Journal of Chromatography A, 1217 (2010) 7956–7965

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry

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ARTICLE INFO

Article history:
Available online 16 July 2010

Keywords:
Isoflavones
Sea algae
Supercritical fluid extraction
Modifier
Sonication pretreatment
Fast chromatography
Tandem mass spectrometry

ABSTRACT

New hyphenated technique for the extraction and determination of isoflavones in sea and freshwater algae and cyanobacteria was developed. The method consists of sonication sample pretreatment, extraction by supercritical CO_2 modified by 3% (v/v) of MeOH/H_2O mixture (9:1, v/v) at 35 MPa and 40 °C for 60 min, fast chromatography analysis by the means of Agilent 1200 Series Rapid Resolution and MS/MS determination. Agilent 1200 Series RRLC was used with Zorbax SB-CN chromatographic column (100 mm × 2.1 mm, particle size 3.5 μm), 3 μl injection volume, mobile phase consisting of 0.2% (v/v) acetic acid in water (solvent A) and acetonitrile (solvent B) and used with linear gradient (30% B at 0 min, from 0 min to 3 min up to 50% B, from 3 to 6 min up to 80% B and from 6 to 10 min down to 30% B). The flow-rate was 0.4 mL/min, column oven temperature 35 °C. MS detector Agilent Technologies 6460 Triple quadrupole LC/MS with Agilent Jet Stream was used in a negative ESI mode under following conditions: gas temperature 350 °C, gas flow 13 L/min, nebulizer gas pressure 50 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min, capillary voltage was 4 kV. Samples were analysed in the multiple reaction monitoring (MRM) mode. Eight isoflavone compounds were found for the first time in seven real samples of sea algae and in three control samples of freshwater algae and cyanobacteria. Usual optimisation study of extraction parameters was performed. Pressure and temperature optima for algae matrix are different from those obtained sooner for other matrices for most of the analytes, but the results of modifier optimisation study are in good accordance with those obtained sooner for spiked samples and red clover matrix. It seems that matrix has very small or no effect on the modifier selection. Two different approaches of sonication pretreatment were tested: sonication bath and the thorn instrument. In longer extraction time experiments, thorn sonication was more efficient and recovery of following supercritical fluid extraction was higher.

1. Introduction

Supercritical fluids are known since 1869 (Andrews) and used in analytical chemistry since 1958 [1]. For a long time, supercritical CO_2 has been used to lower the amount of caffeine in coffee and for industrial-scale extractions of hops and coffee [2]. Thanks to new instrumentation, many analytical-scale applications emerged in 1980s and 1990s [3], mostly in the area on non-polar analytes. This is considered the time of "rise and fall" of supercritical fluids, because when the scientist found that these solvents are either less suitable for high-polarity analytes (CO_2) or too dangerous for common use (N_2O); their initial enthusiasm faded away, as new interesting extraction methods were developed.

However, supercritical CO_2 as a solvent has several still unequalled advantages. Supercritical fluids have dual character—some of their qualities (viscosity, diffusivity) are close to gases, while their solvation power is close to liquids [1]. Thanks to low viscosity and high diffusivity, supercritical fluids can offer both high solvation power and favourable conditions for the mass transfer. Higher speed of transport process, enabled by low viscosity, higher diffusion coefficient and zero surface tension make supercritical fluids very strong extraction agents [4]. In the supercritical part of phase diagram, solvation power of the extraction medium can be changed fluently by the means of extraction pressure, temperature and fluid density, and thus – together with modifier selection – the selectivity of extraction is widely adjustable. Final extracts are clean, low volume and usually do not require complicated cleanup and preconcentration.

Abbreviations: Di, Daidzin; Glyi, Glycitin; Geni, Genistin; Ono, Ononin; Dai, Daidzein; Gly, Glycitein; Sis, Sissotrin; For, Formononetin; Bio, Biochanin A.

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doi:10.1016/j.chroma.2010.07.020

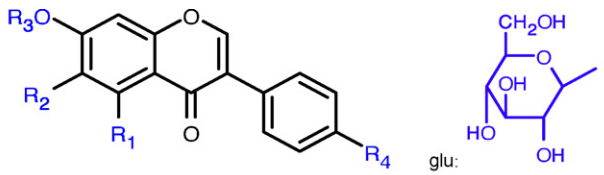
CO_2 is cheap, easy to get in proper purity and it does not create toxic waste like organic solvents. Gaseous CO_2 returns to the atmosphere, where it is plentiful and from where it is recycled easily for another use.

For several decades, studies are performed to find how to use the supercritical CO_2 as a efficient solvent for polar compounds. First of all, a good modifier is necessary. It is known since 1987 that a small amount of MeOH (3.5 mol.% in the extraction fluid) can enhance the solubility of 2-aminobenzoic acid to 620% [5]. Together with the modifier, another substances called entrainers can help a lot. Entrainer substitutes the molecules of analyte, bound in the active centra of matrix, and thus speeds the mass transfer from matrix to the extraction fluid. Trace amount of water seems to be a good entrainer for most soil or plant samples [6]. Nowadays, many types of polar compounds, like lignans, imidazoles, isoflavones [7–9], benzo[c]phenantridine alkaloids [10], phenolic compounds [11] and amino acids [12] are known to be extractable well by the means of supercritical fluids.

Supercritical fluid extraction (SFE) of polar compounds had to make several compromises. Usual consumption of organic solvents went from several mL to several tens mL, what is still very environmental friendly in comparison to liquid extraction methods, but hardly good in comparison with SFE of non-polar compounds. Higher flow-rates are used and the extraction time is usually 40–120 min for polar analytes and real samples instead of ca. 10–15 min we used to get for non-polar analytes and spiked samples. Polar analytes usually don't have problems with stripping effect and the extracts are pre-concentrated on the rotation vacuum evaporator prior to the analysis. The loss of analytes is minimal, but it is another labor-intensive step of sample processing.

In spite of these small disadvantages, in many cases SFE still is the best alternative for the extraction of trace amounts of analytes from complex real matrices, especially thanks to the unique combination of solvation power and adjustable selectivity, that can be further enhanced by proper pretreatment of the sample, like sonication or long-time passive exposition to the entrainer prior to the extraction. Together with modern powerful and sensitive analytical and detection methods, like fast high-pressure liquid chromatography with tandem mass spectrometry detection (HPLC MS/MS), it can be used to look for interesting compounds in matrices, containing very small amount of analytes that however play an important role in the metabolism of the sample specie. Thus, with a large set of powerful tools used together, we can investigate unexplored areas along the metabolic pathways of trace-concentration substances. One such area are isoflavones in lower plants. The aim of this work is to prove the presence of isoflavone compounds in algae by the means of sonication sample pretreatment, supercritical fluid extraction and rapid resolution HPLC with MS/MS detection (structure of isoflavone analytes of interest is given in Fig. 1).

Nowadays, there is growing interest in research, development and commercialization of functional food ingredients, nutraceuticals and dietary supplements. These ingredients are preferred by consumers to have a natural origin (i.e. non-synthetic origin) and are commonly extracted from natural sources, such as plants and other organisms, like algae and microalgae [13]. Isoflavones are a component of functional food and are used in pharmaceutical industry. These phytohormones have also antioxidation effect and are considered beneficial to human health in appropriate concentrations. It is known that aglycones are more rapidly adsorbed than glycosides [14]. In many dietary supplements and extracts, the percentage of aglycones varies in the range 2–15% of total isoflavones, but some samples contain even over 85% of aglycones [15]. On the other hand, farmers call for *Fabaceae* plants with much lower content of isoflavones, as these compounds cause reproduction troubles for the cattle.



Isoflavones		R_1	R_2	R_3	R_4
daidzin	daidzein-7-O-β-D-glucopyranoside	H	H	glu	OH
glycitin	glycitein-7-O-β-D-glucopyranoside	H	OCH_3	glu	OH
genistin	genistein-7-O-β-D-glucopyranoside	OH	H	glu	OH
daidzein	4,7-dihydroxyisoflavone	H	H	H	OH
ononin	formononetin-7-O-β-D-glucopyranoside	H	H	glu	OCH_3
glycitein	4,7-dihydroxy-6-methoxyisoflavone	H	OCH_3	H	OH
genistein	4,5,7-trihydroxyisoflavone	OH	H	H	OH
sissotrin	biochanin A-7-O-β-D-glucopyranoside	OH	H	glu	OCH_3
formononetin	7-hydroxy-4'-methoxyisoflavone	H	H	H	OCH_3
biochanin A	5,7-dihydroxy-4'methoxyisoflavone	OH	H	H	OCH_3

glu: glucopyranosyl

Fig. 1. Structure of isoflavones.

From matrices containing large amount of isoflavones, extracts are usually prepared by the means of Soxhlet extraction, IKA – Soxhlet or sonication, but also SFE was used for the extraction of isoflavones and similar compounds, such as phenolics [16,17]. Nowadays, when the sample preparation often takes a key role in the whole analysis, combinatory and hyphenated techniques are of growing importance, because they often are the only way how to achieve good extraction efficiency and required selectivity at the same time [18], and there already were several attempts to apply hyphenated extraction methods to isoflavone-rich matrices, although the methods were different [19,20]. SFE was already used for both for the extraction of phenolics from a very similar matrix [11] and for the extraction of this type of analytes from more analyte-rich matrices [7–9], but this is the first usage of hyphenated sonication/SPE/SFE method to trace concentrations of isoflavones in algae and cyanobacteria.

There was a good reason to expect the presence of isoflavones in algae and cyanobacteria, because their precursors from the beginning of the metabolic pathway: cinamic acid and p-coumaric acid plus a group of their metabolites were found in freshwater algae and cyanobacteria [11,21]. These phenolic compounds are an important group of antioxidants and one of most wanted component of functional food and food additives. The presence of isoflavones is another logic step along the metabolic pathway. Since we proved in this work that isoflavones are contained in lower plants, we should map their occurrence in all matrices that could possibly act as functional food sources.

2. Materials and methods

2.1. Real samples

Sea algae samples (*Sargassum muticum*, *Sargassum vulgare*, *Hypnea spinella*, *Porphyra* sp., *Undaria pinnatifida*, *Chondrus crispus* and *Halopytis incurvus*) were obtained from the Laboratory of Foodomics, Institute of Industrial Fermentations, Madrid, Spain. Freshwater algae and cyanobacteria samples (*Spongiochloris spongiosa*, *Scenedesmus* and *Nostoc 17*) were obtained from the Department of Autotrophic Microorganisms, Institute of Microbiology, Třeboň, Czech Republic. All real samples were obtained in lyofilized and homogenized form in more than sufficient quantity.

2.2. Chemicals, standards and solvents

HPLC-grade methanol and other organic solvents were obtained from Merck (Prague, CZ). Isoflavones and all other reagents of

ACS purity were purchased from Sigma–Aldrich (Prague, CZ). The stock standard solutions of isoflavones at 10 μg mL^{-1} were prepared in aqueous methanol (1:1, v/v) and stored in the dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions with the aqueous methanol. Except the test of purity of SFE extracts, all solutions were filtered through a 13 mm × 0.45-μm nylon filter (Cronus Filter, Gloucester, UK) prior to HPLC separations. Technical gasses in cylinders (carbon dioxide, nitrogen) were obtained from Siad, Braňany u Mostu, CZ. All other chemicals were of analytical grade.

2.3. Sample pretreatment—sonication

Sample weight was ca. 0.1 g and it was kept in the range 0.1000–0.1020 g. All samples of algae and cyanobacteria were exposed to 300 μL of SFE modifier mixture (MeOH:H_2O 1:9, v/v) for ca. 16 h. Sea algae samples do not require this, because the liquid penetrates the particles of matrix immediately, but for the freshwater algae and cyanobacteria samples the process of complete moisturising is quite slow and might require an hour or two. Because of that, sufficient amount of fresh samples in Eppendorf vials was prepared each afternoon for the next day use. Samples wet with modifier were exposed to sonication for 30 min in the sonic bath (Kraintek, Podhájska, SK) or by the means of the thorn sonication device (Sonopuls, Bandelin Electronic, Germany). Time of exposition to the thorn sonication was optimised for real samples. For sonic bath, the influence of sonication time to extraction recovery is already known from previous experiments. Because of the results obtained for thorn sonication, limited verification series of experiments ($n = 3$ only) were performed for $t = 30$, $t = 45$ and $t = 60$ min with current matrices, but the prolongation of sonication time over 30 min did not cause higher recovery from real samples.

2.4. Supercritical fluid extraction

Solution of isoflavones used for spiked samples was prepared in the laboratory by mixing 1 mg of each compound in aqueous methanol (1:1, v/v) and spiked onto the cleaned algae matrix. Cleaned algae matrix was prepared from real algae sample (*C. crispus* and *S. spongiosa*, data in tables are from *Chondrus* experiments) via extraction of full cell of sample at very high flow-rate (very short restrictor, no analyte trapping), until the concentration of monitored analytes in the matrix decreased under the quantification limit. Matrix was spiked with standard solution and aged for at least 14 days. This matrix was selected because the optimisation studies for spiked glass wool were already performed in previous paper [9] and would not give us any information about the matrix effect. Plus, the aim of the work was the optimisation for algae matrix.

0.1 g of algae or cyanobacteria dust was weighed into a PE Eppendorf vial, 300 μL of SFE modifier mixture was added, and the sonication pretreatment was performed. Then the vial was opened or unsealed and matrix was quantitatively transferred into a clean 0.7-mL extraction cartridge (stainless steel), sealed with a 0.45-μm nylon filter at one end. All samples contained 300 μL of in situ modifier in cartridge (300 μL of solvent is the lowest amount of liquid with which the sonication of 0.1 g sample could be performed and the liquid was transferred quantitatively with the sample).

Blank samples were also prepared by extracting the same amounts of the cleaned matrix. No analytes were found in the blank matrix in concentration over the limit of quantification and there were not higher isoflavone residua found in the instrument after the extractions. Small, but measurable isoflavone residua accumulated in the extraction cartridge, which therefore had to be cleaned in sonic bath after each experiment or extracted again in SFE to get rid of them.

All the extractions were carried out using SE-l instrument (SEKO-K Ltd., Brno, CZ), upgraded with a valve for continuous modifier addition (Rheodyne, IDEX, Germany, 300-μL loop size). The extractions were performed under following conditions: $T = 35–75$ °C, $p = 10–40$ MPa, extraction medium was SFC/SFE grade CO_2 (Siad, Braňany u Mostu, CZ) with 3% (v/v) of the modifier mixture added via the injection valve to the capillary between instrument piston pump and the extraction cell. Thanks to this small change of instrumentation, modifier can't now condense in the piston micropump space and is completely rinsed to the extraction cell. The volume of modifier added after each pump filling is now limited only by parameters of restrictor that could be plugged by too much liquid.

The modified supercritical fluid went through the extraction cartridge with the sample for 60 min (dynamic extraction) and was depressurized via fused silica restrictor (12 cm, 50 μm ID, flow-rate 750–850 mL min^{-1}, MicroQuarz, Munich, Germany) into a liquid trap—a glass flask containing 25 mL of MeOH at the laboratory temperature. About 10 mL of MeOH is necessary for quantitative trapping of isoflavones, but due to the ca. 800 mL min^{-1} flow-rate in this case a 50-mL glass flask with a flexible foil seal is used as a trapping vial to prevent the solvent splashing out of the vial, and 25 mL is the minimum volume necessary to create efficient solvent column. On the other hand, these analytes are non-volatile and can be easily pre-concentrated in the rotary vacuum evaporator at 40 °C and dissolved in 0.5 mL of mobile phase before the fast chromatography/MS–MS analysis.

The common practice is that all obtained extracts are filtered through a nylon disc filter (0.45-μm × 13 mm, LUT syringe filters, Cronus, Gloucester, UK) prior to injections into the HPLC system. In our case, one serie of extracts was not treated this way and analysed especially with a focus to the level of ballast compounds. The extracts are clean enough to be used comfortably without the cleanup, but the effect of filtration is still measurable. Thus, the analyst can choose between lower cost of the analysis and even higher protection of the chromatographic column.

2.5. Fast chromatography and MS/MS

2.5.1. Instrumentation

An Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) consisted of an on-line degaser, a binary pump, a high performance SL autosampler, a thermostated column compartment, a photodiode array UV–vis detector. The system was coupled on-line to an MS detector Agilent Technologies 6460 Triple quadrupole LC/MS with Agilent Jet Stream.

2.5.2. Fast chromatography

The Zorbax SB-CN chromatographic column (100 mm × 2.1 mm, particle size 3.5 μm Agilent Technologies, USA) was used. The injection volume was 0.2–5 μl for standard solutions and 3 μl for real sample extracts. The mobile phase consisted of 0.2% (v/v) acetic acid in water (solvent A) and acetonitrile (solvent B). A linear gradient elution was applied: 30% B at 0 min, from 0 to 3 min up to 50% B, from 3 to 6 min up to 80% B and from 6 to 10 min down to 30% B. The column was equilibrated prior to injection of the next sample with mobile phase containing 30% solvent B (v/v) for 2 min. The flow-rate was 0.4 mL/min and 3 μL aliquots were injected into the column typically. The column oven temperature was set at 35 °C.

A gradient profile for simultaneous separation of isoflavones was selected. For a complete separation of all eight compounds, various concentrations of acetic acid and formic acid as mobile phases were tested. 0.2% (v/v) acetic acid proved to show the best separation efficiency. The selection of this type of column and mobile phase is advantageous also for simultaneous separation of other precursors, like liquiritigenin and naringenin, that are in relation

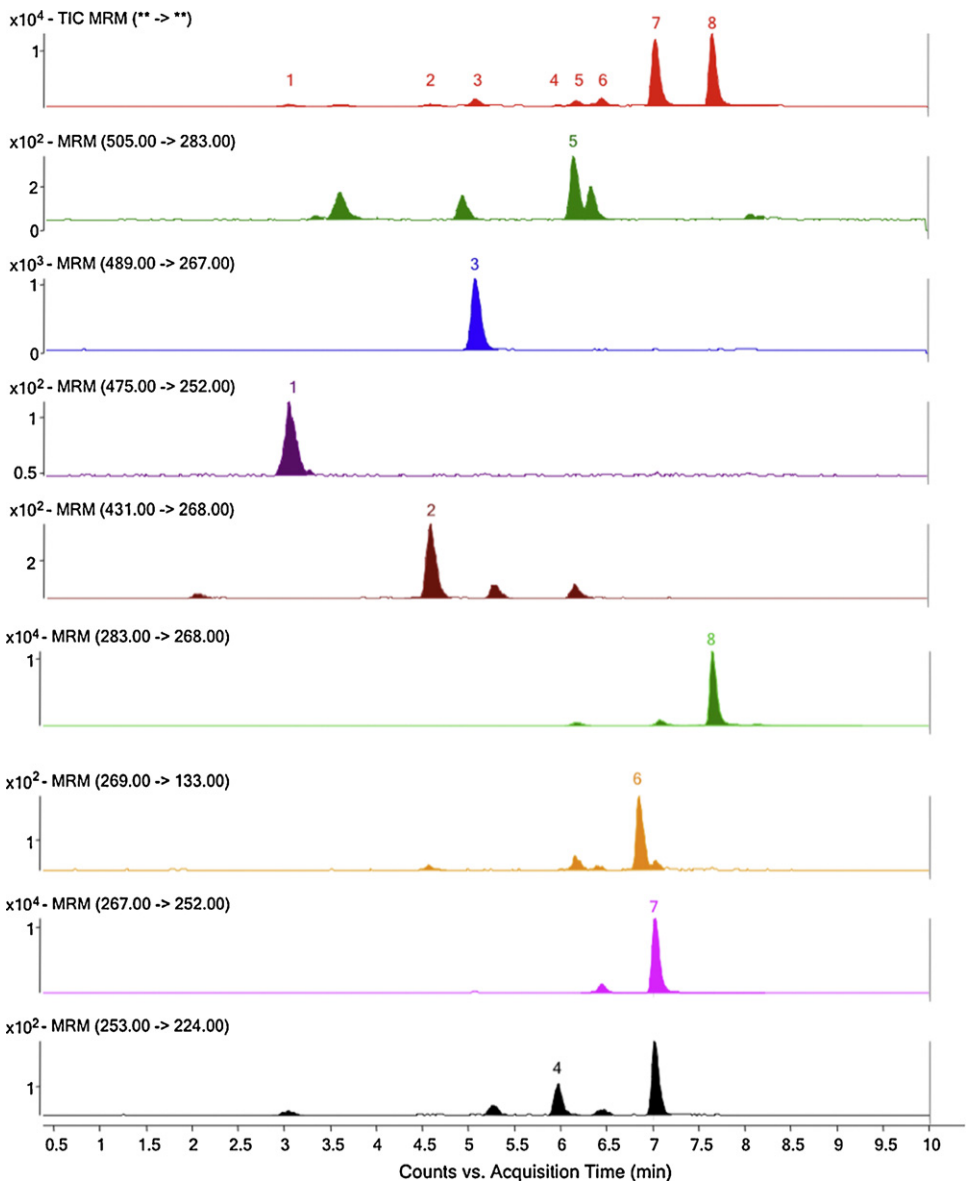


Fig. 2. MRM chromatogram of real sample – Chondrus, sea alga. Peak identification: 1. daidzin, 2. genistin, 3.ononin, 4.daidzein, 5. sissotrin, 6. genistein, 7. formononetin, and 8. biochanin A.

with monitored group of analytes. However, data on these compounds are not presented in the paper.

Retention times and characteristics of individual peaks (peak symmetry, resolution and others) are given in Table 2, as well as corresponding equations for their calculation. Very good resolution, selectivity and symmetry of the peaks were observed for all the compounds of interest. Peak half widths ranged from 0.11 to 0.14 min and peak symmetry varied in the range 0.85–1.07. Parameters of calibration curves in the concentration interval 0.5–2 ng/ml and corresponding limits of detection (LOD for 3 S/N) and limits of quantification (LOQ for 10 S/N) are given in Table 2. The best values of LOD and LOQ were found for biochanin A (LOD = 0.06 ng/ml) and formononetin (LOD = 0.08 ng/ml). Values of correlation coefficients of the calibration straight lines R^2 were in the interval 0.9998–0.9999. Isoflavones were eluted in the same order as in Fig. 2.

2.5.3. Triple quadrupole mass spectrometry

The column effluent was directly introduced into a triple quadrupole mass detector operated in a negative ESI mode. Nitrogen was used as both the drying and sheath gas and collision gas as well. The ESI source parameters were as follows: gas temperature 350 °C, gas flow 13 L/min, nebulizer gas pressure 50 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min, capillary voltage was 4 kV. Fragmentor voltage and collision energy was selected for each compound individually and ranged from 100 to 150 V and 10 to 30 eV, respectively (see Table 1). Samples were analyzed by fast chromatography–MS/MS in the multiple reaction monitoring (MRM) mode to maximize sensitivity. Characteristic transitions (precursor ion → product ion) are shown in Table 1.

2.6. Accuracy, precision, and recovery

Accuracy, precision, and recovery of the determination of the individual isoflavones (daidzin, glycitin, sissotrin, genistin, ononin, daidzein, glycitein, genistein, formononetin and biochanin A) were evaluated with the cleaned matrix spiked with isoflavone standards, the same that we used for optimisation studies, but freshly spiked with isoflavone solutions to avoid the matrix effect. Long-term exposed samples could create matrix–analytes interactions,

Table 1
MRM transition parameters (negative ESI mode).

Compound	Transition	Precursor ion → product ion[a]	Fragmentation [V]	Collision energy [eV]
Daidzin	475 → 252	$[M\text{-}H + CH_3COOH]^- \rightarrow [M\text{-}glc]^{-\cdot}$	100	30
Genistin	431 → 268	$[M\text{-}H]^- \rightarrow [M\text{-}glc]^{-\cdot}$	150	30
Daidzein	253 → 224	$[M\text{-}H]^- \rightarrow [M\text{-}H\text{-}CHO]^{-\cdot}$	150	20
Ononin	489 → 267	$[M\text{-}H + CH_3COOH]^- \rightarrow [M\text{-}glc]^{-\cdot}$	100	10
Sissotrin	505 → 283	$[M\text{-}H + CH_3COOH]^- \rightarrow [M\text{-}glc]^{-\cdot}$	100	10
Genistein	269 → 133	$[M\text{-}H]^- \rightarrow [^{0,3}B^-]$ (r.D.A.)	135	30
Formononetin	267 → 252	$[M\text{-}H]^- \rightarrow [M\text{-}H\text{-}CH_3]^{-\cdot}$	135	10
Biochanin A	283 → 268	$[M\text{-}H]^- \rightarrow [M\text{-}H\text{-}CH_3]^{-\cdot}$	135	10

[a] Nomenclature and some fragmentation patterns are used in accordance with data given in literature [22].

Table 2
Quantitative data obtained by RRLC–MS/MS ($n = 6$); MRM.

R.T. (min)	Compound	Regression equation[a]	R^2[b]	LOD[c] (ng/ml)	LOQ[d] (ng/ml)	RSD[e] (%)	LOD[f] (fmol)
3.03	Daidzin	$6764.93x - 60.74$	0.9998	1.81	6.02	0.32	4.36
4.57	Genistin	$9553.44x - 33.39$	0.9998	1.17	3.88	0.25	2.71
5.06	Ononin	$44683.91x - 163.05$	0.9998	0.25	0.63	0.21	0.58
5.97	Daidzein	$18701.90x - 15.82$	0.9998	0.62	2.05	0.27	2.45
6.17	Sissotrin	$15518.88x - 10.15$	0.9999	0.17	0.55	0.23	0.59
6.85	Genistein	$14875.17x - 39.22$	0.9998	0.63	2.09	0.26	2.34
7.03	Formononetin	$120819.16x - 12.00$	0.9998	0.08	0.26	0.18	0.03
7.65	Biochanin A	$184366.68x - 732.51$	0.9998	0.06	0.19	0.16	0.02

[a] Column: Zorbax SB CN, 100 mm × 2.1 mm (3.5 μm particle size). Concentration range 0.2–5 ng ml^{-1} was used for calibration.
[b] Regression coefficients.
[c] Limit of detection (3 S/N).
[d] Limit of quantification (10 S/N).
[e] Relative standard deviation ($n = 6$).
[f] Limit of detection per column injection (1 μl).

analytes could be trapped in active centra of the matrix or can simply move deep into the particles, from where they can be extracted less easily. This can be avoided either by long-enough ageing period, after which the analyte–matrix interaction is balanced in the whole sample, or by fresh spike, that gives minimum time for these interactions to be created. In our case, cleaned matrix was moisturised like any normal sample and the spike of analytes was added to the eppendorf vial directly before the sonication.

Retention times and regression equations are given in Table 2. All regression coefficients were 0.9998, except for Sis (0.9999). LODs and LOQs varied in the range 0.06–1.81 and 0.19–6.02 ng/ml, respectively. These results are comparable with previous method. For the determination, LODs and LOQs in fmol per injection were used, that are well comparable [7]. Some values were improved apparently—this is caused mostly by new sensitive instrumentation, some values are the same in average, with usual statistic variation. All other data about fast chromatography–MS/MS are given in Table 2. RSDs of the intra-day assay were determined from series of same experiments ($n = 6$) prepared on SFE, analysed via fast chromatography MS/MS with $n = 6$ each. The inter-day precision was determined by six analyses of experiments performed under the same conditions during a 5-day period. Accuracy was evaluated by comparing the determined concentrations with the known concentrations of the individual isoflavones. Inter-day and intra-day RSDs of fast chromatography MS/MS analysis varied in the range 1.54–3.76% and 0.98–3.68%, respectively. RSDs for combined SFE + fast chromatography MS/MS experiments look also very good, max. 2.27 and 2.26%, respectively, but this is caused mostly by a large volume of data from which this results are calculated. In electronic form of the paper, all data are given in Appendix in Tables 8 and 9.

3. Results and discussion

3.1. Optimisation study of SFE conditions

The circle of optimisation studies was performed two times. First of all, small series of preliminary experiments were performed, based on best experimental conditions known for red clover matrix [9]. Although the matrix effect is considerable in SFE and the method is not universal, both matrices are dry lyophilised plant material and thus are quite similar. Preliminary experiments were repeated only once or twice ($n = 2$, $n = 3$) and used to refine the presumption of best experimental conditions for algae matrix.

Under the conditions obtained in the preliminary optimisation experiments, thorough optimisation studies ($n = 6$) were performed. With this experimental approach, the danger of performing optimisation of one parameter using a set of not-yet-optimised parameters was avoided, because it was already proved that with a mild change of other parameters the whole optimisation can be shifted for most of the analytes. Thus the optimisation study was performed mostly as a verification of proper conditions selection.

For all 10 monitored analytes, the best extraction pressure is in the range 30–40 MPa (40 MPa is max. working pressure of the instrument). For Di, Glyi, Dai and For, the pressure optimum is 35 MPa. Gly and Gen prefer 30 MPa, while Geni, Ono, Sis and Bio require 40 MPa to give the best recovery. Although the differences of recovery for individual pressure values are quite low, this is not just a statistic fluctuance, there seem to be small but steady increase or decrease of recovery with the pressure (see Table 3, best recovery values are given in bold). For the whole group of analytes, 35 MPa were selected as the best pressure condition.

There is a question, why the pressure affects the recovery of certain analytes exactly the way that was observed. Basicly, there are two simple factors, that can influence the extraction efficiency: analyte solubility and extraction fluid flow-rate. Under optimum analyte solubility conditions, the mass transfer process would occur as quick as possible and the analytes would be taken from the matrix to the extraction fluid the most effective way. Under maximum pressure and constant restrictor length, the extraction fluid is carried out from the extraction cell as quickly as possible. This way, the concentration of analyte in the extraction fluid in the cell is lowered and the mass transfer of analyte from matrix to the extraction fluid is encouraged, because the extraction is carried out in dynamic mode so the fluid + analytes supercritical phase in

Table 3
SFE—pressure optimization.

	10 MPa		15 MPa		20 MPa		25 MPa		30 MPa		35 MPa		40 MPa	
	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
Di	72.20	1.99	86.11	3.83	94.07	3.29	95.60	2.75	96.41	3.32	**97.17**	4.68	97.09	3.71
Glyi	58.90	3.35	86.43	2.91	95.33	4.71	96.66	4.54	99.45	3.98	**100.30**	2.84	99.37	4.34
Geni	63.25	2.13	74.56	4.36	88.65	4.23	92.51	4.64	96.61	3.47	98.79	2.87	**100.25**	2.55
Ono	55.11	2.88	80.19	5.44	90.45	4.03	94.00	5.05	94.73	1.16	95.55	4.08	**97.08**	2.83
Dai	48.65	3.21	69.64	4.50	86.75	4.21	90.70	3.84	95.64	2.87	**97.88**	4.39	95.24	3.77
Gly	55.31	3.03	70.96	3.34	93.31	3.88	98.51	4.33	**98.93**	4.40	98.57	3.82	96.20	5.24
Sis	52.12	3.28	76.29	3.57	96.71	4.04	96.72	4.48	94.33	2.28	97.06	3.01	**98.48**	4.11
Gen	71.08	2.96	82.04	3.20	91.29	4.27	94.25	1.57	**96.46**	4.46	95.92	4.56	94.54	4.47
For	71.05	2.34	79.10	2.61	90.09	3.05	92.34	2.37	94.02	3.78	**98.54**	3.71	96.03	3.17
Bio	51.36	3.26	67.31	1.30	84.37	3.11	89.61	2.48	90.57	4.12	96.55	3.24	**97.85**	2.00

Table 4
SFE—temperature optimization.

	35 °C		40 °C		45 °C		50 °C		60 °C		75 °C	
	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
Di	85.31	3.15	**96.96**	2.60	89.01	1.17	82.27	1.24	71.93	3.28	70.91	3.42
Glyi	84.51	4.77	**93.13**	3.97	92.97	5.59	82.04	3.98	70.40	3.40	70.51	3.50
Geni	94.28	2.63	**97.99**	4.43	95.53	3.50	87.35	3.73	75.82	2.61	75.16	2.27
Ono	96.01	3.55	**100.42**	5.28	99.33	3.05	84.22	3.05	76.44	3.01	76.07	4.27
Dai	87.54	3.93	91.09	2.58	**97.52**	4.31	91.66	3.45	74.56	4.06	73.73	3.90
Gly	96.77	3.33	**97.48**	4.19	94.50	2.22	88.47	5.10	80.84	3.88	76.34	3.47
Sis	83.14	2.23	**94.39**	3.29	88.79	2.15	85.28	2.61	76.42	4.59	71.34	2.33
Gen	86.05	2.05	**96.36**	3.76	94.97	2.20	86.61	3.41	74.29	4.84	73.05	3.14
For	92.24	3.15	**99.45**	2.77	97.97	4.01	82.95	4.60	86.94	4.07	76.59	3.73
Bio	87.91	2.22	88.41	2.55	**95.23**	3.30	88.36	3.32	77.29	3.89	72.87	3.79

the extraction cell is constantly being "diluted" by freshly pumped clean extraction fluid.

Similar behaviour of analytes according to the changes of extraction pressure was observed by Rostagno et al. [20], who clearly state, that since the density of supercritical fluid increases with pressure, with the increase in density increases also the solvent power of the supercritical fluid and therefore it should be expected to increase recovery of all compounds with the increase in pressure.

Modifier and co-extracted analytes two "less simple" factors of influence. With the change of extraction pressure, the amount of modifier soluble in the extraction phase can change. Large quantities of modifier are used, and if some part of modifier in not soluble in supercritical phase and became liquid, or the whole system drops from supercritical to near-critical state, the extraction fluid would have lower solvation power. Plus, drops of liquid plug the restrictor, decrease the flow-rate of the extraction fluid and thus lower the amount of analytes carried out from the extraction cell. The "ballast compound paradox" works the same way. The more efficient the extraction method is, the more ballast compounds similar to the analytes can be co-extracted. In the restrictor, where the solvation power of extraction fluid decreases, both analytes and ballast compounds form clusters, that can cause not only a restrictor plugging, but also a mild decrease of flow-rate due to narrowing the inner space of restrictor capillary, if there are too many clusters catched on restrictor walls. Because of that, the optimum extraction pressure for each compound is the resultant of maximum positive effects (mass transfer, analyte solubility) and minimum negative effects (like restrictor plugging).

Of course, each analyte also works as a co-extracted ballast compound for all other analytes, but at trace concentrations of target analytes like in algae and cyanobacteria this has almost zero effect.

In the area of temperature optimisation, the situation is simpler. Except Dai and Bio, all analytes are extracted well at 40 °C (see Table 4, best recovery values are given in bold). The last two analytes have clearly better recovery at 45 °C. Thus, temperature optimum for the whole isoflavone group is 40 °C, for application focused Dai or Bio, 45 °C should be used, because the recovery of these two analytes at 40 °C is only 91.09 and 88.41%, respectively. All experiments in this study, except the temperature optimisation, were performed at 40 °C.

In this case, the reason why relatively mild extraction temperature is the best one, is relatively obvious and is common for many types of plant matrices. Although, theoretically, since increasing temperature reduces density, increases the solubility of target compounds in the supercritical fluid, increases the mass transfer kinetics and reduces the matrix effect, in real samples the most important factor is relative stability of matrix and as-low-as-possible co-extraction of ballast compounds, that are always plentiful. There usually is a limit temperature, at which a smooth extraction of wanted analytes becomes possible. Behind that temperature, more and more ballast compounds are loosened from the matrix. This is bad for the extraction selectivity, of course, but it has a unfavourable influence to the extraction efficiency as well. These compounds influence each-other's solubility in the extraction fluid and ballast compounds can contribute a large way to the decrease of extraction fluid flow-rate due to the restrictor plugging.

The system with supercritical CO_2 is highly reactive and the thermal decomposition of various parts of matrix starts at relatively low temperatures. This has always bad influence, not only because of a huge amount of ballast compounds, but there are also new active centra created or unblocked in the matrix that can re-trap the analytes from the fluid and decrease the speed of mass transfer. High selectivity is one of the privileges of SFE, and because of that, conditions that give too ballast-contaminated extracts are not of much interest because of practical reasons.

According to the study of extraction kinetics, 30 min extraction time is insufficient (3 of 10 analytes have recoveries <90%). 45 min should be sufficient for orientation monitoring (all analytes have recoveries >90%) and 60 min seems to be enough for a good analytical determination (most of the analytes have recoveries >98%, except Ono – 96.21%, Gen – 94.67% and For – 97.25%). Combined with 30 min of sonication pretreatment this extraction time is quite

Table 5
SFE—study of extraction kinetics.

	10 min		20 min		30 min		45 min		60 min	
	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
Di	82.22	4.42	89.49	3.73	93.92	2.78	96.02	3.54	98.37	3.08
Glyi	81.10	3.82	88.00	3.91	94.21	3.04	98.24	3.97	101.87	3.57
Geni	86.21	3.49	92.18	0.77	95.51	3.41	96.31	3.12	100.32	2.76
Ono	77.32	3.70	84.71	2.80	87.70	2.78	91.15	3.03	96.21	2.46
Dai	86.63	4.44	92.47	4.92	94.88	3.62	97.02	1.66	98.48	3.33
Gly	86.53	3.06	95.15	3.62	96.73	3.63	98.26	4.83	98.65	3.66
Sis	83.09	2.39	88.11	3.21	90.17	2.90	95.84	4.27	99.92	3.30
Gen	67.33	3.41	79.06	4.64	87.42	2.89	90.33	2.41	94.67	3.19
For	79.78	3.51	86.30	2.87	90.76	3.42	94.10	1.42	97.25	3.57
Bio	74.43	5.24	81.98	3.62	88.05	3.29	92.01	4.56	98.38	4.48

long on gauge standard of SFE. However, the sonication pretreatment of the next sample can be performed simultaneously with the extraction of a previous sample and one sample per hour can be processed (see Table 5).

An attempt was made to shorten the extraction time, but good results were obtained only at the expense of severe decrease of selectivity. If a matrix weighted to the eppendorf vial is left exposed to the solvent (modifier mixture) for 72 h or more, it seems to be equal to the sonication bath pretreatment, that can be reduced to 5–10 min or even skipped. However, many of these samples had to be discarded due to the restrictor plugging, as there was a lot of ballast compounds co-extracted, the increase of recovery was very small or none (mostly because there is not much room left for the recovery increase) and the RSDs were clearly worse, mostly because of troubles with maintaining constant restrictor flow-rate. The exchange of 3 days of passive waiting for 30 min of active extraction seems to be a disadvantage, because the less-comfortable part of the method (weighting the sample not directly to the extraction cell, but to the eppendorf vial, and the quantitative transfer of the sample from the eppendorf vial) had still to be done, the sonication itself is simple, requires no assistance and better selectivity and reproducibility is more important.

The optimisation study of modifier composition and percentage of modifier in the extraction fluid gave really interesting results. Not only the optimum composition of modifier is MeOH/H_2O 9:1 (v/v), what is exactly the same mixture like that one optimised for red clover [9], but also the changes of modifier composition caused similar decrease of recovery for individual analytes. It seems possible, that the matrix effect influences only some extraction conditions, like temperature and pressure, and the modifier composition should be selected according to extracted analytes. Red clover and sea algae are both lyophilised and ground plant material matrices, similar to some extent, but hardly identical, and they seem to have the same effect on optimal modifier composition (or none at all.) If this hypothesis were proved true, it would simplify a lot the selection of modifier.

The current hypothesis does not conclude, that the selection of SFE modifier is influenced only by polarity of compounds and modification of polarity of supercritical fluid and is not affected by the release of analytes from the matrix. More probably, there is a large group of analytes, that are released very similar way from various matrices, because although the matrix is different, the compounds of interest are the same, so is their mechanism of interaction with active centra, and thus they can use the same modifier and, what is more important, the same entraines—other compounds, that substitute molecules of analyte in the active centra or block these troublesome spots in the matrix so that they cannot trap analytes anymore. Small amount of water is a very good entrainer, probably thanks to the matrix swelling [6]—and traces of water are often present in the matrix of in modifier. Plant matrix contains also many other compounds, that compete with the analytes and can block the active centra of matrix efficiently. Because of that, the effect of matrix seems to be very low in these cases. If the matrix does not contain a good entrainer, it must be added, of course—i.e. extraction of spiked amino acids from inert matrix requires small amount of acetic acid as the entrainer, while the extraction of real soil sample

Table 6
The content of isoflavones in real samples.

	Sargassum 3		Sargassum 9		Hypnea		Porphyra		Undaria	
	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]
Daidzin	8.14	3.97	5.14	3.74	1.40	1.90	7.69	3.29	6.05	1.78
Genistin	34.33	4.11	10.60	4.66	8.86	4.30	12.62	4.38	8.25	4.95
Ononin	144.30	2.77	32.75	5.61	24.83	2.90	22.22	2.71	26.54	3.29
Daidzein	7.03	6.31	6.14	4.21	7.39	4.27	2.36	1.45	0.00	–
Sissotrin	59.72	4.45	24.81	4.03	22.98	3.67	17.51	3.47	18.31	4.69
Genistein	9.02	2.63	2.74	5.47	13.14	1.00	1.02	3.87	0.00	–
Formononetin	43.76	4.98	13.70	4.69	13.10	3.60	10.57	3.68	9.86	3.26
Biochanin A	49.69	3.93	15.12	3.27	15.05	3.84	11.05	3.69	10.49	0.75
	Chondrus		Halopytis		Nostoc N17		Spongiochloris sp.		Scenedesmus	
	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]
Daidzin	86.75	5.59	7.19	4.02	17.24	4.48	0.32	3.35	0.96	4.67
Genistin	229.83	4.81	18.70	3.06	18.80	3.64	13.37	5.05	4.91	3.27
Ononin	154.09	2.98	50.80	3.86	88.89	5.62	4.89	4.69	9.14	4.97
Daidzein	101.22	3.41	14.19	4.62	7.05	3.57	0.00	–	10.59	3.45
Sissotrin	111.92	3.48	36.75	3.34	60.92	2.76	2.89	3.54	3.82	4.49
Genistein	144.85	5.30	20.90	4.81	5.91	4.16	4.27	4.79	6.11	3.31
Formononetin	138.11	3.82	24.76	3.27	33.14	3.71	4.29	4.92	5.92	4.27
Biochanin A	146.67	5.14	27.68	3.89	35.78	3.40	4.95	4.40	6.75	4.76

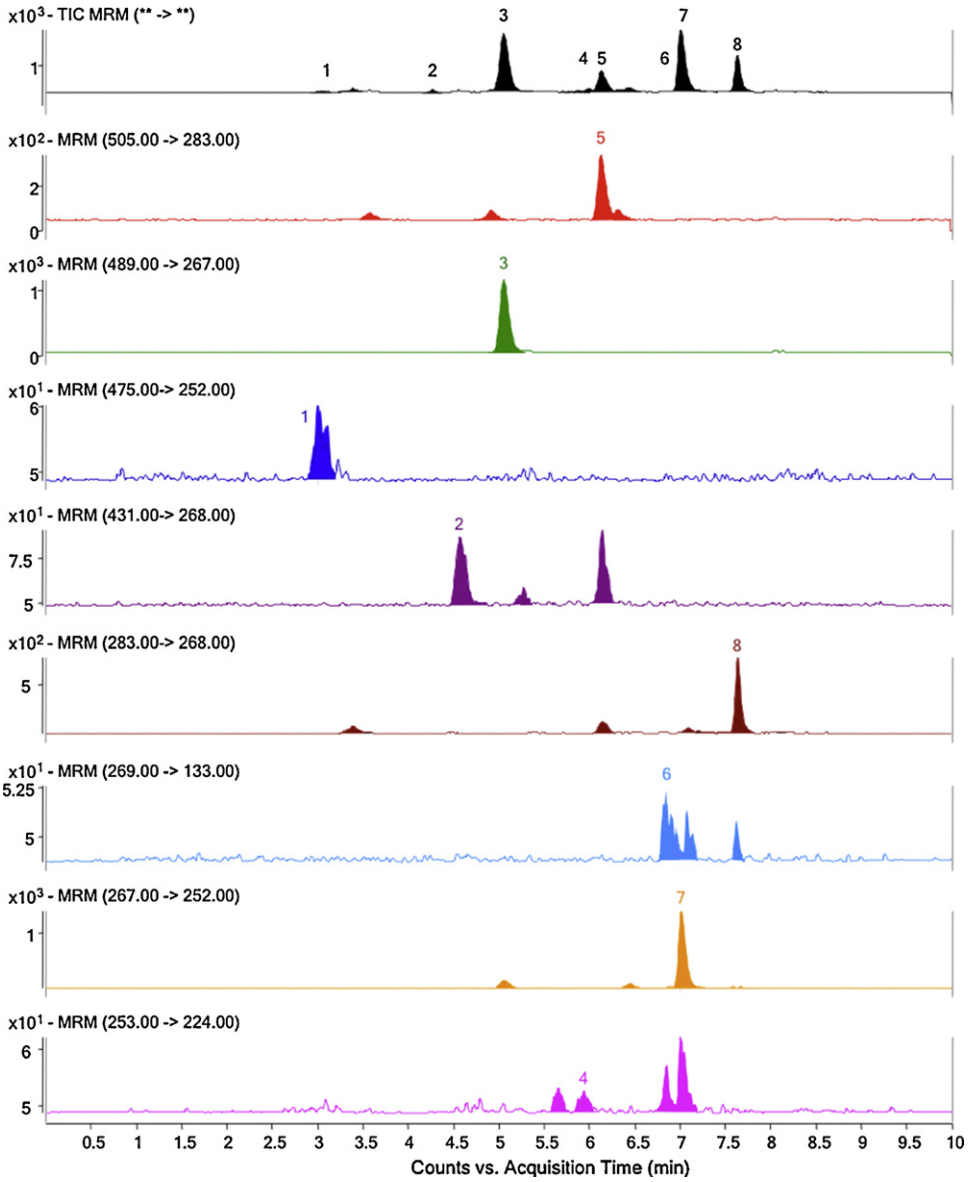


Fig. 3. MRM chromatogram of real sample – Nostoc N 17, cyanobacteria. Peak identification: 1. daidzin, 2. genistin, 3.ononin, 4.daidzein, 5. sissotrin, 6. genistein, 7. formononetin, and 8. biochanin A.

does not need this entrainer as some compounds contained in the soil can do the same job efficiently [12].

The same situation occurred in the area of modifier amount. For red clover, optimum amount of modifier was considered 300 μl, and later was lowered to 200 μl per one pump filling cycle (i.e. 2% of modifier in the extraction fluid). However, this situation was caused mostly by the instrumentation. At the time of measuring data for the paper [7], the modifier was added to the piston micropump, where it mixed with the extraction fluid. This approach is good for lower flow-rates and small percentages of modifier in the stream of CO_2. However, higher amounts of modifier tended to condense in the piston micropump and efficiently lower its working volume. With a new approach, where the modifier is dosed to the capillary with supercritical CO_2 and rinsed completely to the extraction cell, this problem is avoided and the 300 μl dosage can be used without problems. 300 μL of modifier per one pump cycle (3% of modifier in the extraction fluid) was found to be the most efficient concentration, as we expected originally for the red clover matrix as well. This fact supports the hypothesis of very low matrix effect to the modifier selection.

Optimised method was applied to seven samples of sea algae and three control samples of freshwater algae and cyanobacteria. From 10 monitored analytes, 8 analytes were found in most of the samples (see Table 6), although mostly in very low or trace concentrations. This is the first time the presence of isoflavones was proved in algae and cyanobacteria samples. Two samples are documented by the means of MRM chromatograms (see Fig. 2 – *Chondrus*, sea alga rich with analytes, and Fig. 3 – *Nostoc 17*, cyanobacteria with low content of isoflavones).

3.2. Optimisation of sonication pretreatment

Supercritical fluid extraction often works more efficiently with matrices that were put to some pretreatment. For example, the extraction of lignans from plant matrix treated with liquid nitrogen and ground in frozen state is more selective. Sonication is a simple and efficient way how to enhance the recovery. The trick is simple: SFE can be driven either by solubility, or by the mass transfer. In spiked samples, where the analytes do not have time and opportunities to create strong bonds with the matrix, solubility plays

Table 7
The effect of the thorn sonication to recovery.

Chondrus (rich sample)	15 min		30 min		40 min		50 min		60 min	
	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]
Daidzin	68.01	2.61	89.86	2.76	92.59	3.38	89.99	2.41	95.42	2.38
Genistin	168.69	1.44	217.02	2.59	246.84	3.47	264.62	3.8	255.03	2.85
Ononin	123.2	3.05	151.77	2.23	160.49	2.12	161.41	2.04	173.34	2.26
Daidzein	79.52	1.92	103.23	2.13	108.88	2.74	114.24	2.09	123.9	3.13
Sissotrin	79.76	2.61	114.61	2.39	125.03	3.71	130.05	2.06	133.39	2.03
Genistein	102.88	2.74	145.8	3.5	156.44	2.13	163.92	2.76	168.82	3.11
Formononetin	103.46	2.16	136.73	2.36	148.03	2.08	143.44	2.03	148.52	4.43
Biochanin A	107.2	3.15	152.7	3.26	162.76	2.64	167.19	1.79	163.53	2.96
Sargassum 3 (low conc. sample)	15 min		30 min		40 min		50 min		60 min	
	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]	Content [ng/g]	RSD [%]
Daidzin	6.11	3.34	8.33	3.21	8.68	2.58	9.17	2.5	9.23	2.01
Genistin	26.6	2.72	35.01	2.56	36.22	1.85	37.56	2.23	38	2.71
Ononin	110.79	2.56	134.04	2.01	149.85	3.63	157.82	2.48	165.01	2.55
Daidzein	5.27	2.85	6.82	5.02	7.71	1.72	7.69	2.38	7.76	2.01
Sissotrin	42.46	3.04	58.19	1.65	63.46	2.22	69.09	3.18	63.69	1.86
Genistein	6.86	4.26	9.19	3.17	9.91	3.54	9.84	2.27	10.33	2.11
Formononetin	32.54	2.6	42.88	1.34	44.18	2.02	47.47	2.76	49.61	3.2
Biochanin A	38.56	2.57	49.81	1.41	53.04	2.4	53.71	2.3	53.02	2.98

the main role and all analytes are extracted as quick as possible, according to the solubility limitations. In aged or real samples, all "available" analytes are extracted the same way, i.e. quite quickly, but some analytes that are strongly bond to the matrix or hidden deep in matrix particles are limited by the mass transfer process. As soon as the molecules get out of the inner space of matrix or are substituted by entrainer in the active centra, they are extracted too. During the sonication pretreatment, certain parts of matrix – like walls of the cells or organelles in plant material – are damaged, and the SFE mass transfer takes place much more easily.

Two experimental designs for sonication pretreatment are available and both of them were investigated in this paper. Classic method is a sonication bath. This approach is simple, easy to operate and the samples can be closed in Eppendorf vials during the experiments without the risk of contamination or of the loss of the analytes. It also allows to process many samples at once. On the other hand, intensity of sonication is given by parameters of the sonic bath and cannot be changed. This type of pretreatment was used for all samples in all experiments, except the thorn sonication device study. Matrix was sonicated in 300 μl of modifier solution ($MeOH/H_2O$ 9:1, v/v), that was lately quantitative transferred to the extraction cell and used as *in situ* modifier. Pretreatment was carried out for 30 min. This time is always sufficient and further prolongation of pretreatment did not bring any increase of the recovery.

New approach with a thorn inserted directly to the sample and used as a source of ultra-sound was also tested. This device (Sonopuls, Bandelin Electronic, Germany) enables the researcher to adjust the intensity of sonication. The parameters are following: thorn diameter – 2 mm, volume rate – 1–25 ml, amplitude – 285 μmss, max allowed amplitude setting – 97%.

It was found out that the needed process of matrix cell walls destruction occurs (with measurable implications) at ca. 60% of the instrument max. performance. Further increase of the sonication device performance did not bring better results. Time optimisation study was carried out for two different samples. In fact, the aim of original use of this device was shortening the pretreatment time—more efficient sonication should do the job more quickly. However, the erosion of plant cell walls is a slow process in both modes. 15 min is not enough, at 30 min the results are comparable with sonication bath treatment. The interesting result is further increase of isoflavone recovery with longer pretreatment time that wasn't observed for sonication bath (see Table 7). Thus, although the thorn device is more labour-intensive, there is a risk of the loss of analytes in the opened system and only one sample can be processed simultaneously, the recoveries obtained this way are even better at the cost of even longer time of pretreatment.

4. Conclusion

Thanks to the development in modern liquid chromatography instruments, columns, methods and detectors, precise and accurate determination of trace amounts of analytes became possible. We can learn about low-concentration components of complex biological matrices with interesting favourable effects to human health. New scientific branch, foodomics, was established to study functional food and its benefit for people. From all nowadays studied areas, foodomics has one of most promising contributions. However, great analytical tools require new sample preparation techniques: efficient and selective at the same time. This paper describes such method for the supercritical fluid extraction and fast chromatography–MS/MS determination of isoflavones from sea and freshwater algae.

The method is based on previously developed and validated method for red clover matrix and a complete optimisation study for algae matrix was performed. Extraction conditions are following: sample weight – 0.1 g, sample pretreatment – sonication bath or thorn sonication, extraction pressure – 35 MPa, extraction cell temperature – 40 °C, time of extraction – 60 min, modifier: $MeOH/H_2O$ 9:1 (v/v), 300 μl in situ + 3% (v/v) on-line, restrictor 12 cm × 50 μm i.d., trapping solvent – 25 ml MeOH, at laboratory temperature. Two interesting facts emerged during the experiments. First: the matrix effect seems to influence extraction pressure and temperature, but the best modifier amount and composition seems to depend on the type of analytes and not much on the matrix. It is still too soon to say the hypothesis is true based on three types of matrix (spiked glass wool, red clover, 10 sea and freshwater algae samples of similar character), but if this were proved to be true, a database of the most suitable modifiers could be created 1 day only according to the analytes.

Another interesting fact was found out during the experiments with thorn sonication device. This device can't make the extraction time shorter, but at the cost of longer treatment, it can enhance the recovery to the level unreachable by sonic bath treatment. The device requires sealing the top of eppendorf vial with a wax foil, to prevent the loss of analytes, and because of that the quantitative

transfer of sample to the extraction cell is more complicated, but the sonication pretreatment is more thorough and the recovery is higher. This new hyphenated method (thorn sonication–SFE–fast chromatography MS/MS) seems promising and will be applied to other interesting complex matrices.

Acknowledgements

This study was supported by grants No. 525/07/0338 and 525/08/P540 from Czech Science Foundation. Merichel Plaza thanks CSIC for her I3P fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.07.020.

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