γ-T, δ-T	Standard solution	SFE	Capillary SFC	Packed capillary with silica particles coated by polyethyleneglycol (250 × 5 mm)	CO_2 NA	30	FID	NA	1999 [20]
α-T, β-T, γ-T, δ-T, D_3, triacylglycerols	Pulp oil, cloud-berry oil	–	Capillary SFC	SB-Octyl-50 (10 m × 0.5 mm) SB-Cyanopropyl-25 (10 m × 0.5 mm)	CO_2 0.37	55	FID, MS	NA	1995 [21]
α-T, γ-T, δ-T, sterols	Soybean oil	Extraction by heating	Capillary SFC	SP-Octyl-50 (10 m × 0.5 mm)	CO_2 NA	35	FID	NA	1997 [22]
α-T acetate, α-T, β-T, γ-T, δ-T, α-T_3, γ-T_3, δ-T_3	Soybean oil	dilution	SFC	Amine Luna NH_2 (150 × 2.0 mm, 3 μm)	CO_2:EtOH + 0.1% FA 1.5	5	MSMS	50 pmol	2016 [23]
α-T, β-T, γ-T, δ-T, α-T_3, β-T_3, γ-T_3, δ-T_3	Standard solution	–	SFC	Acquity UPC^2 BEH (100 × 3.0 mm, 1.7 μm)	CO_2:MeOH (99.5:0.5) 2.5	5.5	MS-APCI	10.6 mg L^{-1}	2015 [24]
Retinol, retinyl acetate, α-T acetate, α-T, γ-T, δ-T	Olive, sunflower, soybean oil	LLE evaporation	RP-HPLC	Lichrosorb RP-18 (150 × 4.0 mm, 10 μm)	Hexadecyltrimethylammonium bromide:n-propanol NA	15	UV (290–330 nm)	0.12–0.28 mg L^{-1}	2011 [25]
α-T, β-T, γ-T, δ-T, α-T_3, β-T_3, γ-T_3, δ-T_3	Vegetable oils, seeds, eggs, flours	Saponification LLE evaporation	NP-HPLC	LiChrosorb Si60 (250 × 4.0 mm, 5 μm)	Hexane:isopropanol:acetic acid (98.9:0.6:0.5) 1	25	FLD	105–335 ngm L^{-1}	2011 [26]
α-T, β-T, γ-T, δ-T, α-T_3, β-T_3, γ-T_3, δ-T_3	Human plasma, liver tissue	Saponification LLE evaporation	RP-HPLC	Phenomenex Kinetex PFP (150 × 4.6 mm, 2.6 μm)	MeOH:H_2O (85:15) 0.8	15	FLD	92–519 pg	2012 [27]
α-T, β-T, γ-T, δ-T, α-T_3, β-T_3, γ-T_3, δ-T_3	Vegetable sample	Saponification LLE evaporation	RP-HPLC	Kinetex PFP (150 × 3 mm, 2.6 μm); Develosil RP Aqueous 30 (150 × 3 mm, 3 μm)	MeOH:H_2O:Methylbutylether 0.3; 0.5	45; 63	FLD	NA	2015 [28]
α-T, β-T, γ-T, δ-T, α-T_3, γ-T_3, δ-T_3	Fruit, vegetable	PLE DLLME evaporation	RP-HPLC	Ascentis Express F5 (150 × 46 mm, 5 μm)	MeOH:H_2O (85:15) 1	17	FLC MS-APCI [M − H]$^-$	1-5 ng mL^{-1}	2014 [29]
α-T, β-T, γ-T, δ-T, α-T_3, γ-T_3, δ-T_3	Cereals	Saponification DLLME	RP-HPLC	Cosmosil π-NAP (250 × 46 mm, 5 μm)	H_2O:MeOH:ACN (13:80:7) 1	30	FLD	0.05–0.30 μg mL^{-1}	2013 [30]
α-T, γ-T, δ-T, retinol acetate	Infant milk-based formulae	LLE	NP-HPLC	Pinnade II silica (50 × 21 mm, 3 μm)	0.5% ethyl acetate:hexane 0.4	25	DAD	1.8–73.7 μg mL^{-1}	2006 [31]
α-T, β-T, γ-T, δ-T, α-T_3, β-T_3, γ-T_3, δ-T_3, γ-oryzanol	Rice	LLE	NP-HPLC	Inertsil CN-3, SIL-100A (250 × 4.6 mm, 5 μm)	Hexane:isopropanol: ethylacetate: acetic acid (97.6:0.8:0.8:0.8) 0.7–1.5	25	FLD	0.05–0.5 μg mL^{-1}	2011 [32]

α-T, γ-T, retinol	Human plasma	LLE evaporation	RP-HPLC	Waters Symmetry C18 (75 × 4.6 mm, 3.5 μm)	MeOH 1.8	4	UV, FLD	0.03–0.32 mg L^{-1}	2014 [33]
α-T, α-T-d_6	Human plasma	PP LLE evaporation	NP-HPLC	EC Nukleosil (250 × 4.6 mm, 5 μm)	Hexane:isopropanol 1	45	UV	83 pmol mL^{-1}	2003 [34]
α-T, α-T acetate, δ-T, retinol, cholecalciferol, phylloquinone, menaquinone, menadione, xanthophyll	Human blood serum	SPE evaporation	RP-HPLC	Phenomenex Luna C18 (150 × 4.6 mm, 3 μm)	ACN:MeOH 1.3	20	UV	0.1–1 μg mL^{-1}	2004 [35]

for the vitamin E analysis did not need the reconstitution of the sample in different solvent [20].

SFC has been known for more than 50 years. However, it was over shadowed by gas chromatography (GC) and rapidly evolving LC. Recently, SFC made a remarkable comeback. The supercritical fluids exhibit similar properties as the mobile phase used in LC (density and solvating power) and GC (high diffusivity and low viscosity) [36–38]. In modern SFC, mobile phase contains not only CO_2, but also small amount (2–40%) of organic polar modifier (methanol, ethanol, isopropanol, or rarely acetonitrile (ACN)) and additives (volatile organic acids and buffers, possibly water) which affect the polarity, solvating power and eluting power of nonpolar CO_2. Through this modification, SFC can be successfully used for the analysis of molecules with broad spectrum of polarity [39]. Technological advances allow use of the latest trends in the field of stationary-phase technologies such as fully porous sub-2-μm and sub-3-μm core-shell particles and high linear velocity in modern analytical SFC systems [40,41]. This approach, similarly to ultra-high performance liquid chromatography (UHPLC), is known as ultra-high performance supercritical fluid chromatography (UHPSFC). Because of higher sensitivity and selectivity of mass spectrometry (MS), the hyphenation of UHPSFC with MS becomes more popular method. Nowadays, even the cheap single quadrupole instruments become more user-friendly and sensitive which allows to use them as powerful detectors in routine analysis in clinical laboratories. UHPSFC coupled to MS promises not only higher sensitivity in comparison with UV detection but also alternative selectivity to LC-UV and reliability of analysis in complex biological matrices.

The hyphenation of SFC-MS can be made via the pre-BPR flow-splitting with addition of make-up solvent to improve the forming of ions, peak shape and detector response. Proton-donating organic modifiers such as methanol, ethanol, isopropanol and volatile additives (formic acid, acetic acid, ammonium formate, ammonium acetate, water, ammonium hydroxide, etc.) were used as a make-up solvent [42–44].

Due to the low polarity of pure CO_2, similar to hexane or heptane used in NP-HPLC, SFC is suitable for the analysis of non-polar compounds with physico-chemical properties close to pure CO_2. Thus, the high log P of vitamin E is a prerequisite for the use of SFC technique for the analysis of vitamin E isomeric forms. Considering other numerous advantages, such as high separation efficiency, sensitivity, low consumption of organic solvents and good selectivity for separation of the isomers SFC seems to be an appropriate technique for the separation and determination of tocopherols and tocotrienols and application of the method in clinical practice.

The aim of this study was to develop fast, selective and sensitive UHPSFC-MS method for high-throughput analysis of all 8 vitamin E isomeric forms and effective sample preparation step based on LLE for the isolation of vitamin E from human serum.

2. Materials and methods

2.1. Chemicals and reagents

Pressurized liquid CO_2 4.5 grade (99.995%) was purchased from Messer (Prague, Czech Republic). Methanol (MeOH), isopropanol (IpOH), ethanol (EtOH), formic acid, acetic acid and ammonium hydroxide (25%) all of them LC/MS grade and hexane and dichloromethane for HPLC were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium formate (99%) and ammonium acetate (99.8%) for LC/MS were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was prepared by Milli-Q reverse osmosis system (Millipore, Bedford, MA, USA) immediately prior to use.

Reference standards of α-tocopherol (αT; 100 mg mL^{-1}, purity 99.9%), β-tocopherol (βT; 50 mg mL^{-1}, purity 99.0%), γ-tocopherol (γT; 1 mg mL^{-1}, purity 97.3%) and δ-tocopherol (δT; 100 mg mL^{-1}, purity 95.5%) in hexane were provided by Sigma-Aldrich (Steinheim, Germany).

Reference standards of α-tocotrienol (αT_3; 25 mg mL^{-1}, purity 98%), β-tocotrienol (βT_3; 25 mg mL^{-1}, purity 98%), γ-tocotrienol (γT_3; 25 mg mL^{-1}, purity 98%), and δ-tocotrienol (δT_3, 25 mg mL^{-1}, purity 98%) in EtOH were provided by Larodan Fine Chemicals (Malmö, Sweden).

A lyophilized control serum based on human matrix LYO HUM N was provided by Erba Lachema (Brno, Czech Republic).

2.2. Standard solutions

The stock standard solutions of tocochromanols were prepared by dilution of each compound in hexane to give a solution at a concentration of 1 mg mL^{-1}. These solutions were stored at −20 °C and they were prepared fresh every two weeks. The diluent mixture consisting of hexane and IpOH (90:10, v:v) was used for the subsequent dilutions.

2.3. UHPSFC-MS instrumentation and analysis

All the experiments were performed on a supercritical fluid chromatography system Acquity UPC^2 (Waters, Milford, MA, USA), which consisted of a binary pump, an autosampler, a column thermostat, a back pressure regulator (BPR) and PDA detector. The system was coupled to a single quadrupole mass spectrometer with an electrospray ionization (ESI) Acquity QDa Detector (Waters, Milford, MA, USA) via commercial SFC-MS dedicated splitter device (Waters). Additional make-up solvent was brought by an Isocratic Solvent Manager (Waters, Milford, MA, USA). Mixture of 95% MeOH and 5% water was selected as a make-up solvent at flow-rate 0.3 mL min^{-1}.

Four different stationary phases were tested for the method development, namely Waters Acquity UPC^2 BEH, Acquity UPC^2 BEH 2-Ethylpyridine (2-EP), Acquity UPC^2 HSS C18 SB and Acquity UPC^2 CSH Fluoro-Phenyl (PFP). All columns had dimensions of 3.0 × 100 mm and particle sizes of 1.7 μm except for Acquity UPC^2 HSS C18 SB (1.8 μm). The isocratic elution was performed with CO_2 and MeOH or EtOH as modifiers. Various additives in organic modifier were tested, including ammonium acetate (1, 5, 10 and 20 mM), ammonium formate (1, 5, 10 and 20 mM), and water (1, 2, 5%).

Finally, the Acquity UPC^2 BEH 2-EP was selected as the optimum column using CO_2 and MeOH with 10 mM ammonium formate.

Isocratic elution for high resolution method was provided by CO_2 and an organic modifier in ratio 98:2 at a flow-rate 1.5 mL min^{-1}. A column temperature and BPR were set at 40 °C and 23.4 MPa, respectively. High speed method was obtained using isocratic elution with CO_2 and 5% of modifier at a flow-rate 1.5 mL min^{-1}. A column temperature and BPR were set at 50 °C and 13.0 MPa, respectively. The partial loop with needle overfill mode was used to inject of 1 μL of sample. Methanol was selected as a strong and weak wash solvent. The analytes were detected at 290 nm.

The MS conditions were tuned in ESI^+ mode as follows: capillary voltage 0.80 kV, ion source temperature 120 °C and probe temperature 600 °C. Sample cone voltage was optimized for each analyte individually (αT, αT_3 = 10 V; βT, γT, δT, βT_3, γT_3, δT_3 = 5 V). Analyses were performed in SIM (single ion monitoring) experiment using precursor ions $[M+H]^+$: αT = 431.0; βT, γT = 417.0; δT = 403.0; αT_3 = 425.3; βT_3, γT_3 = 411.3; δT_3 = 397.3. MassLynx v4.1 software was employed for MS control and data acquisition and QuanLynx for peak integration and data processing.

2.4. Sample preparation

The combination of PP and LLE was used for sample preparation. 200 μL of EtOH as a precipitating agent was added to 100 μL of human serum for release of analytes from protein binding. The mixture was vortexed for 10 s. 400 μL of-hexane: dichloromethane solution (80:20; v:v) was subsequently added. The sample was centrifuged at 12 000 rpm, 4 °C for 10 min. 300 μL of resulting supernatant were transferred into an insert of a vial. The sample was spinned again and injected onto the BEH 2-EP column.

2.5. Method validation

At first, the repeatability of peak areas and retention times were evaluated at three concentration levels for both high resolution and high speed methods to perform system suitability test (SST) (n = 10). Standard solutions and spiked human serum samples were used for validation of the UHPSFC-MS methods. Both methods were fully validated in terms of linearity, range, limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), precision, accuracy and matrix effects. For the determination of linearity, two calibration curves of all analytes were prepared. Standard calibration curve was prepared from stock standard solutions in the concentration range 0.001–10 μg mL^{-1} for high resolution method and 0.01–10 μg mL^{-1} for high speed method using diluting mixture. Matrix calibration curve was constructed using human reference serum spiked with all analytes and treated with PP and LLE procedure in the concentration range 0.01–10 μg mL^{-1} for high resolution method and 0.05–10 μg mL^{-1} for high speed method. LOQ was determined as the lowest concentration level with S/N ~ 10 and LOD as S/N ~ 3.

For the method precision and accuracy, spiked human serum samples treated by LLE were measured in five replicates at four different concentration levels covering the calibration range: the LLOQ, within five times the LLOQ (low QC), around 30–50% of the calibration curve (medium QC) and 75% of the upper calibration curve range (high QC) according to EMA Guideline on bioanalytical method validation [46]. Matrix effects were evaluated as the comparison of standard solutions and human serum samples, which were first treated with PP and LLE and subsequently spiked with standard solutions at two concentration levels for all isomers of vitamin E [47]. Matrix effects were determined at a low (close to LLOQ) and high (close to ULOQ) concentration level covering the calibration range according to EMA Guideline on bioanalytical method validation [46].

3. Results and discussion

3.1. UHPSFC-PDA method development

All 8 isomers of vitamin E are structurally similar compounds which differ in the number of methyl groups and their position on the chromanol ring. Due to the equal number of methyl substituents in a different position and therefore the same m/z ratio of β- and γ-tocopherols and tocotrienols, the separation of these isomeric forms is the critical point of the method development. This problem was often observed during LC method development. Therefore, it was expected also in SFC. To optimize the SFC separation, PDA detector was set at a selected wavelength of 290 nm. Due to the high log P value of vitamin E, the isocratic elution with 97% CO_2 and 3% MeOH at flow-rate 2.5 mL min^{-1} and column temperature 50 °C was chosen as primary conditions for the column screening. Four different stationary phases were tested (BEH,

BEH 2-EP, CSH PFP and HSS C18). The results are shown in Fig. 2. CSH PFP stationary phase was not suitable for the separation under selected conditions due to coelution of two critical pairs β-; γ-T and β-; γ-T_3 and very low retention (analysis time < 0.8 min) which confirmed our expectations. Two different coelutions (γ-T and β-T_3; γ-T_3 and δ-T) were observed also on HSS C18 stationary phase. BEH stationary phase provided better selectivity, but a coelution of two analytes (δ-T and γ-T_3) and also very low retention of all analytes (analysis time < 1.0 min) were observed which is not desirable for analysis of complex biological matrices. Finally, BEH 2-EP provided the best selectivity and resolution in 2 min analysis time. Only the coelution of δ-T and βT_3 which was less expected was observed instead of predicted coelution of β- and γ-isomers. This column was selected for further optimization. The sensitivity and repeatability of this method were verified at this point. However, determined LOQ (40 μg mL^{-1}) for vitamin E analysis using PDA detector was insufficient for analysis of vitamin E in biological samples. Therefore, the transfer from UHPSFC-PDA to UHPSFC-MS was unavoidable and further optimization steps were performed on UHPSFC-MS system.

3.2. UHPSFC-MS method development

3.2.1. Selection of mobile phase modifier, additive and make-up solvent

Mobile phase composition in SFC-MS is one of the key factor affecting the peak shape, retention time, selectivity and mass spectrometry response similarly to LC-MS. Because of tocotrienol high costs, this large set of experiments was performed only with tocopherol standards. Due to similar physicochemical properties, the behaviour of all 8 compounds in terms of MS response was expected to be analogous. Both, tocopherols and tocotrienols, are non-polar molecules with log P in the range 8–10, pKa typical for neutral compounds (Fig. 1), and few functional groups possessing hydrogen bond donor or acceptor properties (1 H-donor group/2-H acceptor groups). For this reason, the change in retention and selectivity, respectively, was expected in a lesser extent when various additives were used. Despite this fact, several additives, including ammonium acetate (1, 5, 10 and 20 mM), ammonium formate (1, 5, 10 and 20 mM), and water (1, 2, 5%), were tested to verify the influence of individual additives on MS ionization and method sensitivity. To show the results, γ-T was selected as a representative for all tocopherols, as the results for other isomers were identical.

First, MeOH, which is considered to be a modifier of the first choice not only due to its strong elution power, but also due to its cost and availability, was investigated as a co-solvent. The MS response corresponding to pure MeOH was taken as a reference (100%) for the scalling of all other mobile phases (Fig. 3A and B). Subsequently, MeOH and EtOH with different additives were investigated (Fig. 3A and B). When using pure EtOH as an organic modifier, the increase in analysis time from 3 min to 5 min (Fig. 3C, E) was observed. The MS response was similar compared to pure MeOH as shown in Fig. 3B. Therefore, there was no benefit in using EtOH as a co-solvent. When using additives in CO_2/MeOH mobile phase no influence on retention time was observed (Fig. 3D, F) even after significantly long equilibration times (more than 3 h). On the other hand, the influence to signal intensity (evaluated as both peak area and S/N) was significant. As shown in Fig. 3A, an addition of ammonium formate and ammonium acetate led to increase in the MS response in comparison with pure MeOH. When increasing concentration of an additive from 1 mM to 10 mM, the response

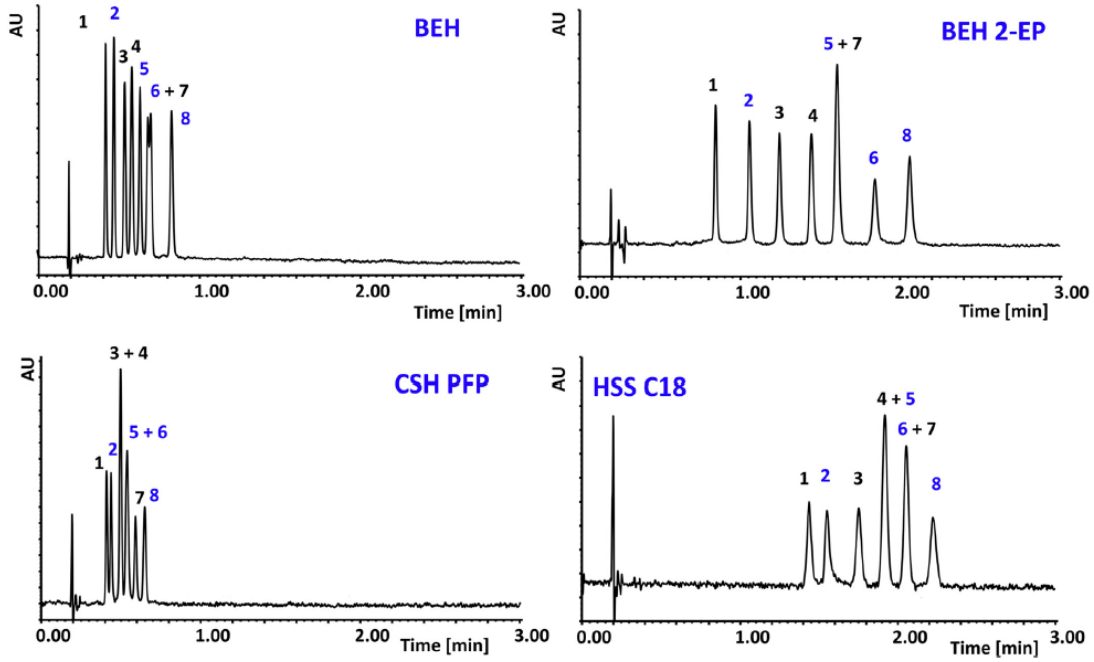


Fig. 2. Column screening to investigate optimal stationary phase for vitamin E analysis. UHPSFC conditions: CO_2: modifier (97: 3), column temperature 50 °C, BPR 13.0 MPa, flow-rate 2.5 mL min^{-1}. (1) α-tocopherol, (2) α-tocotrienol, (3) β-tocopherol, (4) γ-tocopherol, (5) β-tocotrienol, (6) γ-tocotrienol, (7) δ-tocopherol, (8) δ-tocotrienol.

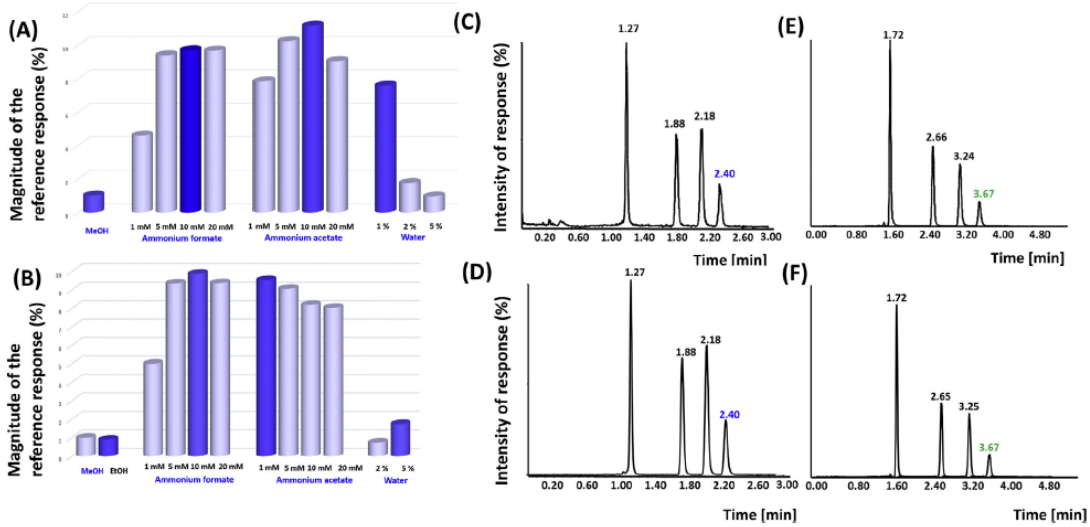


Fig. 3. Optimization of mobile phase composition: Influence of all investigated additives on MS response using (A) MeOH with additives and (B) EtOH with various additives reported for γ-T. TIC chromatograms of tocopherols with (C) MeOH and (D) MeOH + 10 mM ammonium formate, (E) EtOH used as organic modifier and (F) EtOH + 10 mM ammonium formate. UHPSFC conditions: CO_2: modifier (97: 3), column temperature 40 °C, BPR 13.0 MPa, flow-rate 2.0 mL min^{-1}, make-up solvent: MeOH, flow-rate 0.3 mL min^{-1}.

was further increased 4 fold and 10–12 fold, respectively. If 20 mM ammonium acetate or formate was used, the signal of tocopherols was slightly decreased in comparison with 10 mM buffers. Finally, the influence of water addition to MeOH modifier was studied. A significant positive effect (8–10 fold increase of response compared to pure MeOH) on the signal of the analytes was observed when 1% of water was added into MeOH. When the concentration of water increased, the decrease of MS response was observed as is shown in Fig. 3A. When the additives with the highest response (10 mM ammonium formate, 10 mM ammonium acetate and 1% water added to both buffers) were compared, no great difference in MS responses between chosen additives was found. The same set of experiments was repeated using EtOH and the same additives (Fig. 3B). However, no significant difference was observed compared to MeOH based mobile phases.

Finally, due to the shorter analysis time, the highest MS response and still good selectivity, MeOH with 10 mM ammonium formate (Fig. 3D) was chosen as an organic modifier and additive of the CO_2-based mobile phase.

The hyphenation of UHPSFC and MS was implemented via the pre-BPR flow-splitting with addition of make-up solvent. Various organic solvents, including MeOH, EtOH and IpOH, addition of water (2 and 5%) and volatile additives (10 mM ammonium formate, 0.1% formic acid, 0.1% ammonium hydroxide) on MS sensitivity were studied. Pure MeOH was taken as a reference (100%) to compare intensity of response for all tested make-up solvents.

When MeOH was replaced with EtOH or IpOH, the MS signal was reduced for all analytes, typically to 30% of the reference intensity for EtOH, and 10% for IpOH respectively. Consequently, MeOH with various additives was subjected to further testing. Surprisingly, the addition of volatile ammonium formate, formic acid and ammonium hydroxide lead to the decrease of MS response compared to pure MeOH. Only addition of water provided some enhancement of MS signal. The addition of 5% water increased the signal to 180% of reference signal. Therefore, it was chosen as the most suitable make-up solvent (Fig. 4).

3.2.2. The influence of mobile phase composition, column temperature, flow-rate and BPR pressure

In SFC, the analyte retention is not only influenced by the choice of stationary phase and mobile phase composition, but also by column temperature and pressure on which mobile phase density depends. Critical point of CO_2 is changing when it is mixed with polar modifier. Therefore, when using organic modifiers, the mobile phase is not properly in supercritical state but rather in a state which can be called subcritical [36]. Comparing these states, no significant difference in the physical and chemical properties has been described. The organic modifier influences the solvating power of the mobile phase. The temperature and pressure change can also influence the solubility of non-polar analytes and thereby the selectivity and retention time of analytes [36,41,48,49].

At first, a concentration of the organic modifier (95–98% of CO_2) was finely tuned as the most influencing factor in subcritical state. The dependence of the retention and co-solvent concentration is non-linear, with decrease in retention from 0 to 10% of modifier increase, whatever the polarity of the stationary phase. For lipophilic compounds, it was observed that the retention time decreased with modifier amount increase and after the retention reached a minimum value, the retention factor increased again [48,49].

Indeed, in this study, increasing amount of the modifier led to the analysis time decrease, as only low concentrations of the modifier were employed. Due to the various polarities of tocopherols and tocotrienols, the change in selectivity was also observed. Different selectivity was noticed at very low amount of carbon dioxide in mobile phase, namely this effect was observed for critical pair β-T_3 and δ-T (Fig. 5). The baseline separation of all 8 isomeric forms was obtained only when 98% or 96% of CO_2 was used in the mobile phase and temperature was kept at 40 °C. In other cases the peaks were not baseline-separated and δ-T co-eluted with neighboring peaks. Subsequently, column temperature was investigated

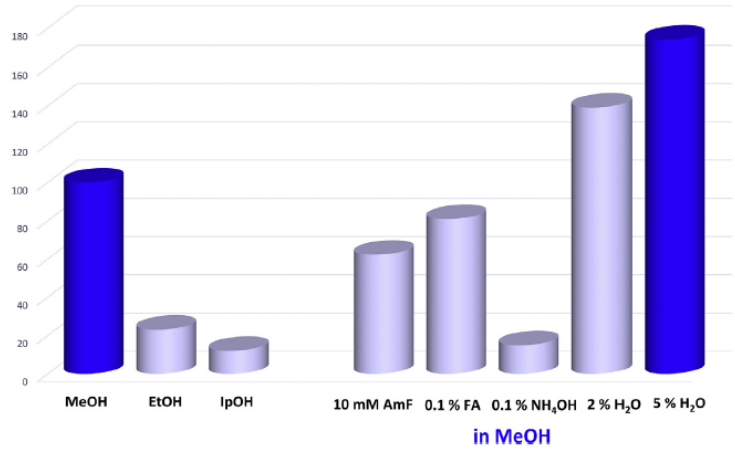


Fig. 4. Optimization of make-up solvent: influence of organic solvents and additives on MS intensity.

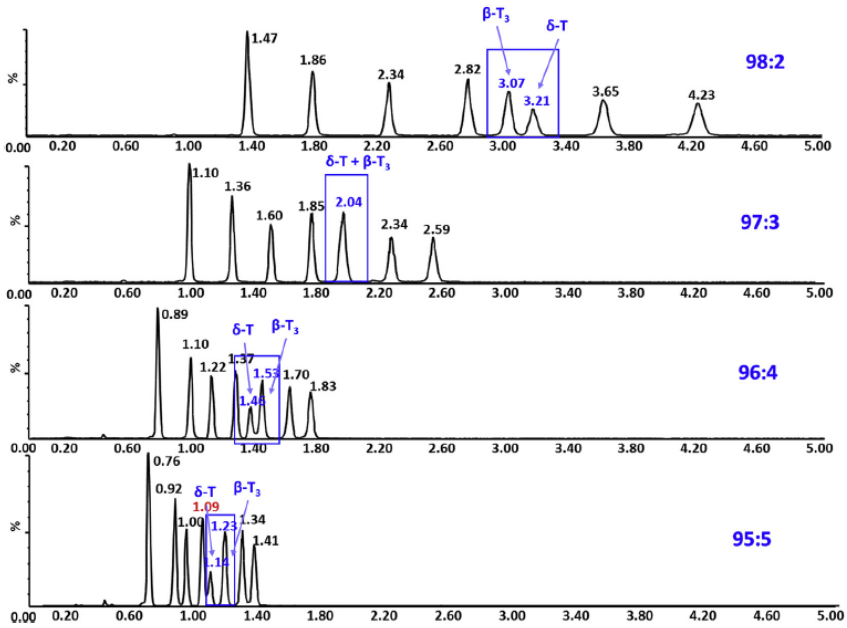


Fig. 5. SIM chromatograms reporting an influence of mobile phase composition on speed of analysis and selectivity. UHPSFC conditions: CO_2: MeOH +10 mM ammonium formate, column temperature 40 °C, BPR 13.0 MPa, flow-rate 2.0 mL min^{-1}, make-up solvent: MeOH +5% H_2O, flow-rate 0.3 mL min^{-1}.

in detail. The analysis time was decreased with decreasing temperature (Fig. 6). The critical pair β-T_3 and δ-T was separated on baseline at 98% of CO_2 in all cases except when the temperature was decreased to 30 °C when partial co-elution was observed. Similarly, at 95% of CO_2, this coelution was observed already at 40 °C. Generally, combination of lower temperature and higher concentration of organic modifier were not favourable for the separation of critical pair β-T_3 and δ-T.

After this optimization, two promising conditions, 98% of CO_2 at 40 °C and 95% of CO_2 at 50 °C, allowing separation of all eight isomers were subjected to further method optimization, where the influence of BPR and flow-rate were further tested. The flow-rate for both methods was investigated in the range 1.5–2.0 mL min^{-1}. When the flow-rate value was decreased from 2.0 mL min^{-1} to 1.5 mL min^{-1}, the sensitivity was increased typically by 60% and run time of separation was prolonged by 50%.

Due to different amount of CO_2 and column temperature setting, the density of mobile phase allowed to test different BPR ranges for

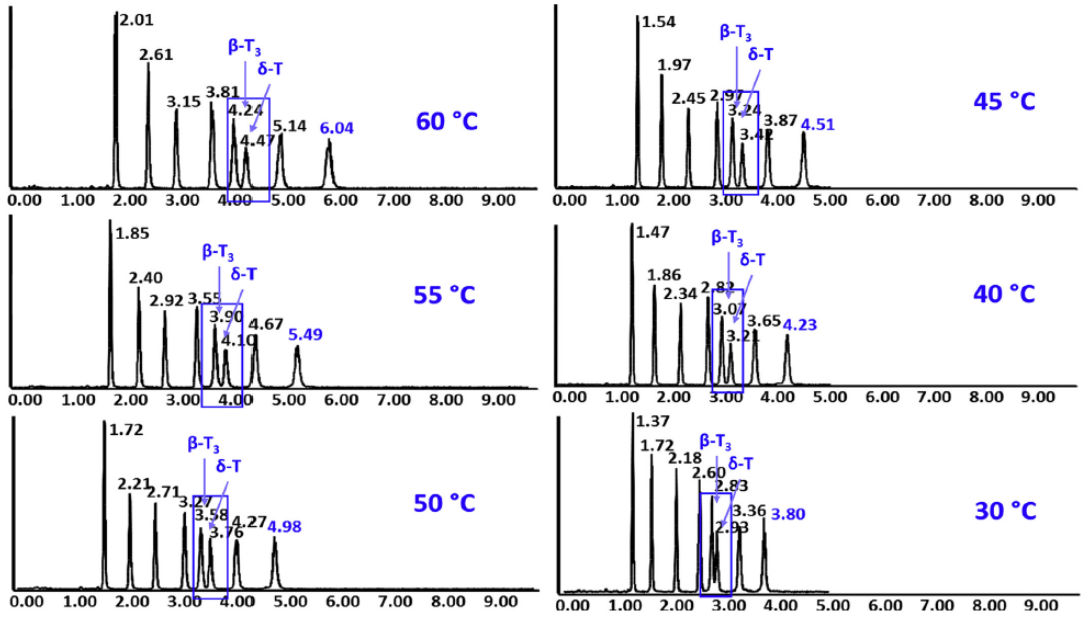


Fig. 6. Effect of temperature on separation of 8 tocochromanols. Critical pair of co-eluting β-T_3 and δ-T is highlighted. UHPSFC conditions: CO_2: MeOH +10 mM ammonium formate (98:2), 13.0 MPa, flow-rate 2.0 mL min^{-1}, make-up solvent: MeOH + 5% H_2O, flow-rate 0.3 mL min^{-1}.

the two promising conditions. Tested BPR range was 13.0–23.4 MPa and 11.4–13.0 MPa for 98% and 95% CO_2, respectively. Generally, when the BPR pressure was increased, the sensitivity of MS response was higher and the analysis time was shorter by 40% and 20%, respectively, as shown in Fig. 7. No influence on separation selectivity was observed probably due to the very little change of analyte solubility in the mobile phase under these conditions [49].

Finally, two high-throughput UHPSFC-MS methods were

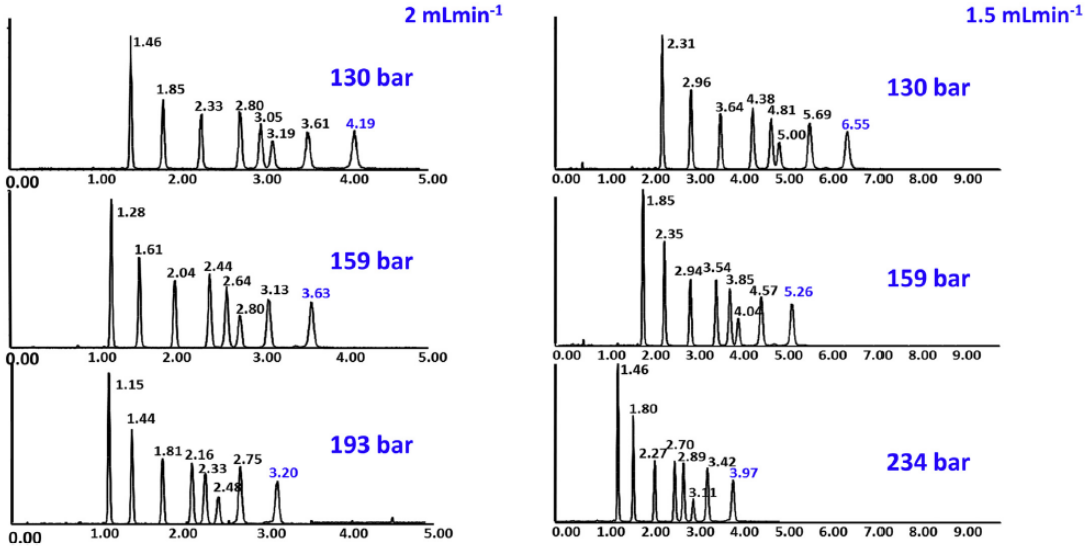


Fig. 7. Effect of flow-rate and BPR pressure on separation of 8 tocochromanols. UHPSFC conditions: CO_2: MeOH +10 mM ammonium formate (98:2), column temperature 40 °C, flow-rate 2.0 mL min^{-1} and 1.5 mL min^{-1} respectively, make-up solvent: MeOH + 5% H_2O, flow-rate 0.3 mL min^{-1}.

obtained – the high resolution method and the high speed method, both with analysis time shorter than 5 min. The high resolution method provided more sensitive analysis and higher resolution which is more suitable for samples originated from complex matrices, while the high speed one is suitable for rapid laboratory screening with still sufficient sensitivity for less complex matrices or for samples after thorough sample preparation. The high resolution method utilized mobile phase composed from 98% CO_2 and 2% of MeOH with 10 mM ammonium formate at flow-rate 1.5 mL min^{-1}, column temperature 40 °C, and BPR pressure 23.4 MPa as the best compromise between sensitivity and separation efficiency. The high speed method was performed at CO_2: MeOH with 10 mM ammonium formate (95:5), column temperature 50 °C, flow-rate 1.5 mL min^{-1} and BPR pressure 13.0 MPa for the maintaining of separation efficiency and short time of analysis (Fig. 8).

3.3. Optimization of sample preparation step

Due to the solubility of vitamin E in non-polar solvents, LLE was chosen for the sample pretreatment as the most suitable approach for the isolation of vitamin E from human serum. As discussed, tocochromanols are strongly binded on plasma proteins which are responsible for their transportation and regulation of their level in plasma [13,14]. Therefore, it was important to release them from protein binding before the LLE using effective protein precipitating (PP) agent.

Different volumes of human serum (100 and 200 μL) as a complex matrix, four organic solvents (ACN, MeOH, EtOH and IpOH) used as a precipitating agent in volumes of 100 and 200 μL, various solvents for LLE (hexane, hexane: dichloromethane (80:20, v:v), hexane: dichloromethane (90:10, v:v) in volumes of 200 and 400 μL and extraction times (5 and 10 min) were investigated in detail.

ACN and MeOH as the most efficient precipitating agents provided very low recoveries (10–35%). When EtOH or IpOH were used as precipitating agents, the recoveries were substantially improved. 100 μL of precipitating agent was not sufficient to break the protein binding of vitamin E. When sample: precipitating ratio was 1:2 instead of 1:1, the recoveries rapidly increased. When using the hexane and mixture hexane: dichloromethane (90:10, v: v) the extraction recoveries were lower (45–65%). Only using 400 μL

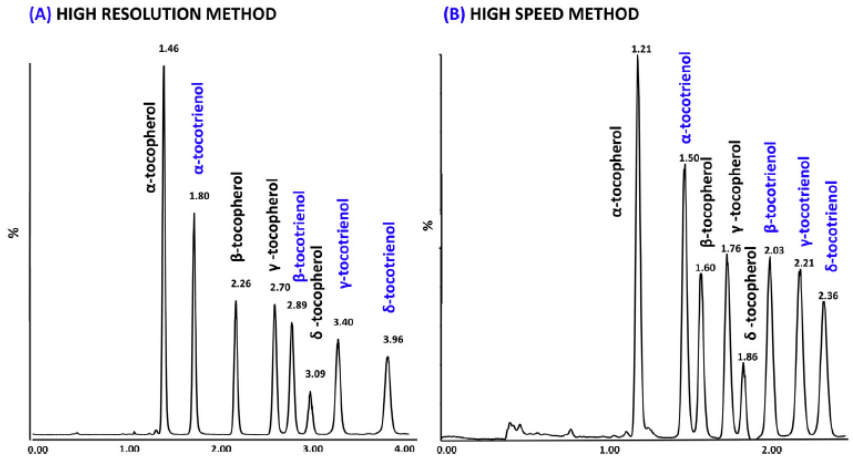


Fig. 8. Final TIC chromatograms for (A) high resolution and (B) high speed UHPSFC-MS method.

Table 2
Peak area and retention time repeatability shown as relative standard deviation (RSD %) for high resolution and high speed method.

			α-T	β-T	γ-T	δ-T	α-T_3	β-T_3	γ-T_3	δ-T_3
High resolution method	RSD (A) [%]	0.01 [μg mL^{-1}]	10.66	14.95	12.56	–	–	–	–	–
		0.05 [μg mL^{-1}]	2.41	3.77	3.04	8.58	6.12	4.51	5.43	7.78
		0.5 [μg mL^{-1}]	1.66	3.29	4.58	5.37	1.75	1.12	1.04	2.62
		5.0 [μg mL^{-1}]	1.20	2.27	2.82	6.13	2.16	1.14	1.00	2.17
	RSD (t_R) [%]	0.01 [μg mL^{-1}]	0.00	0.00	0.00	–	–	–	–	–
		0.05 [μg mL^{-1}]	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
		0.5 [μg mL^{-1}]	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
		5.0 [μg mL^{-1}]	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
High speed method	RSD (A) [%]	0.05 [μg mL^{-1}]	3.22	–	–	–	–	–	5.67	10.37
		0.1 [μg mL^{-1}]	2.09	9.09	–	–	4.79	4.90	3.49	6.23
		0.5 [μg mL^{-1}]	2.90	4.77	4.23	3.93	2.88	2.49	3.22	3.01
		1.0 [μg mL^{-1}]	1.60	1.35	1.59	3.31	3.15	1.48	2.16	1.91
		5.0 [μg mL^{-1}]	1.31	2.08	2.07	2.01	1.48	1.98	1.59	1.95
	RSD (t_R) [%]	0.05 [μg mL^{-1}]	0.24	–	–	–	–	–	0.23	0.19
		0.1 [μg mL^{-1}]	0.00	0.00	–	–	0.00	0.26	0.18	0.12
		0.5 [μg mL^{-1}]	0.24	0.18	0.16	0.21	0.00	0.14	0.13	0.12
		1.0 [μg mL^{-1}]	0.00	0.00	0.16	0.16	0.00	0.00	0.00	0.00
		5.0 [μg mL^{-1}]	0.24	0.18	0.00	0.24	0.00	0.00	0.13	0.12

Table 3
Method linearity, LOD, LLOQ and ULOQ for tocopherols and tocotrienols in standard solution and human serum for high resolution and high speed method.

			α-T	β-T	γ-T	δ-T	α-T_3	β-T_3	γ-T_3	δ-T_3
High resolution method	Standard calibration curve	Method linearity [r^2]	0.9999	0.9995	0.9996	0.9996	0.9987	0.9990	0.9996	0.9997
		LOD [μg mL^{-1}]	0.003	0.003	0.003	0.017	0.017	0.017	0.017	0.017
		LLOQ [μg mL^{-1}]	0.01	0.01	0.01	0.05	0.05	0.05	0.05	0.05
		ULOQ [μg mL^{-1}]	5.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
	Matrix calibration curve	Method linearity [r^2]	0.9930	0.9952	0.9954	0.9931	0.9940	0.9955	0.9927	0.9942
		LOD [μg mL^{-1}]	0.083	0.017	0.017	0.083	0.025	0.025	0.083	0.083
		LLOQ [μg mL^{-1}]	0.25	0.05	0.05	0.25	0.075	0.075	0.25	0.25
		ULOQ [μg mL^{-1}]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
High speed method	Standard calibration curve	Method linearity [r^2]	0.9992	0.9995	0.9998	0.9996	0.9996	0.9996	0.9969	0.9996
		LOD [μg mL^{-1}]	0.017	0.033	0.17	0.17	0.033	0.033	0.017	0.017
		LLOQ [μg mL^{-1}]	0.05	0.10	0.50	0.50	0.10	0.10	0.05	0.05
		ULOQ [μg mL^{-1}]	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
	Matrix calibration curve	Method linearity [r^2]	0.9877	0.9942	0.9912	0.9914	0.9915	0.9941	0.9935	0.9922
		LOD [μg mL^{-1}]	0.167	0.167	0.25	0.167	0.083	0.083	0.25	0.083
		LLOQ [μg mL^{-1}]	0.5	0.5	0.75	0.5	0.25	0.25	0.75	0.25
		ULOQ [μg mL^{-1}]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

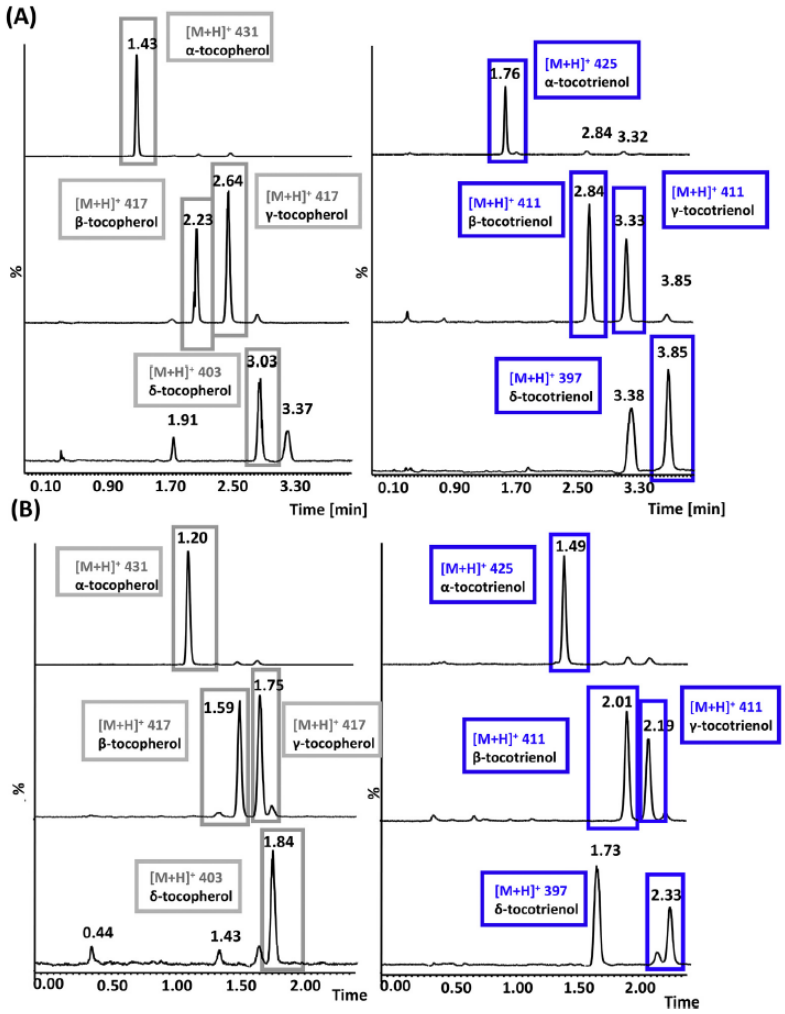


Fig. 9. SIM chromatograms for human serum spiked with all 8 vitamin E isomeric forms for (A) high resolution and (B) high speed method.

hexane: dichloromethane (80:20, v:v) mixture provided results with the recoveries in the range 80–120%.

Finally the method with 10 min extraction time using 100 μL of human serum, 200 μL of EtOH as a precipitating agent and 400 μL of hexane: dichloromethane (80:20, v:v) mixture as an extraction solvent provided results with the highest recovery in the range 80–120%. Due to compatibility of final sample solvent with SFC mobile phase, evaporation and reconstitution of sample before injection could be omitted and sample could be injected in to SFC system directly. While previously described extraction methods for HPLC usually used tens of milliliters of organic solvents (MeOH, hexane, acetone) for vitamin E extraction [25,26,28,31,32], our method uses only 400 μL of hexane: dichloromethane mixture for the tocopherols and tocotrienols extraction from human serum. Thus, advantages of this new method allow not only shortening of analysis time and increasing the throughput of samples in a laboratory, but also lead to reduction of organic solvent consumption. Although hexane: dichloromethane are not green solvents, their consumption in our LLE procedure remains negligible.

3.4. Method validation

Initially, the repeatability of retention times and peak areas expressed as RSD% (n = 10) was evaluated at 4 concentration levels (Table 2) for both high resolution and high speed methods. RSD for retention times was <0.1% for high resolution method and <0.3% for high speed method. RSD for peak areas was <8.5% for high resolution method and <10.0% for high speed method, which is adequate for UHPSFC-MS method. Subsequently, the linearity, LOD and LOQ for standard solutions were determined. The linearity of both methods was assessed for concentration ranges reported at Table 3 with coefficient of determination r^2 better than 0.9969. LOD an LOQ were determined for S/N ≈ 3 and 10, respectively, the results are summarized in Table 3.

PP combined to LLE approach for human serum provided good selectivity and clean-up (Fig. 9). Despite the SIM mode and single quadrupole was used for the detection, the chromatograms were interference free and the method provided very good sensitivity. Both UHPSFC-MS methods using PP and LLE as a sample pretreatment approach which enabled to obtain pure extracts of vitamin E were validated in terms of precision, accuracy, linearity, range, LOD, LOQ and matrix effects using human serum according to EMA Guideline on bioanalytical method validation [46]. Methods showed good linearity expressed as correlation coefficient $r^2 = 0.9930–0.9955$ for all 8 tocochromanols in the different ranges shown in Table 3. LOQ and LOD (determined for S/N ≈ 3 and 10 and providing accuracy in the range 80–120% and precision with RSD < 20%) for all analytes measured by both methods are also summarized in Table 3. High resolution method provided more sensitive results in comparison with high speed method. LOQ of high resolution method was 2–15 times lower than LOQ of the high speed one. When standard and matrix calibration curves were compared, the sensitivity of matrix samples was approximately 4 times lower than the sensitivity of standard calibration curve which corresponded to the sample dilution during the extraction procedure. Thus, no more loss of sensitivity was observed when analysing complex matrices such as human serum. In comparison with previously developed methods for the vitamin E determination, only few methods provided lower limit of quantification which was due to very sensitive fluorescent detection [26,27,29], and quadrupole time-of-flight mass spectrometry detector [23]. The developed high resolution method provided more sensitive results than the methods using MS and UV detection mentioned in Table 1. The developed high speed method provided comparable results with LC-UV and LC-MS methods reported in Table 1.

Both methods provided very good accuracy and precision (Table 4) within the limits of acceptance required by EMA [46]. The results were in the range 80–120% for LLOQ (L4) and 85–115% for three other validation concentration levels (L1, L2, L3) except for one result from L4 (LLOQ) measured by high speed method (128.4% β-T_3). The method precision was found to be similar to methods reported in Table 1 where the precision was typically better than 15%. Accuracy of newly developed method was usually in range 86–115% which is also comparable or better than previously published methods.

Matrix effects were also evaluated for both methods (Table 5).

Table 4
Method accuracy and precision evaluated at 4 concentration levels in the linear range for tocopherols and tocotrienols in human serum for high resolution and high speed method according to EMA Guideline on bioanalytical method validation.

			α-T	β-T	γ-T	δ-T	α-T_3	β-T_3	γ-T_3	δ-T_3
High resolution method	Concentration levels [μg mL^{-1}] (n = 5)	L1	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
		L2	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
		L3	1.00	0.25	0.25	1.00	0.25	0.25	1.00	1.00
		L4	0.25	0.05	0.05	0.25	0.08	0.08	0.25	0.25
	Accuracy [%]	L1	108.0	102.8	106.5	106.6	96.1	93.2	96.9	95.4
		L2	114.0	109.7	113.9	111.8	97.6	99.4	95.9	97.2
		L3	115.4	88.1	102.5	99.2	92.6	101.4	88.3	93.6
		L4	87.9	107.9	78.8	95.0	92.4	105.8	96.7	97.6
	Precision [RSD %]	L1	10.7	4.3	5.7	4.9	4.6	4.0	5.0	4.2
		L2	7.3	6.1	7.8	5.5	5.3	5.1	3.2	4.0
		L3	15.9	5.2	11.2	5.3	9.3	4.2	13.6	8.3
		L4	12.0	9.0	7.0	3.7	5.9	13.4	11.6	7.4
High speed method	Concentration levels [μg mL^{-1}] (n = 5)	L1	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
		L2	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
		L3	1.00	1.00	2.50	1.00	0.75	0.75	2.50	0.75
		L4	0.50	0.50	0.75	0.50	0.25	0.25	0.75	0.25
	Accuracy [%]	L1	109.3	107.8	107.2	103.8	98.9	103.0	97.0	97.5
		L2	101.7	100.8	104.4	104.6	86.2	95.5	89.8	96.9
		L3	89.3	84.9	105.7	87.7	85.9	87.3	96.4	94.1
		L4	114.7	103.0	119.0	102.4	112.3	128.4	86.9	119.8
	Precision [RSD %]	L1	5.3	3.2	3.2	2.2	2.5	2.8	3.0	3.7
		L2	7.5	3.9	9.7	1.9	3.7	3.5	5.7	2.9
		L3	15.2	6.2	4.4	6.8	2.6	5.5	21.7	3.3
		L4	18.5	13.8	20.9	12.4	3.2	1.8	21.0	17.2

Table 5
Evaluated matrix effects for a low (L1, 1.0 μg mL^{-1}) and high (L2, 7.5 μg mL^{-1}) levels of concentration for tocopherols and tocotrienols in human serum for high resolution and high speed method according to EMA Guideline on bioanalytical method validation.

		α-T	β-T	γ-T	δ-T	α-T_3	β-T_3	γ-T_3	δ-T_3
High resolution method	L1	137.0	83.7	129.0	121.6	129.9	125.6	89.8	142.9
	L2	104.4	106.4	109.0	119.1	112.0	93.3	104.9	119.8
High speed method	L1	132.2	107.7	103.4	120.4	106.0	110.0	107.7	113.7
	L2	106.2	104.5	106.0	104.9	104.2	108.8	106.4	108.0

The range of observed matrix effects was 90–138% for a low concentration level (1.0 μg mL^{-1}), 93–119% for a high concentration level (7.5 μg mL^{-1}) which was fully acceptable. Pairwise comparison showed (at the 95% probability level) that the differences between matrix effects determined for high speed and high resolution method were not significant. It is an important fact because various compounds could be eluted in different time during both analytical methods and influence the signal of analytes of the interest. The results of ME evaluation and their comparison between two methods show that the LLE provided appropriate sample clean-up and prevented matrix effects to compromise the results.

4. Conclusions

The aim of this study was to develop fast, simple and selective UHPSFC-MS method for the determination of all 8 isomeric forms of vitamin E. Finally, two selective and sensitive methods involving high-throughput sample preparation step based on PP and LLE and fast UHPSFC-MS were optimized and fully validated for quantitative determination of all tocopherols and tocotrienols in human serum for the first time. Methods used single quadrupole MS which undoubted advantages are price affordability, small size and easy operation in comparison with most frequently used triple quadrupoles. Using additive in mobile phase was beneficial despite neutral character of compounds and few functional groups possessing hydrogen-binding donor/acceptor properties.

The high speed method provided the separation with analysis time of 2.5 min. The high resolution method took 4.5 min and provided more sensitive analysis than the high speed method. The high resolution method is more suitable for analysis of complex matrices due to the longer analysis time and better resolution, while the high speed method is convenient for rapid screening of samples containing higher concentrations of vitamin E in samples. These optimized methods benefit from the combination of LLE and SFC which are based on using of nonpolar extraction agents in LLE and CO_2 as a mobile phase. Consequently, there is no need for evaporation and reconstitution of sample in solvent compatible with used mobile phase which is often needed in HPLC. Moreover, the developed methods are suitable for fast screening due to a significant saving of instrument time, a low consumption of organic solvents, a need of small sample amount and a high throughput of samples in laboratory, which is important for routine bioanalytical laboratories.

Acknowledgement

The authors gratefully acknowledge the financial support of the Charles University Grant Agency for Grant GAUK no. 1948214 and the Project of Specific research, SVV 260 292 (2016). This work was also supported by the Ministry of Education, Youth and Sports of the Czech Republic – grant from the National Program for Sustainability I (LO1204). The authors also wish to gratefully acknowledge Waters, particularly Marek Exner and Petr Butzke for the kind opportunity to use the Waters Acquity UPC^2 coupled to QDa and for the technical support.

References

[1] M.L. Colombo, An update on vitamin E, tocopherol and tocotrienol – perspectives, Molecules 15 (2010) 2103–2113.
[2] H.A. Clemente, H.M.M. Ramalho, M.S.R. Lima, E.C. Grilo, R. Dimensteinn, Maternal supplementation with natural or synthetic vitamin E and its levels in human colostrum, JPGN 60 (2015) 535–537.
[3] T. Plozza, V. Craige Trenerry, D. Caridi, The simultaneous determination of vitamins A, E and β-carotene in bovine milk by high performance liquid chromatography-ion trap mass spectrometry (HPLC-MS^n),, Food Chem. 134 (2012) 559–563.
[4] M. Paz San Andrés, J. Otero, S. Vera, High performance liquid chromatography method for the simultaneous determination of α-, γ- and δ-tocopherol in vegetable oils in presence of hexaseyltrimethylammonium bromide/n-propanol in mobile phase, Food. Chem. 126 (2011) 1470–1474.
[5] A.M. Drotleff, C. Bohnsack, I. Schneider, A. Hahn, W. Ternes, Human oral bioavailability and pharmacokinetics of tocotrienols from tocotrienol-rich (tocotrienol-low) barely oil and palm oil formulations, J. Funct. Foods 7 (2014) 150–160.
[6] H.-Y. Huang, L.J. Appel, Supplementation of diets with α- tocopherol reduces serum concentration of γ- and δ- tocopherol in humans, J. Nutr. 133 (2003) 3137–3140.
[7] C.K. Sen, S. Khanna, S. Roy, Tocotrienols in health and disease: the other half of the natural vitamin E family,, Mol. Asp. Med. 28 (2007) 692–728.
[8] B.B. Aggarwal, C. Sundaram, S. Prasad, R. Kannappan, Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases, Biochem. Pharmacol. 80 (2010) 1613–1631.
[9] H.M. Pinheiro-Sant'Ana, M. Guinazi, D. da Silva Oliviera, C.M. Della Lucia, B. de Lazzari Reis, S.C. Cardoso Brandão, Method for simultaneous analysis of eight vitamin E isomers in various foods by high performance liquid chromatography and fluorescence detection, J. Chromatogr. A 1218 (2011) 8496–8502.
[10] N.I. Krinsky, Human requirements for fat-soluble vitamins, and other things concerning these nutrients, Mol. Asp. Med. 24 (2003) 317–324.
[11] A. Podsedek, Natural antioxidants and antioxidant capacity of Brassica vegetables: a review, LWT 40 (2007) 1–11.
[12] R. Eitenmiller, J. Lee, VITAMIN E: Food Chemistry, Composition and Analysis, Marcel Dekker Inc., New York, 2004, pp. 438–493.
[13] D.H. Blatt, S.W. Leonard, M.G. Traber, Vitamin E kinetics and the function of tocopherol regulatory proteins, Nutrition 17 (2001) 799–805.
[14] A.K. Dutta-Roy, Molecular mechanism of cellula uptake and intracellular translocation of α-tocopherol: role of tocopherol-binding proteins, Food Chem. Toxicol. 37 (1999) 967–971.
[15] L. Packer, J. Fuchs, VITAMIN E in Health and Disease: Biochemistry and Clinical Applications, Marcel Dekker Inc., New York, 1993, pp. 861–862.
[16] M.G. Traber, D. Manor, Vitamin E, Adv. Nutr. 3 (2012) 330–331.
[17] M.G. Traber, J. Atkinson, Vitamin E, antioxidant and nothing more, J. Free Rad. BioL., Med. 43 (2007) 4–15.
[18] M.G. Traber, Mechanisms for the prevention of vitamin E excess, J. Lipid Res. 54 (2013) 2295–2306.
[19] F.J. Rupérez, D. Martín, E. Herrera, C. Barbas, Chromatographic analysis of α-tocopherol and related compounds in various matrices, J. Chromatogr A 935 (2001) 45–69.
[20] E. Ibanez, J. Palacios, G. Reglero, Analysis of tocopherols by on-line coupling supercritical fluid extraction - supercritical fluid chromatography, J. Microcolumn Sep. 11 (1999) 605–611.
[21] P. Manninen, P. Laakso, H. Kallio, Method for characterization of triacylglycerols ad fat-soluble vitamins in edible oils and fats by supercritical fluid chromatography, J. Am. Oil Chem. Soc. 72 (1995) 1001–1008.
[22] G. Galuba, M. Gogolewski, Separation of tocopherols and sterols in soybean oil condensate utilizing supercritical fluid chromatography, Chem. Anal. Wars. 42 (1997) 245–248.
[23] M. Méjean, A. Brunelle, D. Touboul, Quantification of tocopherols and tocotrienols in soybean oil by supercritical-fluid chromatography coupled to high-resolution mass spectrometry, Anal. Bioanal. Chem. 407 (2015) 5133–5142.
[24] P.T. Gee, C.Y. Liew, M.C. Thong, M.,C.L. Gaym, Vitamin E analysis by ultra-performance convergence chromatography and structural elucidation of novel α-tocodienol by high-resolution mass spectrometry, Food Chem. 196 (2016) 367–373.
[25] M.P.S. Andrés, J. Otero, S. Vera, High performance liquid chromatography method for the simultaneous determination of α-, γ- and δ-tocopherol in vegetable oils in presence of hexadecyltrimethylammonium bromide/n-propanol in mobile phase, Food Chem. 126 (2011) 1470–1474.
[26] H.M. Pinheiro-Sant'Ana, M. Guinazi, D. da Silva Oliviera, C.M.D. Lucia, B. de Lazzari Reis, S.C.C. Brandao, Method for simultaneous analysis of eight vitamin E isomers in various foods by high performance liquid chromatography and fluorescence detection, J. Chromatogr. A 1218 (2011) 8496–8502.
[27] N. Grebenstein, J. Frank, Rapid baseline-separation of all eight tocopherols and tocotrienols by reversed-phase liquid chromatography with a solid-core pentafluorophenyl column and their sensitive quantification in plasma and liver, J. Chromatogr. A 1243 (2012) 39–46.

[28] K. Knecht, K. Sandfuchs, S.E. Kulling, D. Bunzel, Tocopherol and tocotrienol analysis in raw and cooked vegetables: a validated method with emphasis on sample preparation, Food Chem. 169 (2015) 20–27.
[29] P. Vinas, M. Bravo-Bravo, I. Lopez-García, M. Pastor-Belda, M. Hernandéz-Córdoba, Pressurized liquid extraction and dispersive liquid-liquid microextraction for determination of tocopherols and tocotrienols in plant foods by liquid chromatography with fluorescence and atmospheric pressure chemical ionization-mass spectrometry detection, Talanta 119 (2014) 98–114.
[30] B. Shammugasamy, Y. Ramakrishnan, H.M. Ghazali, K. Muhammad, Combination of saponification and dispersive liquid-liquid microextraction for the determination of tocopherols and tocotrienols in cereals by reversed-phase high-performance liquid chromatography, J. Chromatogr. A 1300 (2013) 31–37.
[31] J.L. Chávez-Servín, A.I. Castellote, M.C. Lopéz-Sabater, Simultaneous analysis of Vitamins A and E in infant milk-based formulae by normal-phase high performance liquid chromatography-diode array detection using a short narrow-bore column, J. Chromatogr. A 1122 (2006) 138–143.
[32] S.-H. Huang, L.-T. Ng, An improved high-performance liquid chromatographic method for simultaneous determination of tocopherols, tocotrienols and γ-oryzanol in rice, J. Chromatogr. A 1218 (2011) 4709–4713.
[33] C. Yuan, M. Burgyan, D.R. Bunch, E. Reineks, R. Jackson, R. Steinle, S. Wang, Fast, simple, and sensitive high-performance liquid chromatography method for measuring vitamins A and E in human blood plasma, J. Sep. Sci. 37 (2014) 2293–2299.
[34] M. Richellle, I. Tavazzi, L.B. Fay, Simultaneous determination of deuterated and non-deuterated α-tocopherol in human plasma by high-performance liquid chromatography, J. Chromatogr. B 794 (2003) 1–8.
[35] P.F. Chatzimichalakis, V.F. Samanidou, I.N. Papadoyannis, Development of a validated liquid chromatography method for the simultaneous determination of eight fat-soluble vitamins in biological fluids after solid-phase extraction, J. Chromatogr. B 805 (2004) 289–296.
[36] V. Desfontaine, D. Guillarme, E. Francotte, L. Nováková, Supercritical fluid chromatography in pharmaceutical analysis, J. Pharm. Biomed. Anal. 113 (2015) 56–71.
[37] L.T. Taylor, Supercritical fluid chromatography for the 21st century, J. Supercrit. Fluids 47 (2009) 566–573.
[38] K. Kalíková, T. Slechtová, J. Vozka, E. Tesařová, Supercritical fluid chromatography as a tool for enantioselective separation; A review, Anal. Chim. Acta 821 (2014) 1–33.
[39] D. Spaggiari, F. Mehl, V. Desfontaine, A. Grand-Guillaume Perrenoud, S. Fekete, S. Rudaz, D. Guillarme, Comparison of liquid chromatography and supercritical fluid chromatography coupled to compact single quadrupole mass spectrometer for targeted in vitro metabolism assay, J. Chromatogr. A 1371 (2014) 244–256.
[40] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, The use of columns packed with sub-2 μm particles in supercritical fluid chromatography, Trends Anal. Chem. 63 (2014) 44–54.
[41] L. Nováková, A. Grand-Guillaume Perrenoud, I. Francois, C. West, E. Lesellier, D. Guillarme, Modern analytical supercritical fluid chromatography using columns packed with sub-2 μm particles: a tutorial, Anal. Chim. Acta 824 (2014) 18–35.
[42] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Comparison of ultra-high performance supercritical fluid chromatography and ultra-high performance liquid chromatography for the analysis of pharmaceutical compound, J. Chromatogr. A 1266 (2012) 158–167.
[43] L. Nováková, A. Grand-Guillaume Perrenoud, R. Nicoli, M. Saugy, J.-L. Veuthey, D. Guillarme, Ultra-high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. I: investigation of mobile phase and MS conditions, Anal. Chim. Acta 853 (2015) 637–646.
[44] A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, Coupling state-of-the-art supercritical fluid chromatography and mass spectrometry: from hyphenation interface optimization to high-sensitivity analysis of pharmaceutical compounds, J. Chromatogr. A 1339 (2014) 174–184.
[45] http://www.chemicalize.org/10th March 2016.
[46] EMA Guideline on bioanalytical method validation online available 10th March 2016.
[47] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[48] E. Lesellier, C. West, The many faces of packed column supercritical fluid chromatography – a review, J. Chromatogr. A 1382 (2015) 2–46.
[49] A. Tarafder, G. Guiochon, Unexpected retention behaviour of supercritical fluid chromatography at the low density near critical region of carbon dioxide, J. Chromatogr. A 1229 (2012) 249–259.

8.5 Development of matrix effect-free MISPE-UHPLC–MS/MS method for determination of lovastatin in Pu-erh tea, oyster mushroom, and red yeast rice

Journal of Pharmaceutical and Biomedical Analysis 140 (2017) 367–376

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Development of matrix effect-free MISPE-UHPLC–MS/MS method for determination of lovastatin in Pu-erh tea, oyster mushroom, and red yeast rice

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ARTICLE INFO

Article history:
Received 16 February 2017
Received in revised form 24 March 2017
Accepted 27 March 2017
Available online 30 March 2017

Keywords:
Lovastatin
Molecularly imprinted polymers
Matrix effects
Selective sample preparation
UHPLC–MS/MS

ABSTRACT

Matrix effect-free UHPLC–MS/MS method was developed and validated for the determination of cholesterol-lowering lovastatin in food samples represented by Pu-erh tea, oyster mushroom, and red yeast rice. The resulting method was fully validated in terms of intra-day and inter-day precision, accuracy, linearity, range, LOD, LOQ, and matrix effects. The matrix effect phenomenon evaluated by comparison of slopes of calibration curves was completely eliminated by solid-phase extraction based on the technique of molecularly imprinted polymers (MIPs). Comparison of elution profiles obtained on the MIP and corresponding control non-imprinted polymer (NIP) showed selectivity of the extraction procedure. In addition, selectivity of the MIP material and the molecularly imprinted solid-phase extraction (MISPE) was also proved by experiments evaluating retention of analytes physico-chemically similar to the target molecule. Extraction recoveries of these analytes represented by estrogen derivatives (estrone, estriol, 17α-ethinylestradiol, and β-estradiol) were very low or even null. Synthesis and preparation of the resulting MIP sorbent was characterized by excellent repeatability expressed as RSD 7.7% (n = 9) of extraction recoveries. The determined capacity of the MIP material reaching 375 ng/mg is sufficient for analysis of the evaluated statin in its natural sources. Suitability of the resulting MISPE-UHPLC–MS/MS procedure for real sample analysis was verified by the determination of lovastatin in one dietary supplement based on the red yeast rice with a given amount of the target analyte. Finally, three mushroom and fifteen tea samples obtained in Czech food stores and tearooms were subjected to analysis. Low or null amount of lovastatin was found in these samples.

1. Introduction

Statins act as competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase which represents the key enzyme of cholesterol biosynthesis [1]. All the marketed statins possess common side chain in the form of 3,5-dihydroxypentanoic acid which interacts with the HMG binding domain of the active site of HMG-CoA reductase [2]. Besides hypolipidemic action, statins are associated with extra-lipid effects such as antitumor activity, modulation of bone formation, immunomodulation, inhibition of platelet aggregation, stabilization of atherosclerotic plaques, restoration of endothelial function, and anti-inflammatory activity [3–9]. Nevertheless, they cause several side effects, most commonly muscle pain, liver damage, and allergic reaction [10,11]. They may also interact with other pharmaceuticals and also food components [12,13]. Statins exist in lactone (prodrug) and open hydroxy-acid (active) forms and interconversion between them occurs both *in vivo* and *in vitro*. Therefore, both forms of statins have to be monitored simultaneously [14,15].

Lovastatin originally manufactured by Merck represents the first marketed statin that was approved by the Food and Drug Administration (FDA) in 1987 [16]. It is produced by several fungal species of the genus *Trichoderma* (*Hypocreaceae*), *Monascus* (*Elaphomycetaceae*), *Penicillium*, and *Aspergillus* (*Trichocomaceae*) [17–19]. These fungal species were isolated from traditional fermented Chinese food such as Pu-erh tea and red yeast rice. Lovastatin producing fungal species were also found in oyster mushroom (*Pleurotus ostreatus*).

Pu-erh tea is manufactured from tea leaves of *Camellia sinensis* (Linn.) var. *assamica* (Masters) Kitamura. This large leaf tea species

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http://dx.doi.org/10.1016/j.jpba.2017.03.058
0731-7085/

originates from Yunnan Province that is located in the south of China [20]. Popularity of this fermented tea is currently increasing especially due to the health beneficial effects such as antiobesity, hypocholesterolemic, and hypolipidemic activities and also due its unique taste and flavour [21]. According to the post-harvest processing of tea leaves Pu-erh tea is divided into raw and ripe type. Raw Pu-erh tea (also known as green Pu-erh and Shengcha) is turned into ripe Pu-erh (also known as black Pu-erh and Shou-cha) after natural aging including long-term storage. Ripe Pu-erh can also be prepared directly by microbial post-fermentation at higher temperature and higher humidity [22]. The long-term storage, post-fermentation process, and their conditions have a significant impact on lovastatin content in Pu-erh teas [20,23]. It usually ranges from tens up to hundreds of nanograms per gram of dried tea leaves [14,20].

Dietary supplements based on red yeast rice extracts were promoted in the late nineties to be efficient in cholesterol reduction as much as drugs containing statins. Cholesterol-lowering action is provided by polyketide compounds known as monacolins that are produced by red mold *Monascus purpureus*. The main component is represented by monacolin K which is actually natural form of lovastatin [24]. Therefore, due to the content of active ingredient equal to the Merck's Mevacor (lovastatin) FDA banned the sale of a red yeast rice extract Cholestin™ supplied by Pharmenex in 2001 [25]. The amount of lovastatin observed in the red yeast rice is commonly in the range of hundreds of micrograms and several milligrams per gram of dried sample [26–28].

Pleurotus ostreatus (*Pleurotaceae*) (Jacq. ex. Fr, Kummer) represents a common edible mushroom that is grown all over the world. This wood-decaying fungus is well known for its health beneficial activity and also common culinary use. It is characterized by antioxidant, antihypercholesterolemic and immunomodulatory effects [29,30]. Content of lovastatin quantified in *Pleurotus ostreatus* by Gunde-Cimerman et al. [31] and Chen et al. [32] ranged from hundreds of micrograms up to milligrams per gram of dried mushroom samples.

Although LC–MS instrumentation is well-established and widely used analytical tool in the field of quantitative analysis, it suffers from a major drawback represented by matrix effects. The matrix effect phenomenon is caused by co-eluting compounds that affect the process of ionization of a target analyte in the ion source of mass spectrometer [33]. Their assessment is usually performed by qualitative and quantitative approaches such as post-extraction addition, post-column infusion, and comparison of slopes of calibration curves [34]. The compounds responsible for ion suppression or enhancement can be brought from complex matrices of analysed samples, solvents, reagents, and materials used for sample preparation or solvents, buffers, and additives contained in the mobile phase [35]. Matrix effects can be reduced or fully removed by several approaches using different mechanisms which can be applied during all steps of the whole LC–MS analysis. It includes sample preparation, chromatographic separation, mass-spectrometric detection, and data evaluation employing various calibration methods [36]. The use of sample preparation for matrix effect elimination involves especially selective approaches [37] such as molecularly imprinted polymers, aptamers, and antibodies. These materials are tailor-made for a selective recognition of the target analyte in complex matrices. Concerning laboratory preparation of a material with an ability of selective recognition of the target analyte in a complex matrix, MIPs mostly represent the fastest, the easiest, and also the cheapest way.

MIP can be applied in various fields of analytical chemistry such as binding assays, enantiomer separation in liquid chromatography or capillary electrochromatography, sensors, and finally, their most common and also the most advanced use is a sorbent for solid-phase extraction [38].

The purpose of this work was to develop and validate a selective, sensitive, and matrix effect-free UHPLC–MS/MS method for the determination of lovastatin in common food samples represented by oyster mushroom, red yeast rice, and Pu-erh tea leaves.

2. Materials and methods

2.1. Chemicals and reagents

Lactone forms of lovastatin (LOV) and simvastatin (SV) were supplied by Sigma-Aldrich (Steinheim, Germany). Open hydroxy-acid form of lovastatin (sodium salt, LOVa) was purchased from Cayman Chemical (Ann Arbor, MI, USA) and hydroxy-acid form of simvastatin (ammonium salt, SVa) from Toronto Research Chemicals (Toronto, Canada). Acetonitrile and mobile phase additives (acetic and formic acid), all of them LC–MS grade, were obtained from Sigma-Aldrich (Steinheim, Germany) as well as estrogen derivatives (estriol, β-estradiol, 17α-ethinyl estradiol, and estrone). LC–MS grade water was prepared by Milli-Q reverse osmosis system (Millipore, Bedford, MA, USA) immediately before the use. Solvents (methanol, toluene, and dichloromethane) and reagents (azobisisobutyronitrile, methacrylic acid, and ethylene glycol dimethacrylate) employed for the synthesis of MIP material were supplied by Sigma-Aldrich (Steinheim, Germany). In order to remove inhibitor monomethyl ether hydroquinone (MEHQ), both methacrylic acid (MAA), and ethylene glycol dimethacrylate (EGDMA) were distilled under reduced vacuum. In addition, before the distillation, EGDMA was washed twice with 10% NaOH solution, twice with saturated NaCl solution, twice with distilled water, then dried using anhydrous sodium sulfate and finally filtered. EGDMA, MAA, and azobisisobutyronitrile (AIBN) were stored in the freezer until use.

2.2. Instrumentation and analytical conditions

ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) consisting of binary solvent manager and sample manager was coupled with Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters). All UHPLC-MS/MS analyses were performed using analytical column BEH C18 (50 mm × 2.1 mm, 1.7 μm) (Waters) and the column was maintained at 40 °C. Analytes were separated using gradient elution consisted of 0.5 mM ammonium acetate (AmAc) in water, pH 4 (solvent A) and acetonitrile (solvent B). The gradient started with 30% of solvent B, increased to 70% over 3.7 min, and in 4.0 min the percentage of solvent B ramped to initial conditions (30%). The flow rate was set at 0.35 ml/min. The chromatographic runtime took 5.0 min including equilibration of the system and the injected volume was 5 μl. Injected samples were dissolved in a mixture that corresponds to the initial conditions of the gradient elution. The ESI ion source and ion optics parameters were optimized and set as follows: capillary voltage 3.0 kV in positive mode of ionization and 2.5 kV in negative ionization mode, extractor voltage 1.0 V, hexapole voltage 1.0 V, cone voltage 25 V (LOVa, SVa) and 20 V (LOV and SV), cone gas flow rate 100 l/h, source temperature 130 °C, desolvation temperature 350 °C, and desolvation gas flow rate 600 l/h. Triple quadrupole mass spectrometer was operating in SRM mode by monitoring the following transitions: 420.9 → 319.2 (LOVa; collision energy: 15 eV), 434.9 → 319.2 (SVa; 15 eV), 405.2 → 285.2 (LOV; 10 eV) and 419.0 → 285.2 (SV; 10 eV). Acid forms of statins were ionized in negative mode while lactone forms in positive mode. Data were acquired and processed using MassLynx™ software version 4.1 (Waters, Milford, MA, USA).

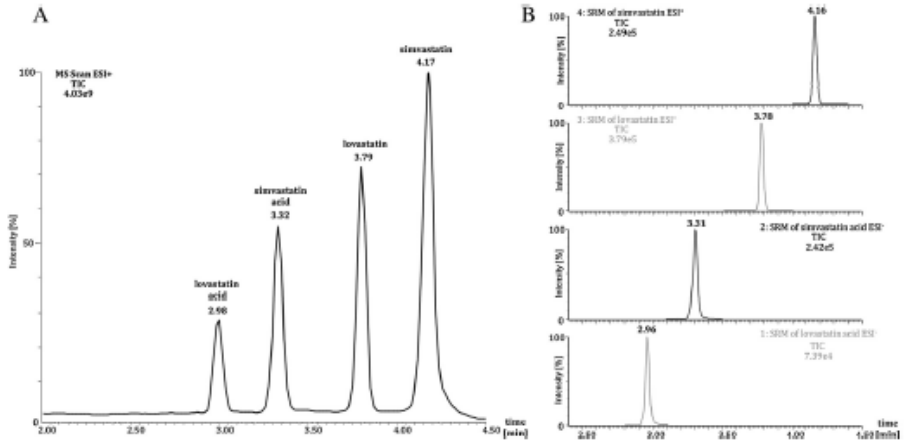

Fig. 1. Full-scan chromatogram of the statins (1 μg/ml) in positive ionization mode (A) and the chromatograms of individual SRM transitions (100 ng/ml) (B).

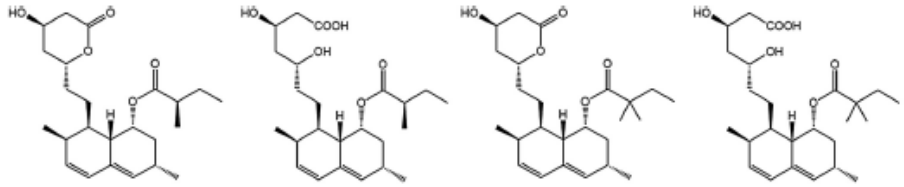

Fig. 2. Structures of the target analyte (lovastatin), its interconversion product (lovastatin acid), template molecule (simvastatin) and its interconversion product (simvastatin acid).

2.3. Standard solutions

Stock standard solutions of statin lactone forms were prepared in acetonitrile while the hydroxy-acid forms in the mixture consisting of 0.5 mM AmAc in water, pH 4 and acetonitrile (70/30, v/v) to give a solution with a concentration of 1.0 mg/ml. Stock standard solutions were stored at 4 °C and prepared fresh every two weeks.

2.4. Mushroom, tea, and red yeast rice sample treatment

All the food samples were treated identically as follows: dried mushrooms, dried Pu-erh tea leaves or red yeast rice were taken from the bottom, middle, and top of the package, put together, blended, and rubbed using mortar and pestle to homogenize the sample. 1 g of the formed homogenized sample was dissolved in 10 ml of acetonitrile, sonicated for 30 min, and stirred at 100 rpm at room temperature for 24 h. The acetonitrile extract (500 μl) was diluted with buffer (4500 μl) to get a 90/10 (0.5 mM AmAc, pH 4/ACN) mixture. 1 ml of the resulting sample (without any other treatment) was percolated through the MIP sorbent and subjected to optimized molecularly imprinted solid-phase extraction (MISPE).

2.5. Synthesis and preparation of the MISPE sorbent

MISPE sorbent was prepared by radical polymerization in bulk. The template molecule (simvastatin; 1 mM) was weighed in a 7 ml-glass vial (i.d. 20 mm) and dissolved in a porogenic solvent. Subsequently, the functional monomer (MAA; 4 mM), the cross-linker (EGDMA; 20 mM), and finally initiator (AIBN; 0.25 mM) were added. When adding each reagent, the formed solution was thoroughly mixed. The resulting polymerization mixture was immediately placed in an ice-bath and purged with nitrogen for 10 min. The glass vial containing a magnetic stir bar was sealed and immersed in a silicon oil bath heated at 65 °C and stirred for 24 h. The formed block of polymer was crushed, grounded, and manually sieved to get particles with size between 25–36 μm. The MIP particles were additionally subjected to sedimentation (performed in triplicates) using MeOH/H_2O (80/20; v/v) mixture to eliminate the finest particles. 20 mg of the MIP material was packed into a 1 ml-polypropylene cartridge (Sigma Aldrich, Steinham, Germany) between two polyethylene frits (Sigma Aldrich, Steinheim, Germany). The template molecule was removed by washing step using acetonitrile. The obtained fractions were analysed by UHPLC–MS/MS method to evaluate the presence of the template. Control of the imprinting effect (i.e. cavity forming) was

Table 1
Conditions used for the synthesis of all tested MIP materials.

MIP	Template molecule	Functional monomer	Cross-linker	Initiator	Molar ratio	Porogenic solvent
MIP1	Simvastatin	MAA	EGDMA	AIBN	1:4:20	acetonitrile
MIP2	Simvastatin	MAA	EGDMA	AIBN	1:4:20	dichloromethane
MIP3	Simvastatin	MAA	EGDMA	AIBN	1:4:20	toluene
MIP4	Simvastatin	MAA	EGDMA	AIBN	1:4:20	methanol
MIP5	Simvastatin	MAA	EGDMA	AIBN	1:4:20	acetonitrile/H_2O; 80/20 (v/v)
MIP6	Simvastatin	MAA	EGDMA	AIBN	1:4:20	methanol/H_2O; 80/20 (v/v)
MIP7	Simvastatin	MAA	EGDMA	AIBN	1:4:20	0.1% formic acid in acetonitrile/H_2O 80/20 (v/v)
MIP8	Simvastatin	MAA	EGDMA	AIBN	1:4:20	0.1% formic acid in methanol/H_2O 80/20 (v/v)
MIP9a, b, c	Simvastatin	MAA	EGDMA	AIBN	1:4:20	0.1% formic acid in acetonitrile
MIP10	Simvastatin	MAA	EGDMA	AIBN	1:4:20	0.1% formic acid in methanol
MIP11	Simvastatin	MAA	EGDMA	AIBN	1:4:20	1% formic acid in acetonitrile

performed by comparing extraction profiles obtained on MIP and corresponding non-imprinted polymer (NIP) at the same time. The control NIP material was prepared according to the same procedure as MIP, except that no template molecule was employed for its synthesis.

2.6. MISPE procedure

Conditioning of the MISPE sorbent was performed with 2 ml of ACN and 5 ml of the mixture consisting of 0.5 mM AmAc, pH 4 and ACN (95/5; v/v). Samples dissolved in 1 ml of 0.5 mM AmAc, pH 4/ACN (90/10; v/v) were loaded onto the MIP sorbent. The washing step was carried out with 4 ml of the mixture consisted of 0.5 mM AmAc, pH 4 and acetonitrile (77/23; v/v). Due to the low extraction recovery observed when evaluating retention of lovastatin in real samples, the volume of washing mixture was decreased to 3 ml. The elution step was performed with 1 ml of acetonitrile and the elution fraction was evaporated. When necessary to increase sensitivity of the UHPLC–MS/MS method, the evaporated elution fraction was 10-fold concentrated by reconstitution in lower volume (100 µl instead of 1 ml). After finishing the MISPE procedure, SPE cartridge was rinsed with 8 ml of acetonitrile to wash out the MISPE sorbent. Then it was filled with mixture consisting of 0.5 mM AmAc, pH 4 and acetonitrile (95/5; v/v) and kept at room temperature until further use.

2.7. Validation of the MISPE-UHPLC–MS/MS method

At first, to perform the system suitability test (SST) repeatability of retention times and peak areas were evaluated at three concentration levels 5 ng/ml, 50 ng/ml, and 500 ng/ml in ten replicates for all four statins. Then lower concentration levels (1 ng/ml, 10 ng/ml, and 100 ng/ml) were tested for lovastatin. Both standards solutions and spiked lovastatin-free oyster mushroom, Pu-erh tea, and red yeast rice samples were employed for validation of the UHPLC–MS/MS method including the MISPE procedure. Method validation was performed in terms of linearity, range, intra-day and inter-day precision, accuracy, LOD, LOQ, and matrix effects. Linearity was established over the range 1 ng/ml–500 ng/ml. Oyster mushroom, red yeast rice, and Pu-erh tea samples spiked with lovastatin standard at 1 ng/ml, 10 ng/ml, and 100 ng/ml were used for determination of intra-day and inter-day precision and accuracy. Limit of quantification (LOQ) was determined as the lowest level of calibration curve with S/N ~10. Limit of detection (LOD) was established as the level with S/N ~3. Matrix effects were determined in oyster mushroom, Pu-erh tea and red yeast rice samples. Their evaluation was carried out by comparison of the slopes of calibration curves constructed in the diluent mixture and in the food matrix treated with and without MISPE procedure. Samples, which were not subjected to the MISPE procedure, were filtered through a 0.22 µm membrane filter before the UHPLC–MS/MS analysis. Six concentration levels of lovastatin were used for this purpose.

3. Results and discussion

Stability of statins is an important issue to be considered. Reversible interconversion between the lactone and the open hydroxy-acid form represents the manifestation of the instability. It is necessary to ensure conditions allowing to avoid this phenomenon during the whole MISPE-UHPLC-MS/MS analysis *i.e.* storage of standards, sample preparation, and UHPLC-MS/MS analysis. Ammonium acetate buffer (pH 4) was used for this purpose.

UHPLC–MS/MS method was developed for determination of both lovastatin forms to evaluate the suitability of the chosen conditions to avoid the interconversion. Simvastatin was analysed to monitor the process of elimination of the template from the MIP material. SRM transition of the open hydroxy-acid form of simvastatin was added to the method to observe the possible interconversion of the template during the synthesis of the MIP material. Chromatographic separation of these four statins was based on UHPLC–MS/MS method published by our research group (Vlčková et al.) [39]. The LC conditions were modified to shorten the separation time. The resulting LC–MS chromatogram is shown in Fig. 1A and the SRM transitions in Fig. 1B.

3.1. Synthesis of MIP sorbent

Simvastatin was chosen as a template molecule for the synthesis of the MIP material due to its physico-chemical properties (logP and pKa shown in Fig. 2; predicted by chemicalize.com) which are very close to that of the target analyte. Generally, tens of milligrams of the template molecule are used to prepare a MIP material, thus, the affordability has also to be taken into account. Simvastatin is a semisynthetic analogue of lovastatin and the only structural difference lies in the substituent represented by methyl group occurring at C-2 position of the 2-methylbutanoic acid that is ester-bound to hexahydronaphthalene ring. The analyte of interest was not employed for the synthesis of the MIP material in order to avoid potential false positive results caused by the template bleeding. To explore the effect of polarity of the porogenic solvent on the properties of the resulting MIP material, identical reagents were used for the synthesis of all the MIPs, except the porogenic solvent. Thus, methacrylic acid was used as a functional monomer, ethylene glycol dimethacrylate as a cross-linking agent and azobisisobutyronitrile as an initiator of the polymerization. The molar ratio of 1/4/20/0.25 (template/monomer/cross-linker/initiator) was the same during the synthesis of all the MIP sorbents. Detailed conditions for synthesis of the MIPs are shown in Table 1.

When looking for a most suitable MIP sorbent, selectivity of the MISPE procedure and retention of the target analyte were studied. Process of selectivity and retention evaluation is described in the

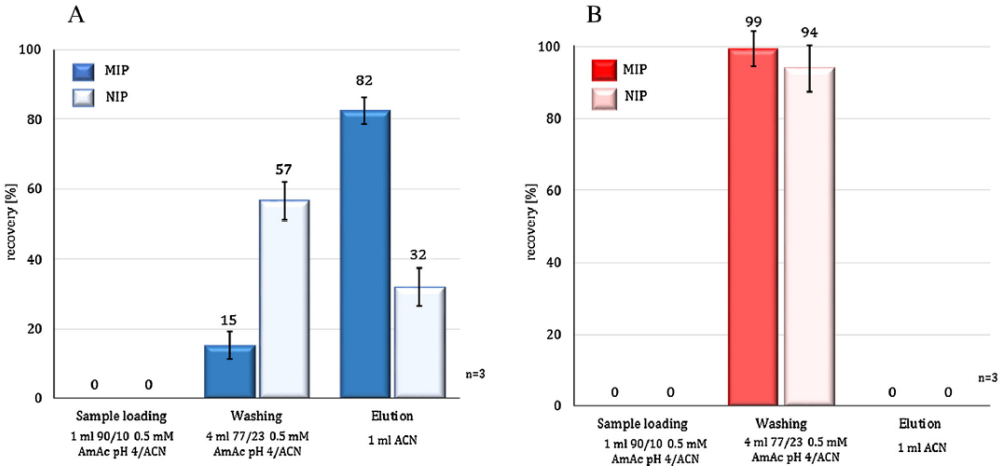

Fig. 3. Evaluation of selectivity and repeatability of the MISPE procedure in standard samples on MIP9a and control NIP9a for lovastatin (100 ng/ml) (A) and lovastatin acid (100 ng/ml) (B).

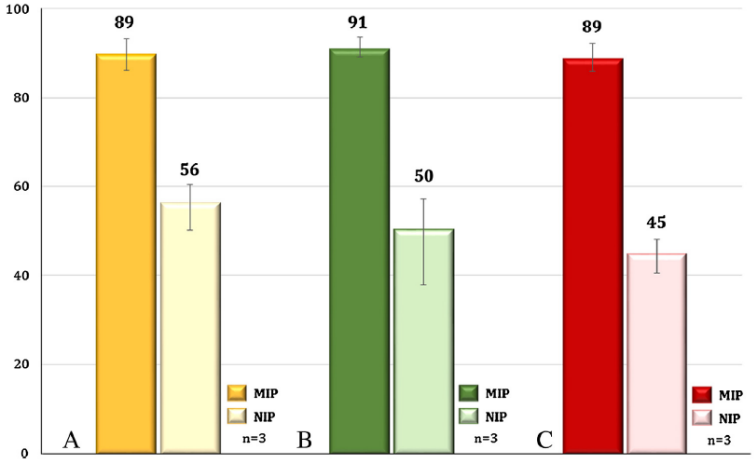

Fig. 4. Repeatability of the MISPE procedure in food samples on MIP9a and control NIP9a for lovastatin (100 ng/ml) in oyster mushroom (A), Pu-erh tea (B) and red yeast rice (C) extracts.

next Section 3.2. MIP1 was prepared based on the procedure published by Wang et al. [40] using acetonitrile. Then, dichloromethane (MIP2) and toluene (MIP3) were employed for synthesis to explore the effect of non-polar solvent. Subsequently, polarity of the porogen was increased to induce different interactions. Methanol (MIP4), acetonitrile/water (80/20; MIP5), and methanol/water (80/20; MIP6) were used for this purpose. Methanol and water-organic solvent mixtures were reported to induce interconversion of statins. Moreover, the interconversion product of simvastatin was detected when eliminating the template. However, it was not possible to quantify the product of the interconversion precisely because sedimentation step partially removing both forms of template was performed before the template elimination itself as described in section 2.5. In order to avoid the interconversion of the template formic acid (FA) was added to the porogenic solvent. Therefore, acidified mixtures acetonitrile/water (80/20, 0.1% FA; MIP7) and methanol/water (80/20, 0.1% FA; MIP8) were used for the synthesis. Pure acetonitrile (MIP9) and methanol (MIP10) acidified with formic acid were also examined. MIP9 provided the highest retention of lovastatin when employing increasing amount of organic solvent in the washing mixture. Moreover, only MIP9 showed selectivity between the MIP and the control NIP. Therefore, the MIP9 was employed for further experiments. Effect of higher amount of formic acid dissolved in acetonitrile was studied. However, particles of the formed MIP material (MIP11) were too small. They completely fell through 25 μm sieve, and thus MIP11 was not subjected to further experiments.

3.2. Development of MISPE procedure

At first, the most suitable solvent for sample loading step was sought. Acetonitrile, dichloromethane, toluene, methanol, water,

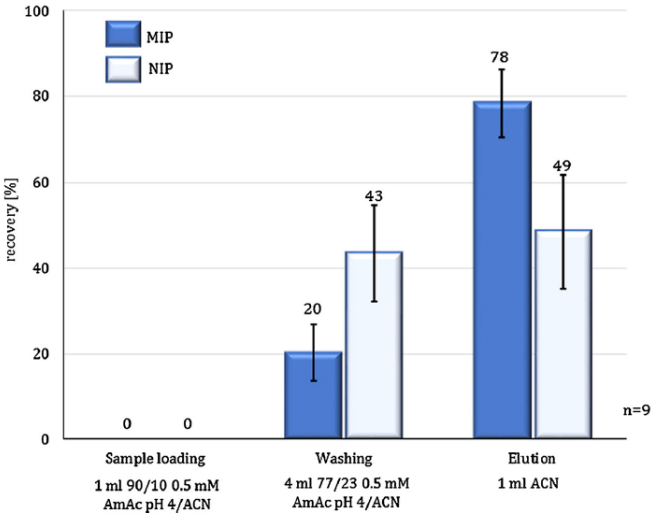

Fig. 5. Repeatability of the synthesis of the resulting MIP sorbent expressed as extraction recovery of nine MISPE procedures performed in standard samples (100 ng/ml) on MIP9a, MIP9b, and MIP9c and corresponding NIP9a, NIP9b, and NIP9c.

0.5 mM ammonium acetate, pH 4, and their mixtures were tested for this purpose. When percolating lovastatin dissolved in water and 0.5 mM ammonium acetate, pH 4, it was fully retained on all the prepared MIP materials (MIP1-10). When using pure organic solvents, the target analyte was not retained at all. However, due to the low solubility of lovastatin in water a certain amount of organic solvent had to be added to the sample loading mixture, whose resulting composition was 0.5 mM AmAc, pH 4/acetonitrile; 90/10 (v/v). Ammonium acetate was added due to the stability reasons. Then, mixtures used for selectivity evaluation and optimization of the washing step were consisted of 0.5 mM AmAc, pH 4 and increasing content of organic solvents corresponding to the porogens including acetonitrile, methanol, toluene, and dichloromethane (85/15, 80/20, 75/25, 70/30, 65/35; v/v). Only MIP9 provided selectivity when using corresponding NIP as a control material. Selectivity of the MISPE procedure and acceptable extraction recovery were observed when percolating mixtures with ratio 80/20 and 75/25. Then the ratio of 77/23 (v/v) was examined and finally also chosen as the most suitable one. Regarding solvent for elution, all the mixtures containing more than 60% of ACN allowed to elute the total amount of the retained lovastatin. Finally, 1 ml of 100% ACN was used for the elution of the target analyte to speed up the evaporation step. After SPE procedure, the MIP sorbent was washed with acetonitrile to remove all the matrix components, filled with the mixture used for conditioning step, and kept at room temperature until further use.

When optimizing the MISPE procedure open hydroxy-acid form of lovastatin was also monitored. No traces of this form were detected during the process. Therefore, these conditions were found to be suitable in terms of avoiding the interconversion phenomenon of lovastatin. When the standard of hydroxy-acid form of lovastatin was subjected to the optimized MISPE procedure, its whole amount was removed during the washing step as demonstrated in Fig. 3B. It is another manifestation of MISPE selectivity which, however, represents disadvantage in lovastatin determination. On the other hand, Yang et al. [14] did not detect the hydroxy-acid form of lovastatin in Pu-erh tea analysis. Quantification of monacolins in products based on red yeast rice showed that lovastatin (monacolin K) predominated over its interconversion product in most of the analysed samples [28].

Recovery of the MISPE procedure observed in pure acetonitrile medium was 82% (n = 3) as presented in Fig. 3A. However, when applying the MISPE procedure for the first time in Pu-erh tea, oyster mushroom, and red yeast rice matrices, the obtained recovery was much lower (55–57%). Based on these results, volume of the washing step mixture was decreased from 4 ml to 3 ml. Thereafter, the extraction recovery obtained using the modified MISPE procedure was increased in all three tested matrices to be higher than 88% as shown in Fig. 4.

3.3. Extraction of lovastatin from food samples

Ethyl acetate represents the most frequently used solvent for lovastatin extraction from food samples. Thus, this solvent was employed for initial experiments with spiked lovastatin-free food samples. The formed ethyl acetate extract was subsequently evaporated to dryness, reconstituted in sample loading mixture (0.5 mM AmAc, pH 4/ACN; 90/10) and subjected to the optimized MISPE procedure. However, the obtained recovery was too low (<30%). Therefore, other solvents which also represent common extraction solvents for lovastatin had to be considered. Acetonitrile was used as an extraction agent for determination of lovastatin in various mushrooms by Chen et al. [32]. Finally, acetonitrile was preferred to methanol, because of its compatibility with the MISPE procedure. However, no significant improvement was observed when using acetonitrile in the same experiment as ethyl acetate. The drying step, high complexity of the real samples, and low content of organic solvent in the MISPE sample loading mixture appeared to be critical for the reconstitution of the dried extracts. Therefore, the formed acetonitrile extract was directly diluted with 0.5 mM AmAc buffer to form a sample that may be loaded onto the MIP sorbent without any other treatment. This procedure did not cause any loss of extraction recovery. To overcome the decrease in sensitivity of the UHPLC–MS/MS method caused by sample dilution, the evaporated elution fraction of the MISPE procedure was concentrated by reconstitution in lower volume (100 μl instead of 1 ml).

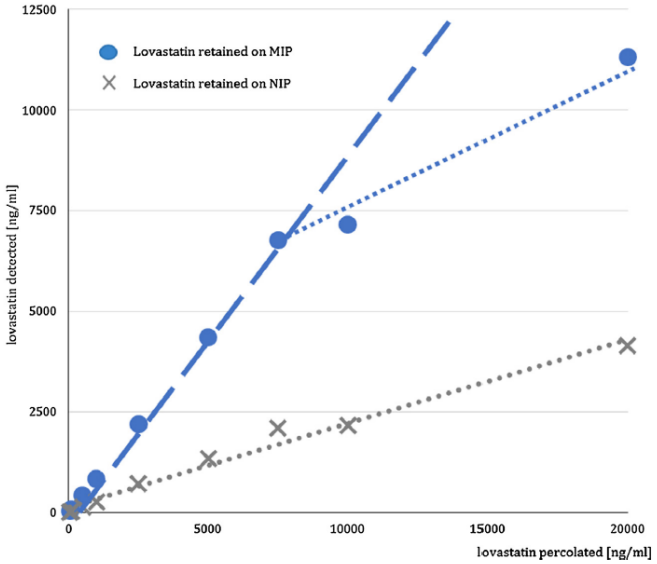

Fig. 6. Determination of the capacity of the MIP9a sorbent for lovastatin in standard samples.

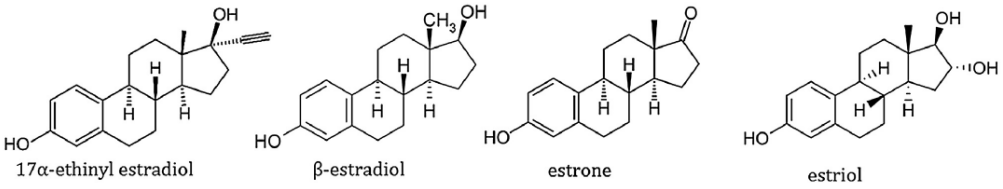

Fig. 7. Structures of estrogens used for additional evaluation of selectivity of the MISPE procedure.

3.4. Repeatability of the MIP procedure and repeatability of the MIP sorbent synthesis

The optimized MISPE procedure was characterized by very good repeatability expressed as RSD of the extraction recovery observed in standard medium (4.6%; n = 3) as presented in Fig. 3A and also in real media (≤5%; n = 3) as showed in Fig. 4. To evaluate repeatability of the synthesis and preparation of the resulting MISPE material, three different MIP sorbents were synthesized using the same procedure at three different days within two months by two different analysts. Then, three SPE procedures were performed on each of these three different MIPs (MIP9a, MIP9b and MIP9c). The MIP9 showed excellent repeatability of synthesis expressed as RSD value 7.7% (n = 9) of extraction recoveries evaluated in standard sample as demonstrated in Fig. 5. These experiments also revealed more non-specific interactions involved in MISPE extraction as the control NIP sorbents synthesized in triplicates showed increased extraction recovery of lovastatin.

3.5. Determination of capacity of the MIP sorbent

Capacity of the MIP sorbent was determined to assess its applicability in real sample analysis. The capacity represents the maximum amount of lovastatin that can be retained on the MIP material under optimized conditions without any decrease of extraction recovery when washing the sample. 1 ml of sample containing increasing concentration (50, 100, 500, 1000, 2500, 5000, 7500, 10 000 and 20 000 ng/ml) of lovastatin was percolated through the MIP sorbent (20 mg of material) to determine its capacity. Elution fractions of the higher concentrations had to be diluted to fit into the linear range of the UHPLC–MS/MS method. The curve plotted in Fig. 6 shows a linear part up to 7.5 μg when employing 20 mg of MIP material. Therefore, the resulting maximum capacity of the MIP material for lovastatin is 375 μg/g. The maximum amount of lovastatin observed in food samples is in the range of units of milligrams. As the food samples are subjected to acetonitrile extraction resulting in 10-fold dilution, the obtained capacity is sufficient for analysis of lovastatin commonly contained in tea, mushroom and red yeast rice samples. Anyway, samples with higher amount of lovastatin exceeding capacity of the MIP sorbent need to be subjected to a dilution because of linear range of the UHPLC–MS/MS method.

3.6. Selectivity of the MISPE procedure

Selectivity of the MISPE procedure was evaluated by comparing elution profiles obtained on MIP and on the control NIP. As presented in Fig. 3A, the resulting MIP material was characterized by

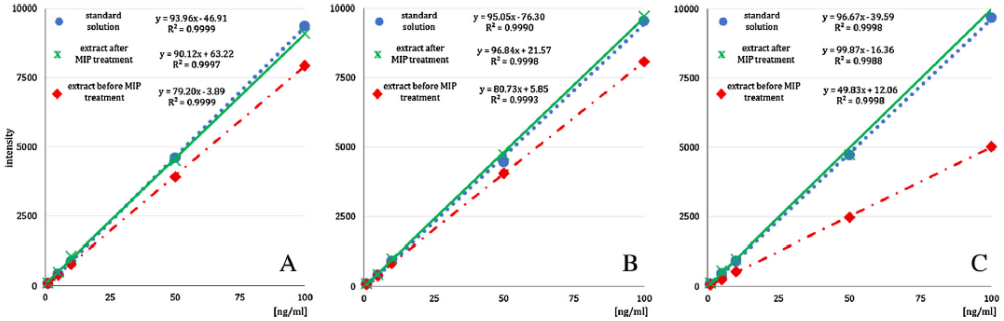

Fig. 8. Evaluation of matrix effects in oyster mushroom (A), Pu-erh tea (B) and red yeast rice (C) extracts by comparing slopes of calibration curves constructed in diluent mixture and in extracts before and after MISPE procedure.

Table 2
Validation of the MISPE-UHPLC–MS/MS in all three tested food matrices.

	Intra-day precision RSD (%)			Inter-day precision RSD (%)			Accuracy (%)		
	1 ng/ml	10 ng/ml	100 ng/ml	1 ng/ml	10 ng/ml	100 ng/ml	1 ng/ml	10 ng/ml	100 ng/ml
Pu-erh tea	7.1	3.4	3.2	13.1	9.2	5.1	105.6	94.2	94.2
Oyster mushroom	5.5	6.8	3.8	12.4	11.3	9.9	126.0	97.8	89.3
Red yeast rice	4.2	3.2	2.5	11.2	4.8	6.5	125.8	105.7	87.0

the ability to selectively recognize the target analyte. Extraction recoveries were 82% (RSD 4.6%; n = 3) obtained on the MIP and 32% (RSD 17.0%; n = 3) on the NIP. The elution profile of the control NIP material pointed on non-specific interactions taking part during the lovastatin retention. To confirm the selectivity of the MISPE procedure another approach was employed. Retention of different molecules characterized by close lipophilicity and acidity to the target analyte was studied. Estrogen derivatives were chosen for this purpose (Fig. 7, predicted by chemicalize.com): estriol, β-estradiol, 17α-ethinylestradiol, and estrone. Elution fractions obtained using the MISPE procedure (n = 3) developed for lovastatin were subsequently analysed by UHPLC–MS/MS method for the determination of estrogen steroids developed and validated by our group. BEH Phenyl (1.7 μm; 2.1 × 50 mm) stationary phase and mobile phase consisted of ACN and 0.01% NH_4OH solution were employed for chromatographic separation. Estriol was completely eliminated from the MIP sorbent during the MISPE procedure. Extraction recoveries of both β-estradiol and estrone were lower than 15% while 17α-ethinylestradiol was retained much more (43.8%, RSD = 7.6%). Extraction recovery obtained on NIP for lovastatin was close to that of 17α-ethinylestradiol on MIP. Thus, these results suggest that the MISPE procedure is selective for lovastatin.

3.7. Validation of the MISPE-UHPLC–MS/MS method, matrix effects, and clean-up efficiency

At first, repeatability of retention times and peak areas were evaluated at three concentration levels (5 ng/ml, 50 ng/ml, and 500 ng/ml) to perform system suitability test (SST). It was expressed as RSD (%) of ten measurements of the mixture of four statins. RSD of retention times was <0.2%, RSD of peak areas was <3.9% in the case of lactone forms, and <8.9% for the hydroxy-acid forms of statins. Due to the low amount of the target analyte observed in preliminarily examined food samples, it was necessary to evaluate also lower concentrations of lovastatin. Therefore, SST experiments were executed for lovastatin at 1 ng/ml, 10 ng/ml, and 100 ng/ml to evaluate the suitability of the UHPLC–MS/MS method. RSD of retention times was <0.2% and RSD of peak areas <4.3% for lovastatin observed at all three tested concentration levels. The resulting UHPLC-MS/MS method was validated in terms of linearity, limit of detection (LOD), and limit of quantification (LOQ) for all four statins whereas intra-day and inter-day precision, accuracy, and matrix effects were evaluated only for lovastatin. The resulting method provided excellent linearity expressed as correlation coefficient $r^2 > 0.9990$ in the range of 1–500 ng/ml for simvastatin, lovastatin and their interconversion products. LOQ was 1 ng/ml for lactone forms and 5 ng/ml for open hydroxy-acid forms of statins, and LOD 0.3 ng/ml and 1.5 ng/ml, respectively. Results of the experiments evaluating intra-day and inter-day precision and accuracy in Pu-erh tea, oyster mushroom, and red yeast rice are shown in the Table 2.

Matrix effects were evaluated by comparing the slope of calibration curve constructed in the diluent mixture with the slopes of calibration curves that were prepared by spiking the extracts of food matrices with increasing concentration level of lovastatin (1, 5, 10, 50, and 100 ng/ml). The first extract was treated by the MISPE procedure and the second one was only percolated through PTFE filter (0.22 μm). All the extracts without MIP treatment were affected by ion suppression and the deviation between the calibration curve slopes was in the case of red yeast rice 47% and 49% (one red yeast rice sample measured twice), oyster mushroom 16% and 11% and Pu-erh tea 17% and 15% (two different sample were measured). The examples of calibration curves constructed in food extracts are shown in Fig. 8. The matrix effects observed in all the studied food extracts treated by MISPE procedure were negligible. They were decreased under 5% in all three examined matrices as shown in Fig. 8.

3.8. Determination of lovastatin in food samples

Lovastatin was analysed in three dried oyster mushroom and fifteen Pu-erh tea samples that were bought in Czech food stores and Czech tearooms. The analysed mushroom samples were grown in the Czech Republic and no trace of lovastatin was detected. Regarding determination of lovastatin in Pu-erh tea, both raw and ripe and loose leaf and compressed forms were analysed, altogether 15 samples. Lovastatin was detected in four Pu-erh tea samples on and below LOQ. Limit of quantification of the method was 1 ng/ml

(i.e. 10 ng/g dry weight; because of the dilution by extraction solvent). Popularity and consumption of Pu-erh tea is getting higher worldwide. Therefore, it is very difficult to meet the demand for this special tea type when preparing it by traditional time consuming procedure. The unique flavour, taste, and also compound composition are formed during the storage that takes several years. To circumvent this time issue new procedures of Pu-erh tea manufacturing lasting only tens of days were developed. Nevertheless, the resulting tea differs from that prepared by traditional method. These facts could be the reason of the null or low amount of lovastatin in the tested samples. Moreover, positive effect of long-term storage on lovastatin content was demonstrated by Zhao et al. [20]. Dietary supplement containing red yeast rice powder was subjected to the determination of lovastatin to confirm the suitability of the resulting MISPE-UHPLC–MS/MS method. The declared amount of lovastatin (5 mg) was found in the sample. Because the MISPE-UHPLC–MS/MS method was developed and optimized to determine low amount (nanograms) of lovastatin, the dietary supplement had to be diluted to fit into the linear range of the UHPLC–MS/MS method.

4. Conclusions

Reliable, selective, sensitive and matrix effect-free UHPLC–MS/MS method was developed and validated for determination of lovastatin in food samples. Sample preparation step involved SPE extraction based on the molecularly imprinted polymers. MIP material brought important selectivity into this process, which is in great demand particularly for LC–MS analysis due to occurrence of matrix effect phenomenon. High repeatability of the MISPE procedure alongside with the complete matrix effect elimination allowed to reduce costs of the whole analysis because of no need for stable isotopically labelled internal standards and reusability of the MISPE sorbent. Synthesis of the MIP material was characterized by very good repeatability expressed as RSD = 7.7% (n = 9) of three extraction procedures that were performed on each of three different MIP sorbents prepared according to the same procedure within two months by two different analysts. The selectivity of the MISPE procedure was evaluated by comparing the elution profiles obtained on the MIP and on the corresponding control NIP. In addition, experiments with estrogen derivatives proved the selectivity of the resulting MIP sorbent in a different way. The determined capacity is sufficient for determination of lovastatin that is commonly found in food samples. The optimized MISPE procedure showed its efficiency in purification of the real matrix. Matrix effects evaluated by comparing slopes of the calibration curves were completely eliminated. Oyster mushroom and Pu-erh tea samples analysed by the resulting MISPE-UHPLC–MS/MS showed null or very low lovastatin amount. Suitability of the whole procedure was confirmed by analysis of a dietary supplement with declared amount of lovastatin.

Acknowledgements

The authors gratefully acknowledge the financial support of the Grant Agency of the Charles University (Grant No. 274216), Charles University, project SVV 260 412/2017 and project STARSS reg. no.: CZ.02.1.01/0.0/0.0/15_003/0000465 funded by ERDF.

References

[1] E.S. Istvan, Structural mechanism for statin inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, Am. Heart J. 144 (2002) 27–32, http://dx.doi.org/10.1067/mhj.2002.130300.
[2] A. Endo, K. Hasumi, HMG-CoA reductase inhibitors, Nat. Prod. Rep. 10 (1993) 541–550, http://dx.doi.org/10.1039/NP9931000541.
[3] J.A. Farmer, Pleiotropic effects of statins, Curr. Atheroscler. Rep. 2 (2000) 208–217, http://dx.doi.org/10.1007/s11883-000-0022-3.
[4] D. Pella, R. Rybar, V. Mechirova, Pleiotropic effects of statins, Acta Cardiol. Sin. 21 (2005) 190–198.
[5] J.K. Liao, U. Laufs, Pleiotropic effects of statins, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 89–118, http://dx.doi.org/10.1146/annurev.pharmtox.45.120403.095748.
[6] A.S. Wierzbickia, R. Poston, A. Ferro, The lipid and non-lipid effects of statins, Pharmacol. Ther. 99 (2003) 95–112, http://dx.doi.org/10.1016/S0163-7258(03)00055-X.
[7] S. Bellosta, N. Ferri, F. Bernini, R. Paoletti, A. Corsini, Non-lipid-related effects of statins, Ann. Med. 32 (2000) 164–176, http://dx.doi.org/10.3109/07853890008998823.
[8] M. Osmak, Statins and cancer: current and future prospects, Cancer Lett. 324 (2012) 1–12, http://dx.doi.org/10.1016/j.canlet.2012.04.011.
[9] S. Chuengsamarn, S. Rattanamongkoulgul, S. Suwanwalaikorn, S. Wattanasirichaigoon, L. Kaufman, Effects of statins vs. non-statin lipid-lowering therapy on bone formation and bone mineral density biomarkers in patients with hyperlipidemia, Bone 46 (2010) 1011–1015, http://dx.doi.org/10.1016/j.bone.2009.12.023.
[10] B.A. Golomb, M.A. Evans, Statin adverse effects: a review of the literature and evidence for a mitochondrial mechanism, Am. J. Cardiovasc. Drugs 8 (2008) 73–418, http://dx.doi.org/10.2165/0129784-200808060-00004.
[11] D.N. Kiortsis, T.D. Filippatos, D.P. Mikhailidis, M.S. Elisaf, E.N. Liberopoulos, Statin-associated adverse effects beyond muscle and liver toxicity, Atherosclerosis 195 (2007) 7–16, http://dx.doi.org/10.1016/j.atherosclerosis.2006.10.001.
[12] S. Bellosta, R. Paoletti, A. Corsini, Safety of statins focus on clinical pharmacokinetics and drug interactions, Circulation 109 (2004) 50–57, http://dx.doi.org/10.1161/01.CIR.0000131519 15067.1f.
[13] M.B. Bottorff, Statin safety and drug interactions: clinical implications, Am. J. Cardiol. 97 (2006) 27–31, http://dx.doi.org/10.1016/j.amjcard.2005.12.007.
[14] D.J. Yang, L.S. Hwang, Study on the conversion of three natural statins from lactone forms to their corresponding hydroxy acid forms and their determination in Pu-Erh tea, J. Chromatogr. A 1119 (2006) 277–284, http://dx.doi.org/10.1016/j.chroma.2005.12.031.
[15] L. Nováková, D. Šatínský, P. Solich, HPLC methods for the determination of simvastatin and atorvastatin, Trends Anal. Chem. 27 (2008) 352–367, http://dx.doi.org/10.1016/j.trac.2008.01.013.
[16] T.P. Stossel, The discovery of statins, Cell 134 (2008) 903–905, http://dx.doi.org/10.1016/j.cell.2008.09.008.
[17] S.M. Samiee, N. Moazami, S. Haghighi, F.A. Mohseni, S. Mirdamadi, M.R. Bakhtiari, Screening of lovastatin production by filamentous fungi, Iran. Biomed. J. 7 (2003) 29–33.
[18] S. Goswami, A.S. Vidyarthi, B. Bhunia, T. Mandal, A review on lovastatin and its production, J. Biochem. Technol. 4 (2012) 581–587.
[19] R.S. Upendra, P. Khandelwal, Z.R. Amiri, L. Shwetha, M.S. Ausim, Screening and molecular characterization of natural fungal isolates, J. Microb. Biochem. Technol. 5 (2013) 25–30, http://dx.doi.org/10.4172/1948-5948.1000094.
[20] Z.-J. Zhao, Y.-Z. Pan, Q.-J. Liu, X.-H. Li, Exposure assessment of lovastatin in Pu-erh tea, Int. J. Food. Microbiol. 164 (2013) 26–31, http://dx.doi.org/10.1016/j.ijfoodmicro.2013.03.018.
[21] L.K. Lee, K.Y. Foo, Recent advances on the beneficial use and health implications of Pu-Erh tea, Food Res. Int. 53 (2013) 619–628, http://dx.doi.org/10.1016/j.foodres.2013.02.036.
[22] H.-P. Lv, Y.-J. Zhang, Z. Lin, Y.-R. Liang, Processing and chemical constituents of Pu-erh tea: a review, Food Res. Int. 53 (2013) 608–618, http://dx.doi.org/10.1016/j.foodres.2013.02.043.
[23] K.-Ch. Jeng, Ch.-S. Chen, Y.-P. Fang, R.-W.Ch. Hou, Y.-S. Chen, Effect of microbial fermentation on content of statin, GABA, and polyphenols in Pu-Erh tea, J. Agric. Food Chem. 55 (2007) 8787–8792, http://dx.doi.org/10.1021/jf071629p.
[24] J. Ma, Y. Li, Q. Ye, J. Li, Y. Hua, D. Ju, D. Zhang, R. Cooper, M. Chang, Constituents of red yeast rice, a traditional chinese food and medicine, J. Agric. Food Chem. 48 (2000) 5220–5225, http://dx.doi.org/10.1021/jf000338c.
[25] M. Journoud, P.J.H. Jones, Red yeast rice: a new hypolipidemic drug, Life Sci. 74 (2004) 2675–2683.
[26] H.-N. Huang, Y.-Y. Hua, G.-R. Bao, L.-H. Xie, The quantification of monacolin K in some red yeast rice from fujian province and the comparison of the other product, Chem. Pharm. Bull. 54 (2006) 687–689, http://dx.doi.org/10.1248/cpb.54.687.
[27] A. Mornar, M. Sertic, B. Nigovic, Development of a rapid LC/DAD/FLD/MSn method for the simultaneous determination of monacolins and citrinin in red fermented rice products, J. Agric. Food Chem. 61 (2013) 1072–1080, http://dx.doi.org/10.1021/jf304881g.
[28] Y.-G. Li, F. Zhang, Z.-T. Wang, Z.-B. Hu, Identification and chemical profiling of monacolins in red yeast rice using high-performance liquid chromatography with photodiode array detector and mass spectrometry, J. Pharm. Biomed. Anal. 35 (2004) 1101–1112, http://dx.doi.org/10.1016/j.jpba.2004.04.004.
[29] K. Deepalakshmi, S. Mirunalini, Pleurotus ostreatus: an oyster mushroom with nutritional and medicinal properties, J. Biochem. Technol. 5 (2014) 718–726.
[30] Y. Patel, R. Naraian, V.K. Singh, Medicinal properties of pleurotus species (Oyster mushroom): a review, World J. Fungal Plant Biol. 3 (2012) 1–12, http://dx.doi.org/10.5829/idosi.wjfpb.2012.3.1.303.

[31] N. Gunde-Cimerman, A. Comerman, Pleurotus fruiting bodies contain the inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme a reductase-lovastatin, Exp. Mycol. 19 (1995) 1–6, http://dx.doi.org/10.1006/emyc.1995.1001.
[32] S.-Y. Chen, K.-J. Ho, Y.-J. Hsieh, L.-T. Wang, J.-L. Mau, Contents of lovastatin, g-aminobutyric acid and ergothioneine in mushroom fruiting bodies and mycelia, LWT-Food Sci. Technol. 47 (2012) 274–278, http://dx.doi.org/10.1016/j.lwt.2012.01.019.
[33] A. Cappiello, G. Famiglini, P. Palma, H. Trufelli, Matrix effects in liquid chromatography-mass spectrometry, J. Liq. Chromatogr. Relat. Technol. 33 (2010) 1067–1081, http://dx.doi.org/10.1080/10826076.2010.484314.
[34] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1217 (2010) 3929–3937, http://dx.doi.org/10.1016/j.chroma.2009.11.060.
[35] A. Furey, M. Moriarty, V. Bane, B. Kinsella, M. Lehane, Ion suppression; A critical review on causes, evaluation, prevention and applications, Talanta 115 (2013) 104–122, http://dx.doi.org/10.1016/j.talanta.2013.03.048.
[36] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, Mass Spectrom. Rev. 30 (2011) 491–509, http://dx.doi.org/10.1002/mas.20298.
[37] I. González-Marino, J. Be. Quintana, I. Rodríguez, R. Rodil, J. González-Penas, R. Cela, Comparison of molecularly imprinted, mixed-mode and hydrophilic balance sorbents performance in the solid-phase extraction of amphetamine drugs from wastewater samples for liquid chromatography-tandem mass spectrometry determination, J. Chromatogr. A 1216 (2009) 8435–8441, http://dx.doi.org/10.1016/j.chroma.2009.09.069.
[38] V. Pichon, F. Chapuis-Hugon, Role of molecularly imprinted polymers for selective determination of environmental pollutants—a review, Anal. Chim. Acta 622 (2008) 48–61, http://dx.doi.org/10.1016/j.aca.2008.05.057.
[39] H. Vlčková, P. Svoboda, O. Novák, P. Solich, L. Nováková, Development of MEPS-UHPLC–MS/MS multistatin methods for clinical analysis, Bioanalysis 8 (2016) 333–349, http://dx.doi.org/10.3390/ijms12095908.
[40] X.-J. Wang, Z.-L. Xu, J.-L. Feng, N.-C. Bing, Z.-G. Yang, Molecularly imprinted membranes for the recognition of lovastatin acid in aqueous medium by a template analogue imprinting strategy, J. Membr. Sci. 313 (2008) 97–105, http://dx.doi.org/10.1016/j.memsci.2007.12.067.