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RESEARCH ARTICLE

On-Line Solid Phase Extraction Liquid Chromatography-Mass Spectrometry Method for Multiplexed Proteins Quantitation in an Ecotoxicology Test Specie: *Gammarus fossarum*

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OBJECTIVES: A fully automated on-line solid phase extraction procedure was developed and validated for the analysis of 30 key proteins biomarkers in *Gammarus fossarum*.

METHODS: After protein extraction and tryptic digestion, peptides were cleaned-up onto an on-line SPE cartridge (Oasis HLB, 2.1 mmx20 mm, 25 µm particle size) coupled to a LC-MS/MS system. The SRM assay was performed on a quadrupole-trap mass spectrometer.

RESULTS: The method targeted 30 proteins in *G. fossarum* (46 reporter peptides used in the SRM assay) was developed and validated. The method duration was 30 min including the on-line SPE step saving up to 6h per sample. The method's performance was validated according to FDA guidelines.

CONCLUSIONS: Our method substitutes valuably conventional methods like off-line SPE performed beforehand to the LC-MS/MS system. This assay offers higher sensitivity with no loss and/or degradation of reporter peptides and reached good specifications (linearity, precision and accuracy).

KEYWORDS: multiplex, protein quantification, mass spectrometry, biomarkers, sample preparation, ecotoxicology.

INTRODUCTION

Although chemical analysis is a useful tool to address ecosystem quality assessment, the quantification of all chemical compounds and their associated degradation products remain challenging to assess their bioavailability, and to predict their conjugate effects on biota. For that reason, sub-individual biological indicators have been proposed to link the presence of chemical compounds and their effects on biota by detecting sublethal changes [1]. These indicators are designed to be an early warning signal for ecosystem degradation. As human medical diagnoses, key proteins involved in the molecular response mechanisms related to xenobiotic toxicity or homeostasis can be used to provide evidence of exposure or effects to one or more chemical pollutants on sentinel organisms [2]. Biomarkers based on protein measurements are particularly relevant since proteins are the molecular effectors of biological processes. Due to numerous and various biological processes that could be relevant for status health, many protein biomarkers need ideally to be evaluated.

Enzyme-linked immunosorbent assays are the gold standard method for a reliable and sensitive protein quantification. However, the development of such an assay is expensive and time consuming. In addition, the main limitation is related to the antigen-antibody reaction, which is based on their amino acid sequence and tri-dimensional conformation. Consequently, this method is sensitive to phylogenetic distance among species and poorly transferable, which is problematic in a biomonitoring perspective [3]. Thus, the development of protein biomarkers, mainly in invertebrates, is restricted to a rather limited set of enzymatic proteins [4,5], via indirect strategy assay. Furthermore, the results of such functional assays are expressed in nmol of substrate hydrolysed per minute ($nmol.min^{-1}$) or $nmol.min^{-1}.mg\ proteins^{-1}$ [6,7], often making difficult inter-laboratory comparisons. In addition, distinct enzyme isoforms may have different sensitivities to substrates and inhibitors. Thus, transposition and generalization of well-known biomarkers to a diversity of organisms is limited by the difficulties to adapt methodologies to non-model species [8].

For an integrative approach and a relevant interpretation in terms of impact in regard to an exposure or in term of health status, a multi-biomarker strategy must be used [9]. Recently, an integrative index called the integrated biomarker response (IBR), has been developed and proposed for the assessment of ecological risk [10,11]. In the context of a multi-biomarker deployment in routine biomonitoring, a multiplex methodology to monitor a panel of biomarkers in a single biological sample and in a single run, will allow high-throughput analyses at less cost. However, for any biomarker, a specific analytical procedure in terms of homogenisation buffer, reaction medium or wavelength measurement is required. The multiplication of protocols used is extremely resource consuming (time, personnel, cost and biological material).

Mass spectrometry is the method of choice for the quantitation of low-abundance proteins in biological research [12–15]. Single protein detection and quantification methods have been used sequentially. Although these methods are both well-established and validated, sample-preparation revealed to be time-consuming and costly when numerous markers per sample must be monitored. A significant number of protein candidates can be multiplexed and simultaneously targeted for quantitative detection in biological matrix in a single measurement with modern triple quadrupole mass spectrometer operated in Selected Reaction Monitoring mode (SRM) [16,17]. For example, we recently proposed a multiplex method for the quantification of several proteins in the amphipod crustacean *Gammarus fossarum*, commonly employed as model organism in ecotoxicological assessment [18,19]. Multiplex assays are often used in high-throughput screening settings where many proteins can be analysed simultaneously. Strictly speaking, a multiplex assay is not necessarily performed in high-throughput point of view. When the execution of a single multiplex assay generates data for many analytes, it is considered high-throughput. However, it is rather the ability to rapidly process multiple specimens in an automated fashion that characterizes high-throughput techniques.

For SRM-MS multiplexed protein quantitation, sample purification after enzymatic digestion is one of the bottleneck of the systems, thus limiting the high throughput acquisition of data necessary for environmental monitoring. Solid Phase Extraction (SPE) is more often used as sample preparation prior to liquid chromatography (LC) and tandem mass spectrometry (MS/MS) analyses. In general, SPE is a manual off-line procedure increasing significantly the time of the procedure and generates errors in the laboratory workflow (analyte loss, degradation and/or adsorption during solvent evaporation, errors during tube manipulation). On-line SPE automates the sample clean up step and analyte enrichment process and therefore overcomes all issues mentioned above.
The aim of this work was to investigate and develop an on line SPE-LC/MS/MS method to address some of the limitations of current sample preparation methods for multiplexed protein biomarkers in *G. fossarum*. The goal was to provide full automation, on-line coupling to MS detection, short sample preparation time and to increase the multiplexing capacity. The developed method was validated and could successfully be applied for the quantification of potential protein biomarkers in a relevant model organism in ecotoxicology.

MATERIALS AND METHODS

Chemicals and reagents

Water, methanol and acetonitrile (LC-MS grade) were purchased from Fisher Scientific (Strasbourg, France). Iodoacetamide (IAM), dithiothreitol (DTT), formic acid (FA) (LC–MS grade), TCPK – treated, TRIS, urea, EDTA, Triton X, sodium chloride, leupeptin and aprotinin were obtained from Sigma–Aldrich (St Quentin-Fallavier, France). Labelled peptides (purity > 97%) containing either a C terminal [$^{13}C_6$, $^{15}N_2$] arginine or lysine were synthetized by Fisher Scientific (Strasbourg, France). Absolute ethanol and ethylic ether were obtained from Carlo Erba (Val de Reuil, France). XBridge C18 column (2.1 x 100 mm, 3.5 µm) and Oasis HLB column (2.1 x 20 mm, 25 µm) were purchased from Waters (Ireland).

Protein selection

Protein sequences were obtained from a *G. fossarum* proteogenomics database [20] which contains 1873 proteins and their presumed function as deduced from sequence similarities searches. **Table 1** reports a listing of 30 proteins interesting for quantification. **Table 1** includes proteins identified as sex-specific (copine-8; yolk proteins including vitellogenins; Prophenoloxidase and Ca-transporting ATPase, in addition to proteins related hormonal regulation and moult (juvenile hormone esterase carboxylesterase (JHE carboxylesterase); Cytochromes; Farnesoic acid methyltransferase (FaMET); and Chitinase), immunity (Hemolectin) and proteins with annotation linked to biomarkers presently used in ecotoxicology (Catalase; glutathione-S-transferase GST; Na^+K^+ATPase and Cellulase)

Sample extraction and preparation

Protein extraction and digestion conditions have been optimized in previous studies [3,18,21]. Briefly *G. fossarum* whole bodies were homogenized in Tris buffer (Tris-HCL 50mM; 100mM NaCl; 1mM EDTA; Triton X-100 0.1 % v/v; adjusted to pH 7.8, and containing leupeptin and aprotinin at 10 µg/mL), in a volume of 25 µL per mg of specimen. Then, samples were centrifuged at 10000 x *g* for 15 min at 4 °C. After collection of 250 µL of clear supernatant, a delipidation step was performed by adding 750 µL of ethanol/diethylether mixture (1/1, v/v). After extraction, the solution was vortexed and replaced on ice for 10 min. After a centrifugation of 10 min at 10000×g at 4 °C, clear supernatant was removed, and the remaining bottom volume was mixed with 250 µL of Tris buffer.
Protein denaturation and cysteine reduction was performed with 3 mL of ammonium bicarbonate (50 mM) and DTT 15 mM (final concentration). Denaturation and reduction steps occurred simultaneously for 40 min at 60 °C. After cooling to room temperature,

Table 1. Cont'd. List of proteins with their biological function and corresponding light and heavy reporter peptide and their MS/MS transitions and MS parameters monitored for the most intense transition. Biological functions grouped in six general functions: RF: Female reproduction; RM: male reproduction; I: Immunology; RH: Hormonal regulation; BM: General biomarker and Molt. (ND: no detected), (*used to indicate labeled amino acid).

Protein #	Protein name (function) - ID	Peptide sequence	Retention time (min)	m/z of transition		4000Q trap		Precursor charge	Ion fragment (charge)
				Q1	Q3	DP (V)	CE (V)		
1	Vitellogenin (RF) - 64	HIEIFSPITK*	12.3	398.2	466.3	62	36	3+	y4 (1+)
		HIEIFSPITK	12.3	395.6	458.3				
2	Hemolymph clottable protein (RF) - 276	HAEFSVNPPLDSTQAVK*	11.9	616.7	80.0	63	26	3+	y10 (2+)
		HAEFSVNPPLDSTQAVK	11.9	614.0	528.3				
		IIYPAEALTIVIEK*	16.5	791.0	93.0	83	36	2+	y12 (2+)
		IIYPAEALTIVIEK	16.5	787.0	673.9				
3	Transglutaminase (RM/I) - 1917	GTLAVIPVQNR*	12.2	589.3	623.3	61	29	2+	y5 (1+)
		GTLAVIPVQNR	12.2	584.3	613.3				
4	Copine-8 (RM) - 2308	EVLEELPAQYMEFTR*	14.5	933.0	229.1	92	50	2+	b2 (1+)
		EVLEELPAQYMEFTR	14.5	928.0	229.1				
5	Prophenoloxidase (RM/I) - 2562	APILEGYFSK*	13.6	566.8	531.3	70	28	2+	y9 (2+)
		APILEGYFSK	13.6	562.8	527.3				
		GIDIIGDAFEADR*	14.2	701.3	286.1	68	45	2+	b3 (1+)
		GIDIIGDAFEADR	14.2	696.3	286.1				
		ATQPSYTVAQLELPGVNITR*	14.3	723.4	766.4	80	30	3+	y7 (1+)
		ATQPSYTVAQLELPGVNITR	14.3	720.1	756.4				
		GIDFGTTQSVR*	11.7	595.8	758.4	72	29	2+	y7 (1+)
		GIDFGTTQSVR	11.7	590.8	748.4				
6	Ca transport ATPase sarcoplasmic/endoplasmic reticulum type-like isoform 1 (RM) - 4227	KAEIGIAMGSGTAVAK*	10.7	504.6	683.4	56	23	3+	b7 (1+)
		KAEIGIAMGSGTAVAK	10.7	501.9	683.4				
		LLEFEITGSTYEPIGDVFLGGQR*	16.4	851.1	687.4	97	33	3+	y6 (1+)
		LLEFEITGSTYEPIGDVFLGGQR	16.4	847.8	677.4				
7	Transglutaminase (RM/I) - 7169	VLAVDILAK*	13.3	475.3	737.5	55	23	2+	y7 (1+)
		VLAVDILAK	13.3	471.3	729.5				
8	Hemolectin (I) - 11145	VEC[CAM]IAGFILPLEFK*	17.0	822.5	389.2	85	42	2+	b7 (2+)
		VEC[CAM]IAGFILPLEFK	17.0	818.4	389.2				
9	Chitinase (Molt) - 12415	GGQWFGYDDISMIR*	14.6	827.9	1079.5	69	43	2+	y9 (1+)
		GGQWFGYDDISMIR	14.6	822.9	1069.5				
10	Prophenoloxidase (RM/I) - 15561	IVIDLLQQSTTVAQLR*	14.8	904.5	213.2	80	47	2+	b2 (1+)
		IVIDLLQQSTTVAQLR	14.8	899.5	213.2				

Table 1. Cont'd. List of proteins with their biological function and corresponding light and heavy reporter peptide and their MS/MS transitions and MS parameters monitored for the most intense transition.

Protein #	Protein name (function) - ID	Peptide sequence	Retention time (min)	m/z of transition		4000Q trap		Precursor charge	Ion fragment (charge)
				Q1	Q3	DP (V)	CE (V)		
11	Clottable protein 2 (RF) - 17046	IATGMQSALPEYAGTGIK*	12.4	908.5	943.5	62	26	2+	y9 (1+)
		IATGMQSALPEYAGTGIK	12.4	603.3	468.2				
		IFNVLQPIAESK*	14.0	683.9	652.4	67	35	2+	y6 (1+)
		IFNVLQPIAESK	14.0	679.9	644.4				
		ITMQEDGSGEVQLK*	10.7	771.9	681.4	82	40	2+	y6 (1+)
		ITMQEDGSGEVQLK	10.7	767.9	673.4				
		TSEVFLPLTNELYQQTK*	15.0	673.7	671.9	56	28	3+	y11 (2+)
		TSEVFLPLTNELYQQTK	15.0	671.0	667.9				
12	Copine-8 (RM) - 18473	KFLPSSGVDDLIK*	12.8	476.3	389.3	55	27	3+	b3 (1+)
		KFLPSSGVDDLIK	12.8	473.6	389.3				
		SYQILLLITNGGLSDIDATKK*	16.0	757.8	379.2	71	31	3+	b3 (1+)
		SYQILLLITNGGLSDIDATKK	16.0	755.1	379.2				
13	Epididymal sperm-binding protein 1 (RM) - 18609	VLSVVQNIITR*	13.6	626.4	213.2	74	31	2+	b2 (1+)
		VLSVVQNIITR	13.6	621.4	213.2				
14	Na/K-ATPase a1 subunit (BM) - 32234	FVGLISLIDPPR*	16.4	668.9	379.2	76	40	2+	y3 (1+)
		FVGLISLIDPPR	16.4	663.9	369.2				
		NLAFFSTNAVEGTAR*	13.6	804.4	228.1	74	41	2+	b2 (1+)
		NLAFFSTNAVEGTAR	13.6	799.4	228.1				
15	Copine-8 (RM) - 34845	AVAEIVQDYDSDGFFPALGFGGK*	12.5	804.4	754.4	84	64	3+	y8 (1+)
		AVAEIVQDYDSDGFFPALGFGGK	12.5	801.7	746.4				
16	Clotting protein precursor (RF) - 39606	ISPLINSPSDLPK*	12.4	694.9	594.8	58	30	2+	y11 (2+)
		ISPLINSPSDLPK	12.4	690.9	590.8				
17	Catalase (BM) - 45375	NLPADQAAALASSDPDYAIR*	12.5	690.3	744.4	77	28	3+	y6 (1+)
		NLPADQAAALASSDPDYAIR	12.5	687.0	734.4				
18	Cytochrome P450 enzyme, CYP4C39 (Molt) – 100255	ILEDFVDVFNR*	15.3	688.9	227.2	67	35	2+	b2 (1+)
		ILEDFVDVFNR	15.3	683.9	227.2				
		VYAEVIEVAGSGPIGLDQLR*	14.7	699.4	711.4	78	29	3+	y6 (1+)
		VYAEVIEVAGSGPIGLDQLR	14.7	696.0	701.4				

Table 1. Cont'd. List of proteins with their biological function and corresponding light and heavy reporter peptide and their MS/MS transitions and MS parameters monitored for the most intense transition.

Protein #	Protein name (function) - ID	Peptide sequence	Retention time (min)	m/z of transition		4000Q trap		Precursor charge	Ion fragment (charge)
				Q1	Q3	DP (V)	CE (V)		
19	Hemolectin (I) - 109695	NAGPVLLPSNTSPVLR*	13.6	823.0	243.1	85	47	2+	b3 (1+)
		NAGPVLLPSNTSPVLR	13.6	818.0	243.1				
20	Na+/K+ ATPase (BM) - 110907	LGAIVAVTGDGVND-SPALK*	12.6	903.0	242.1	80	52	2+	b3 (1+)
		LGAIVAVTGDGVNDSPALK	12.6	899.0	242.1				
		VIMVTGDHPITAK*	10.3	463.9	589.3	64	21	3+	y11 (2+)
		VIMVTGDHPITAK	10.3	461.3	585.3				
20	Catalase (BM) - 110912	ADPALGQAIQER*	10.4	639.8	546.8	25	30	2+	y10 (2+)
		ADPALGQAIQER	10.4	634.8	541.8				
		LADNIAGHVINTQEFIR*	13.8	641.0	697.9	74	30	3+	y12 (2+)
		LADNIAGHVINTQEFIR	13.8	637.7	692.9				
21	Cytochrome P450 CY-P12A2 (Molt) - 122081	FNNNLINTR*	10.6	558.3	400.2	60	30	2+	y3 (1+)
		FNNNLINTR	10.6	553.3	390.2				
		TLEELSNEALR*	11.9	642.8	215.1	64	32	2+	b2 (1+)
		TLEELSNEALR	11.9	637.8	215.1				
22	Farnesoic acid O-methyl-transferase (RH) - 134275	EVFIGGWSNQNSAIR*	13.4	844.4	376.2	86	44	2+	b3 (1+)
		EVFIGGWSNQNSAIR	13.4	839.4	376.2				
23	Glutathion S transférase (BM) - 142711	LSAWLAAC[CAM]K*	11.9	514.3	201.1	67	25	2+	b2 (1+)
		LSAWLAAC[CAM]K	11.9	510.3	201.1				
24	JHE-like carboxylesterase 1 (RH) - 144144	AFWGSLPLR*	14.4	528.8	652.4	68	26	2+	y6 (1+)
		AFWGSLPLR	14.4	523.8	642.4				
		ILTTMWADFAR*	15.0	667.8	775.4	76	34	2+	y6 (1+)
		ILTTMWADFAR	15.0	662.8	765.4				
26	Farnesoic acid O-methyl-transferase (RH) - 166723	EFWIATDHNEVR*	12.0	509.6	476.2	67	23	3+	y8 (2+)
		EFWIATDHNEVR	12.0	506.2	471.2				
27	Farnesoic acid O-methyl-transferase (RH) - 166723	EFWIATDHNEVR*	12.0	509.6	476.2	67	23	3+	y8 (2+)
		EFWIATDHNEVR	12.0	506.2	471.2				
27	Chitinase (Molt) - 181833	LVLGTATYGR*	11.0	530.8	848.5	66	26	2+	y8 (1+)
		LVLGTATYGR	11.0	525.8	838.4				

Table 1. Cont'd. List of proteins with their biological function and corresponding light and heavy reporter peptide and their MS/MS transitions and MS parameters monitored for the most intense transition.

Protein #	Protein name (function) - ID	Peptide sequence	Retention time (min)	m/z of transition		4000Q trap		Precursor charge	Ion fragment (charge)
				Q1	Q3	DP (V)	CE (V)		
28	Vitellogenin (RF) - 200426	ELTSAAEVVSSLLK*	15.9	727.9	377.3	82	43	2+	y7 (2+)
		ELTSAAEVVSSLLK	15.9	723.9	373.2				
		TLGALELDVFSEAGK*	15.1	779.4	272.2	80	50	2+	b2 (1+)
		TLGALELDVFSEAGK	15.1	775.4	272.2				
29	Clotting protein precursor (RF) - 206469	AAIETAFVNHLK*	12.2	441.2	590.3	65	20	3+	y10 (2+)
		AAIETAFVNHLK	12.2	438.6	586.3				
30	Cellulase (BM) - 213317	ELFDFADAHR*	13.5	410.9	421.2	61	20	3+	y7 (2+)
		ELFDFADAHR	13.5	407.5	416.2				

alkylation and Iodoacetamide (IAM) (final concentration of 15 mM) quenching was performed at room temperature in the dark for 40 min. Proteolysis was achieved with 300 µg of trypsin and incubation for 1 h at 37 °C. Finally, reaction was stopped by the addition of 20 µL of formic acid (FA). 10 µL of a solution containing 1 µg/mL of all isotopically labelled peptides was added to the sample.

Off-line Solid phase extraction comparison

The sample was loaded onto an Oasis HLB (3 mL, 60 mg) extraction cartridge (Waters), pre-conditioned with 1 mL of methanol and 1 mL of water acidified with 0.5% FA. 3 mL samples were then loaded on the cartridges. Following rinsing with 1 mL of solution of water/methanol (95/5 v/v) acidified at 0.5 % of FA, analytes were eluted with a mixture of 1 mL of methanol acidified at 0.5 % of FA into an Eppendorf tube. The eluate was then evaporated to dryness under a gentle stream of nitrogen until a volume of 10 µL, which was diluted with 90 µL of water/acetonitrile (90/10, v/v) with 0.1 % of FA. After vortexing, the samples were transferred to glass vials.

Analytical part

The HPLC system consisted of an Agilent 1200 system (Agilent Technologies, Waldbronn, Germany) with a high-pressure binary pump (pump A, for LC), an autosampler and a column oven with a programmable 10 ports/2 positions valve, a second-high pressure binary (pump B, for one-line SPE). Online SPE was performed on a Waters (Millford, MA) Oasis HLB cartridge (2.1 mm × 20 mm, 25 µm particle size). The cartridge was preconditioned with methanol and water acidified with 0.5 % FA. Samples (100 µL) were then injected with pump B with 100% of water containing 0.5 % FA for 2 min at a flow rate of 1 mL/min. Afterwards, cartridge was rinsed for 2 min with a solution of water/methanol (95/5 v/v) acidified at 0.5 % of FA and the analytes were then eluted with the chromatographic gradient.

HPLC is coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer API 4000 QTRAP® from SCIEX (Concord, Canada) equipped with a Turbo VTM ion source connected to the HPLC system as an MS/MS detector. Instrument control, data acquisition and processing were performed using the Analyst 1.5 software. A Xbridge C18 column (100 mm × 2.1mm, particle size 3.5 µm) from Waters was used for HPLC separation with 100 µL injected sample.

The analytes were transferred from on-line SPE to the C18 column with pump 1 at a flow rate of 300 µL/min. The mobile phase consisted of water containing 0.1% (v/v) formic acid as eluent A and acetonitrile containing 0.1% (v/v) formic acid as eluent B. A gradient elution was used from 2 % B to 33 % B in 12 min, followed by a 6 min second linear gradient from 33 % B to 64 % Pump 2 was delivering 100 % of acidified MeOH for 19 min at 100 µL/min. Then, column rinsing, and equilibration was performed for 8 min, with switching of the valve in right position at 23 min. The injection duty cycle was 30 min, considering the column equilibration time. The operational procedure is shown in **Table 2**.

MS analysis was carried out in positive ionization mode using an ion spray voltage of 5500 V. The curtain gas (nitrogen) and the nebulizer (nitrogen) flows were set at 50. The Turbo VTM ion source was set at 550 °C with the auxiliary gas flow (nitrogen) set at 40 psi. The software Skyline v3.1 (MacCoss Lab Software, USA) was used to analyse the results. From the MRM transitions, three transitions by peptide were selected for the detection of the peptides but only the most intense transition was used to quantify a peptide. The MRM transitions were reported in **Table 1**. They were monitored and acquired at unit resolution, with a dwell time of 10 msec used for each transition, to obtain 10 data points per chromatographic peak minimum.

Standard solutions and quality controls

Stock of isotopically labelled peptides solutions were prepared by dissolving accurately

Table 2. Operational procedure for the on-line SPE-LC-MS/MS system with a column C18.

Step	Time (min)	Valve position	Pump A (C18)			Pump B		
			%A	% B	Flow (mL/min)	%A	%B	Flow (mL/min)
1	0	R	98	2	0.1	100	0	1
2	2							
3	2.10					95	5	
4	4							
5	4.10	L	98	2	0.3	0	100	0.1
6	16		67	33				
7	22		36	64				
8	23		0	100				
9	23.1	R	0	100	0.1	0	100	1
10	25							
11	25.1							
12	27					100	0	
13	27.1		98	2				
14	30		98	2				

Pump A [A: Water 0.1% formic acid; B: ACN 0.1% formic acid], Pump B [A: Water 0.5% formic acid; B: MeOH 0.5% formic acid].

weighed standard compounds in a mixture of H_2O/ACN/formic acid (50/50/0.1, v/v/v), to yield a concentration of 20 µg/mL. Solutions at 50, 100, 200, 400, 500, 1000 and 5000 ng/mL were prepared from the stock solutions at 20 µg/mL and diluted further with either an H_2O/ACN/formic acid (90/10/10, v/v/v) mixture or the extracted and homogenized whole-body *G. fossarum* matrix. These solutions were used to build the calibration curves. Quality controls at 250 ng/mL (i.e. QC1), 625 ng/mL (i.e. QC2) and 2500 ng/mL (i.e. QC3). were prepared from the stock solutions. Dilutions were done with either an H_2O/formic acid (90/10/0.1, v/v/v) mixture or the extracted and crushed G. fossarum matrix.

Assay validation

A standard curve was produced, based on seven samples containing equal amounts of *G. fossarum* protein extract digests as background matrix in order to determine the LOD, LOQ and linearity of the method. Each sample analyzed three times was spiked with an increasing amount labelled peptide between 50 and 5000 ng/mL, covering a 100-fold range. Signal-to-noise ratio was estimated by comparing measured signals from samples with known low concentrations of labelled peptide with those of blank samples. Several approaches for determining the detection limit and quantification limits are possible. The approach based on comparison measured signals from samples with known low concentration of analyte with those of blank sample was selected. A signal-to-noise ratio between 3:1 and 10:1 was considered acceptable for estimating the LOD and LOQ. These ratios are respectively the lowest level an analyte can be detected, not necessarily quantitated under the analytical conditions and the lowest level an analyte can be quantitated with an acceptable level of precision and accuracy. Three runs on three separate days consisted of one set of calibration standards, three (intra-batch) or nine (inter-batch) replicates of each QC concentration (250, 625 and 2500 ng/mL) were used for evaluation of method precision and accuracy. To plot the curve of the calibration standards least square linear regression with a weighting factor of $1/x^2$ was used.

There is no guidance for analytical method validation in ecotoxicology. So, we were inspired by the FDA bioanalytical method validation guidance for industry with small extension of performance criteria for precision and accuracy. A criterion of precision determined at each concentration level should not exceed a percent CV above 20 % and an average accuracy in determining the expected concentration within 80–120 %. If standard points for any level fell outside these ranges, the entire level would be removed from the curve and the linear regression equation would be recalculated. Calibration standards and the final calibration line will contain at least 5 calibration concentrations. The determination coefficient (r^2) will be greater than 0.98. Finally, the matrix effect was evaluated by comparing the relative area of peptides in the pure solvent and that in *G. fossarum* extract. It was spiked with the isotopically labelled peptides after digestion at two concentrations (250 and 2500 ng/mL).

RESULTS AND DISCUSSION

Armengaud and his collaborators have shown that combination of genomics and proteomics, the so-called proteogenomics approach, is a straightforward strategy for discovering proteins in non-model organisms employed in environmental science [22]. Based on a large proteogenomic survey of *G. fossarum*, a list of interesting proteins, detected and specific of *G. fossarum*, has been generated. From this list, 30 proteins (**Table 1**) representative of different biological functions (sex-specific proteins, proteins related to moult and hormonal regulation, immunity), some of which with annotation related to biomarkers currently used in ecotoxicology, have been retained for quantification with the aim of enlarging the catalogue of new potential biomarkers. Selection of the best reporter peptides in regards of sensibility, selectivity and biological specificity was previously reported [18] and the relevance of this set of protein biomarkers for ecotoxicological test was demonstrated [19]. Since in *G. fossarum*, some proteins and consequently reporter peptides are found at low concentration levels, a clean-up step with solid phase extraction (SPE) is necessary to achieve the lowest possible sensitivity.

The sample extraction is usually the bottleneck of the whole analytical procedure and only the implementation of on-line SPE made possible the effective development of faster methods by reducing the analysis time. To increase the analytical throughput, an on line SPE-LC/MS/MS method has been developed. The benefits of on-line SPE are illustrated in **Figure 1**. On-line SPE allows clean-up, concentration and direct elution to the analytical column, which eliminates manual intervention, decreased risk of contamination, elimination of analyte losses by degradation or by evaporation during solvent evaporation and plasticware or glassware transfers. As already reported, peptides are subject to adsorption during the drying step and sample preparation [23,24]. Greater sensitivity is observed in on-line SPE since the totality of the extract is transferred into the LC column. In off-line configuration only, an aliquot of the extract is injected into the column. The analysis of the integral sample leads to lower limits of detection or reduce sample volume to obtain similar sensitivity. Injection of 100 µL of digested protein extract corresponds to 1000 µL used in off-line SPE with a time saving more than 5h corresponding to SPE and solvent evaporation.

Several experimental variables, such as sample volume, flow rate, valve switch time and solvent composition for purification and elution should be optimized in an on-line SPE procedure to achieve the maximum extraction recovery, salt elimination, and prevention of carryover. Indeed, the trapped peptides should be eluted and refocused onto the HPLC column by the elution gradient by the time the SPE column is switched into the analysis flow path. For peptides, the gradient elution in reversed-phase separations usually starts at high content of aqueous in the mobile phase, and the moderate elution from the pre-concentrating column could result in peak broadening, which results in decreased efficiency and thereby sensitivity. For these reasons, the elution gradient was optimized before method validation.

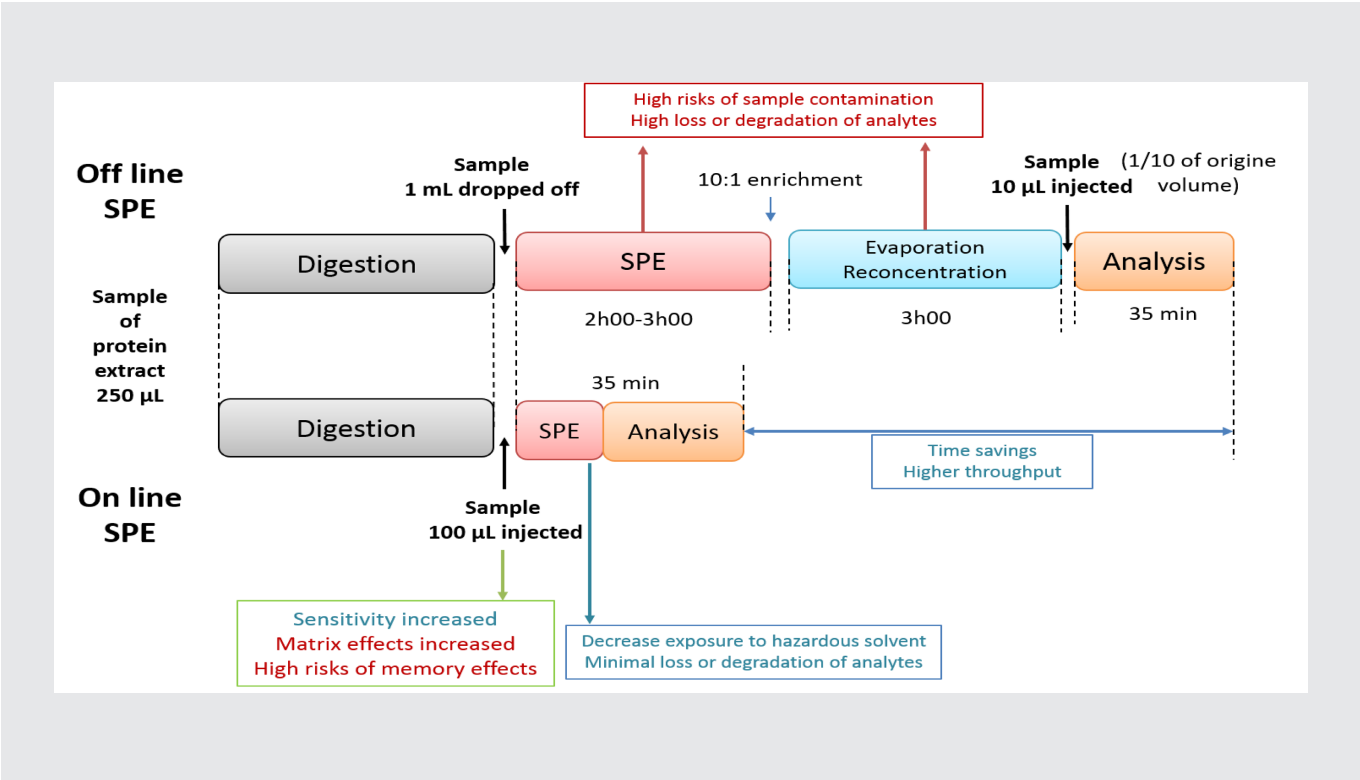

Figure 1. Comparison between off-line and on-line SPE-LC.

Optimisation of the HPLC Elution Gradient

The first attempt was to use the same HPLC gradient, as optimised in the off line SPE procedure [18]. To obtain lower peak broadening and better separation, the gradient profile was re-optimized. The final elution gradient started with 2 % of ACN/0.1 % formic acid and 98 % raising up to 33 % in 12 min, then 36 % in 6 minutes and a fast increase to 100 % in 1 minute to rinse the HPLC column before valve switching. The total gradient duration was therefore 19 min.

Optimisation of the on-line SPE steps

During the loading step, flow rates and sample volume are important in front of the online SPE cartridge void volume and may exert an influence on overall sensitivity. Different loading flow rates caused great differences in injection time. Therefore, different loading flow rate were tested and a flow rate of 1 mL/min was finally retained. As illustrated in supplementary **Figure 1**, for three peptides an injection volume up to 100 µL can be used without loss of linearity.

The on-line SPE column preconcentrates not only targeted peptides but also organic and inorganic impurities. Organic solvents are needed to elute hydrophobic impurities and high content water would rinse out the hydrophilic matter like ionic compounds. For the rinsing step, water and acidified methanol were used. Proportion of 5 %, 10 %, 15 % and 20 % of methanol or 1, 2, 3 min duration were compared. The maximum peak heights were obtained using 5 % of MeOH which allowed to flush enough of the matrix and to retain all of the analytes. Higher proportion of methanol showed lower signal and the loss of a few analytes that exhibit a low hydrophobic character. The rinsing time step was found optimal for 2 min. The resulting parameters of all these optimizations are reported in **Table 2**.

Comparison between off-line SPE and on-line SPE

After development of the on-line SPE method, 46 peptides corresponding to 30 proteins of interest were detected. Samples spiked with synthetic peptides corresponding to the 46 isotopically labelled proteotypic peptides of biological interest were purified in triplicate with off-line and on-line SPE and comparatively analysed in reversed phase chromatography. Results are reported in **Figure 2**. Histograms represent the number of peptides per interval of gain/loss factor. The abscissa represents peak height gain intervals (in %) of chromatographic peak for the 46 peptides most intense SRM transitions between off-line and on-line SPE are represented in abscissa. Improvement in peak height is associated with a better sensitivity. For example, a 100 % gain is equivalent to a doubled signal. **Figure 2** shows that there are 21 peptides for which the height gain is between 250 % and 500 %. The curve shows that 38 % of the peptides have a height gain and are more intense with an on-line SPE than with an off-line SPE. Half of the peptides (46 %) are not influenced by the modification of the purification protocol. Strikingly, the on-line process allowed the detection of one more peptide than the off-line process: peptide IVIDLLQQSTTVAQLR of the protein 15561 (Prophenoloxidase). **Figure 3** illustrates the chromatograms of some peptides from the mixture. The lower and upper panels present on-line and off-line SPE coupled C18 separation. Between the two panels, 3 SRM transitions for 3 peptides are extracted after separation in reversed phase LC and intensity gains are illustrated. For some peptides, intensity is higher after an on-line SPE extraction with transitions that can be ten times more intense in maximum.

Figures 2 and 3 results point out the analytical biases that can be generated during sample preparation especially during solvent evaporation and solubilisation of sample after evaporation. On-line SPE is faster and fewer losses are observed. Moreover, fewer samples are needed as the totality of the extract is injected. In off-line mode, only a small

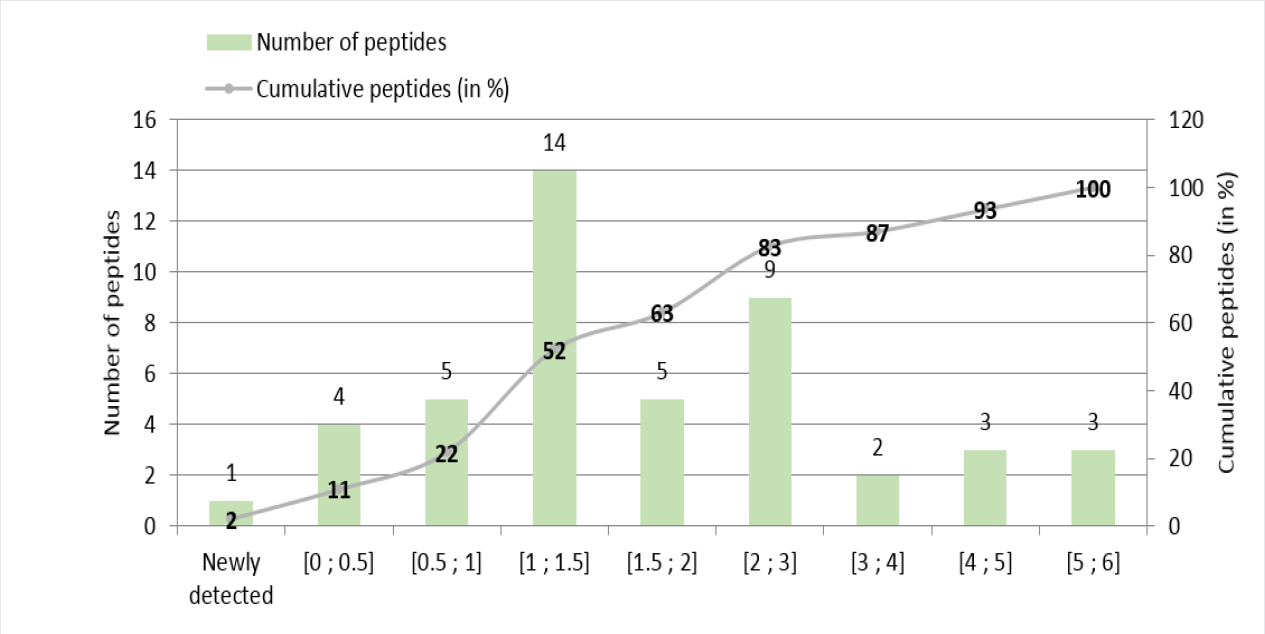

Figure 2. Height gain/loss factor between off-line SPE and on-line SPE for 46 tryptic peptides.

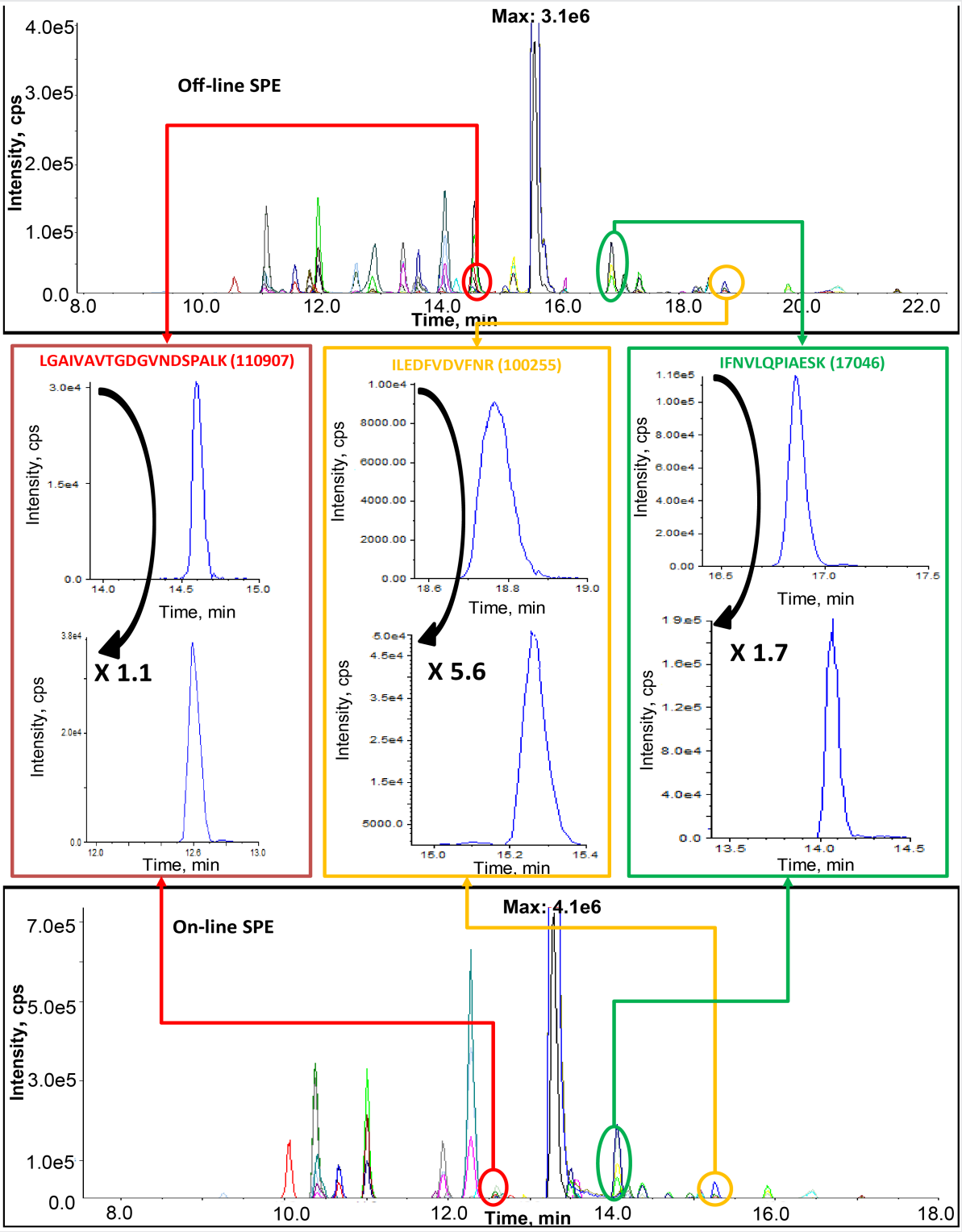

Figure 3. C18 separation with off-line SPE (upper panel) and on-line SPE (lower panel) of a set of tryptic peptides (3 SRM transitions are represented per peptide). Sensitivity enhancements are shown for 3 peptides (LGAIVAVTGDGVNDSPALK, ILEDFVDVFNR and IFNVLQPIAESK) with the most intense transition.

aliquot of extracted sample is injected. This is interesting in a multi-omics context analysis to perform multiple analyses with different operation procedures, such as Lipidomics, Metabolomics, and Proteogenomics.

Assay performance evaluation

After optimization of the on-line solid phase extraction, the next step is to assess the performance of the assay. Quantitation of proteins was performed by comparing peak height

Table 3. MRM assay linearity, sensibility and intra-day, inter-day precision, accuracy for each peptide.

Protein #	Target peptide sequence	Coefficient of Determination (R^2)	Multiplication factor (0.10-10 g/mmol) to have limits in mmol/mL	LOQ (ng/mL)	LOD (ng/mL)
1	HIEIFSPITK	0.996	8.443	21	6
2	HAEFSVNPPLDSTQAVK	0.995	5.435	160	48
	IIYPAEALTIVIEK	0.997	6.358	220	66
3	GTLAVIPVQNR	0.990	8.566	240	72
4	EVLEELPAQYMEFTR	0.990	5.391	240	72
5	APILEGYFSK	0.996	8.895	180	54
	GIDIIGDAFEADR	0.997	4.633	30	9
	ATQPSYTVAQLELPGVNITR	0.996	8.473	60	18
	GIDFGTTQSVR	0.989	7.187	70	21
6	KAEIGIAMGSGTAVAK	0.994	6.650	60	18
	LLEFEITGSTYEPIGDVFLGGQR	0.998	3.934	260	78
7	VLAVDILAK	0.999	10.625	30	9
8	VEC[CAM]IAGFILPLEFK	0.999	6.333	90	27
9	GGQWFGYDDISMIR	0.989	6.080	100	30
10	IVIDLLQQSTTVAQLR	0.993	5.561	370	111
11	IATGMQSALPEYAGTGIK	0.992	5.531	40	12
	IFNVLQPIAESK	0.998	7.361	60	18
	ITMQEDGSGEVQLK	0.997	6.516	70	21
	TSEVFLPLTNELYQQTK	0.997	4.972	70	21
12	KFLPSSGVDDLIK	0.989	7.049	140	42
	SYQILLLITNGGLSDIDATKK	0.992	4.418	80	24
13	VLSVVQNIITR	0.997	8.055	30	9
14	FVGLISLIDPPR	0.998	7.538	70	21
	NLAFFSTNAVEGTAR	0.993	6.259	130	39
15	AVAEIVQDYDSDGFFPALGFGGK	0.995	4.160	227	68

Proteins #: 1. Vittelogin (RF) - 64; 2. Hemolymph clottable protein (RF) - 276; 3. Transglutaminase (RM/I) - 1917; 4. Copine-8 (RM) - 2308; 5. Prophenoloxidase (RM/I) - 2562; 6. Ca transport ATPase sarcoplasmic/endoplasmic reticulum type-like isoform 1 (RM) - 4227; 7. Transglutaminase (RM/I) - 7169; 8. Hemolectin (I) - 11145; 9. Chitinase (Molt) - 12415; 10. Prophenoloxidase (RM/I) - 15561; 11. Clottable protein 2 (RF) - 17046; 12. Copine-8 (RM) - 18473; 13. Epididymal sperm-binding protein 1 (RM) - 18609; 14. Na/K-ATPase a1 subunit (BM) - 32234; 15. Copine-8 (RM) - 34845; 16. Clotting protein precursor (RF) - 39606; 17. Catalase (BM) - 45375; 18. Cytochrome P450 enzyme, CYP4C39 (Molt) – 100255; 19. Hemolectin (I) - 109695; 20. Na+/K+ ATPase (BM) - 110907; 21. Catalase (BM) - 110912; 22. Cytochrome P450 CYP12A2 (Molt) - 122081; 23. Farnesoic acid O-methyltransferase (RH) - 134275; 24. Glutathion S transférase (BM) - 142711; 25. JHE-like carboxylesterase 1 (RH) - 144144; 26. Farnesoic acid O-methyltransferase (RH) - 166723; 27. Chitinase (Molt) - 181833; 28. Vitellogenin (RF) - 200426 ; 29. Clotting protein precursor (RF) - 206469; 30. Cellulase (BM) - 213317. (Six general functions: RF: Female reproduction; RM: male reproduction; I: Immunology; RH: Hormonal regulation; BM: General biomarker and Molting).

Table 3 Cont'd. MRM assay linearity, sensibility and intra-day, inter-day precision, accuracy for each peptide.

Protein #	Target peptide sequence	Coefficient of Determination (R^2)	Multiplication factor (0.10-10 g/mmol) to have limits in mmol/mL	LOQ (ng/mL)	LOD (ng/mL)
16	ISPLINSPSDLPK	0.981	7.243	60	18
17	NLPADQAAALASSDPDYAIR	0.992	4.856	140	42
18	ILEDFVDVFNR	0.995	7.318	20	6
	VYAEVIEVAGSGPIGLDQLR	0.999	4.793	50	15
19	NAGPVLLPSNTSPVLR	0.998	6.117	40	12
20	LGAIVAVTGDGVNDSPALK	0.999	5.565	90	27
	VIMVTGDHPITAK	0.998	7.238	70	21
21	ADPALGQAIQER	0.995	7.884	50	15
	LADNIAGHVINTQEFIR	0.994	5.233	100	30
22	FNNNLINTR	0.998	9.048	100	30
	TLEELSNEALR	0.999	7.847	40	12
23	EVFIGGWSNQNSAIR	0.991	5.960	110	33
24	LSAWLAAC[CAM]K	0.998	10.393	30	9
25	AFWGSLPLR	0.999	9.558	60	18
	ILTTMWADFAR	0.988	7.550	20	6
26	EFWIATDHNEVR	0.995	6.594	30	9
27	LVLGTATYGR	0.995	9.522	30	9
28	ELTSAAEVVSSLLK	0.998	6.913	30	9
	TLGALELDVFSEAGK	0.999	6.453	40	12
29	AAIETAFVNHLK	0.989	7.613	210	63
30	ELFDFADAHR	0.996	8.195	70	21

Proteins #: 1. Vittelogin (RF) - 64; 2. Hemolymph clottable protein (RF) - 276; 3. Transglutaminase (RM/I) - 1917; 4. Copine-8 (RM) - 2308; 5. Prophenoloxidase (RM/I) - 2562; 6. Ca transport ATPase sarcoplasmic/endoplasmic reticulum type-like isoform 1 (RM) - 4227; 7. Transglutaminase (RM/I) - 7169; 8. Hemolectin (I) - 11145; 9. Chitinase (Molt) - 12415; 10. Prophenoloxidase (RM/I) - 15561; 11. Clottable protein 2 (RF) - 17046; 12. Copine-8 (RM) - 18473; 13. Epididymal sperm-binding protein 1 (RM) - 18609; 14. Na/K-ATPase a1 subunit (BM) - 32234; 15. Copine-8 (RM) - 34845; 16. Clotting protein precursor (RF) - 39606; 17. Catalase (BM) - 45375; 18. Cytochrome P450 enzyme, CYP4C39 (Molt) – 100255; 19. Hemolectin (I) - 109695; 20. Na+/K+ ATPase (BM) - 110907; 21. Catalase (BM) - 110912; 22. Cytochrome P450 CYP12A2 (Molt) - 122081; 23. Farnesoic acid O-methyltransferase (RH) - 134275; 24. Glutathion S transférase (BM) - 142711; 25. JHE-like carboxylesterase 1 (RH) - 144144; 26. Farnesoic acid O-methyltransferase (RH) - 166723; 27. Chitinase (Molt) - 181833; 28. Vitellogenin (RF) - 200426 ; 29. Clotting protein precursor (RF) - 206469; 30. Cellulase (BM) - 213317. (Six general functions: RF: Female reproduction; RM: male reproduction; I: Immunology; RH: Hormonal regulation; BM: General biomarker and Molting)

and/or peak area of extracted signal (peak height or peak area of the heavy and native forms of the proteotypic peptides). Generally, isotope dilution-based quantification methods display good linearity and excellent precision, whatever the quantification standard used. Standards correspond to synthetic peptides that can be spiked into the samples after the proteolysis step. The ratios of both peak areas are used to determine the precise amount of proteins in the sample extract because an absolute amount of labelled synthetic peptides is added. The internal standard is present after digestion as native peptides are formed, so that peptide extraction efficiency, absolute losses during sample handling (including sample concentration), and variability during introduction into the SPE-LC–MS/MS system do not normally affect the determined ratio of native and labelled peptide abundances. A wide range of concentrations for the standard was evaluated.

Table 3 Cont'd. MRM assay linearity, sensibility and intra-day, inter-day precision, accuracy for each peptide.

Protein #	QC1 (250 ng/mL)				QC2 (625 ng/mL)				QC3 (2500 ng/mL)			
	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)
1	17.8	103.8	17.9	85.2	10.5	99.3	15.8	105.0	6.2	100.1	4.6	99.2
2	18.1	89.3	19.7	114	15.6	110.7	18.3	106.0	10.5	94.2	15.9	98.8
	19.1	100.5	20.2	111.4	14.4	97.3	19.2	108.6	5.5	101.2	4.9	97.2
3	16.7	103.5	14.6	83	15.9	113.1	17.6	108.0	15.5	108.6	14.7	99.3
4	15.2	95.3	12.5	116	13.3	95.2	15.4	109.0	11.9	98.7	17.7	97.6
5	12.8	90.8	15.9	114	11.9	107.1	18.4	109.7	10.7	84.6	15.4	95.6
	12.3	104.9	16.8	85.1	9.0	98.2	11.3	107	10.9	112.3	9.7	101.1
	19.4	86.8	14.5	119	12.5	105.8	7.5	113.8	15.9	99.9	11.2	93.1
	21.2	99.1	19.5	85	19.7	99.8	15.6	88.9	17.8	99.8	16.1	97.2
6	20.9	100.1	17.5	81	18.7	89.9	17.5	116.1	9.9	102.6	7.6	98.0
	-	-	-	-	15.6	86.4	20.3	103.3	9.4	102.5	19.2	104.2
7	13.1	98.3	11.4	81.6	8.2	102.4	7.0	99.0	3.2	99.1	2.5	102.3
8	20.5	102.7	18.9	111.4	18.3	99.6	14.6	106.2	2.9	100.9	3.6	94.0
9	11.9	121.7	9.0	119.3	10.8	95.4	3.6	112.0	2.7	101.6	9.9	108.0
10	-	-	-	-	15.2	94.4	13.9	89.9	8.4	102.8	10.9	105.2
11	16.6	84.0	11.5	81.0	13.1	108	18.2	90.5	13.7	98.3	15.9	106.3
	16.5	102.4	18.6	118	8.6	94.6	17.3	107.8	3.0	101.7	4.5	96.0
	19.6	79.3	14.6	81	17.5	120	13.5	117.0	11.8	90.8	16.9	107.0
	15.9	105.3	19.1	118.7	15.6	96.6	17.2	107.0	4.6	101.6	10.9	93.1
12	14.9	106.7	20.7	118.0	14.5	94	19.6	111.0	9.5	101.1	18.2	98.0
	16.4	105.9	18.8	102.1	16.2	87.1	19.2	110.7	7.6	102.1	6.5	104.9

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	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)
13	18.4	88.4	19.5	88.2	17.7	98.7	21.1	110.1	7.8	99.5	9.6	107.0
14	19.7	93.6	18.2	85.3	13.5	102.7	16.8	114.9	3.0	100.6	5.9	95.0
	18.0	98.9	18.4	117.2	17.7	93.2	18.2	91	9.5	102.8	17.0	103.4
15	16.2	112.6	12.4	80.3	15.4	85.1	18.5	115.4	13.7	89.2	17.2	97.1
16	19.5	82.6	20.0	115.7	19.4	117.6	18.6	92.5	18.9	83.9	17.5	104.6
17	17.2	94.2	15.4	84.9	19.2	102.8	17.5	106.0	12.7	84.6	15.6	103.7
18	16.8	105.2	17.8	113.3	14.5	91.5	15.1	116.7	4.9	102.3	7.7	96.5
	19.7	109.2	18.4	104.9	14.9	99.8	12.7	92.5	13.5	99.9	5.6	99.6
19	15.6	88.6	14.1	121.2	15.1	100.5	15.9	92.9	4.6	100.1	10.3	101.4
20	15.9	105.1	20.7	113.5	9.0	96.1	8.5	93.0	4.0	101.1	5.9	96.2
	7.9	101.2	15.3	118.0	6.1	95.3	11.5	109.5	4.8	101.5	4.1	97.6
21	11.7	110.5	19.5	117.7	10.3	91.3	19.0	118	4.1	102.2	6.0	98.0
	15.0	95.0	17.2	84.0	13.7	106.6	16.1	89.9	13.4	97.3	14.6	109.0
22	20.7	96.3	15.2	88.2	10.1	104.3	18.4	96.6	9.8	98.7	9.2	103.0
	16.3	107.4	19.0	107.7	15.2	97.8	7.6	95.9	3.4	100.9	4.8	99.8
23	19.2	89.2	15.4	111.0	18.5	114.2	15.6	91.0	18.0	87.9	19.3	102.3
24	14.6	96.6	20.0	119.0	14.3	96.6	13.5	108.5	3.5	101.4	4.8	95.9

Proteins #: 1. Vittelogin (RF) - 64; 2. Hemolymph clottable protein (RF) - 276; 3. Transglutaminase (RM/I) - 1917; 4. Copine-8 (RM) - 2308; 5. Prophenoloxidase (RM/I) - 2562; 6. Ca transport ATPase sarcoplasmic/endoplasmic reticulum type-like isoform 1 (RM) - 4227; 7. Transglutaminase (RM/I) - 7169; 8. Hemolectin (I) - 11145; 9. Chitinase (Molt) - 12415; 10. Prophenoloxidase (RM/I) - 15561; 11. Clottable protein 2 (RF) - 17046; 12. Copine-8 (RM) - 18473; 13. Epididymal sperm-binding protein 1 (RM) - 18609; 14. Na/K-ATPase a1 subunit (BM) - 32234; 15. Copine-8 (RM) - 34845; 16. Clotting protein precursor (RF) - 39606; 17. Catalase (BM) - 45375; 18. Cytochrome P450 enzyme, CYP4C39 (Molt) – 100255; 19. Hemolectin (I) - 109695; 20. Na+/K+ ATPase (BM) - 110907; 21. Catalase (BM) - 110912; 22. Cytochrome P450 CYP12A2 (Molt) - 122081; 23. Farnesoic acid O-methyltransferase (RH) - 134275; 24. Glutathion S transférase (BM) - 142711; 25. JHE-like carboxylesterase 1 (RH) - 144144; 26. Farnesoic acid O-methyltransferase (RH) - 166723; 27. Chitinase (Molt) - 181833; 28. Vitellogenin (RF) - 200426 ; 29. Clotting protein precursor (RF) - 206469; 30. Cellulase (BM) - 213317. (Six general functions: RF: Female reproduction; RM: male reproduction; I: Immunology; RH: Hormonal regulation; BM: General biomarker and Molting)

Table 3 Cont'd. MRM assay linearity, sensibility and intra-day, inter-day precision, accuracy for each peptide.

Protein #	QC1 (250 ng/mL)				QC2 (625 ng/mL)				QC3 (2500 ng/mL)			
	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy %)	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)	Intra-day precision (RSD%)	Intra-day accuracy (%)	Inter-day precision (RSD%)	Inter-day accuracy (%)
26	19.2	101.8	15.9	107.8	19.1	96.4	17.5	105	6.1	100.9	6.0	99.1
	12.9	95.9	19.8	116.9	11.7	85.2	15.6	112.2	7.4	103.0	10.4	102.8
26	8.9	87.8	11.4	115.9	3.6	92.7	9.9	108.6	3.8	101.3	11.1	105.3
27	12.3	99.1	17.9	82.3	9.2	91.4	16.1	115.4	3.1	102.5	2.3	96.2
28	17.0	97.1	13.8	118.6	19.3	94.9	14.9	110.2	5.2	101.4	4.0	98.5
	9.6	103.0	18.2	108.9	10.7	96.3	11.3	105.6	2.6	101.1	6.9	97.9
29	18.8	90.9	10.0	110.0	4.9	106.3	6.5	90.3	3.4	94.1	1.4	97.7
30	12.2	101.5	18.2	107.0	11.7	90.8	18.3	106.3	5.7	101.4	6.0	104.7

Proteins #: 1. Vittelogin (RF) - 64; 2. Hemolymph clottable protein (RF) - 276; 3. Transglutaminase (RM/I) - 1917; 4. Copine-8 (RM) - 2308; 5. Prophenoloxidase (RM/I) - 2562; 6. Ca transport ATPase sarcoplasmic/endoplasmic reticulum type-like isoform 1 (RM) - 4227; 7. Transglutaminase (RM/I) - 7169; 8. Hemolectin (I) - 11145; 9. Chitinase (Molt) - 12415; 10. Prophenoloxidase (RM/I) - 15561; 11. Clottable protein 2 (RF) - 17046; 12. Copine-8 (RM) - 18473; 13. Epididymal sperm-binding protein 1 (RM) - 18609; 14. Na/K-ATPase a1 subunit (BM) - 32234; 15. Copine-8 (RM) - 34845; 16. Clotting protein precursor (RF) - 39606; 17. Catalase (BM) - 45375; 18. Cytochrome P450 enzyme, CYP4C39 (Molt) – 100255; 19. Hemolectin (I) - 109695; 20. Na+/K+ ATPase (BM) - 110907; 21. Catalase (BM) - 110912; 22. Cytochrome P450 CYP12A2 (Molt) - 122081; 23. Farnesoic acid O-methyltransferase (RH) - 134275; 24. Glutathion S transférase (BM) - 142711; 25. JHE-like carboxylesterase 1 (RH) - 144144; 26. Farnesoic acid O-methyltransferase (RH) - 166723; 27. Chitinase (Molt) - 181833; 28. Vitellogenin (RF) - 200426 ; 29. Clotting protein precursor (RF) - 206469; 30. Cellulase (BM) - 213317. (Six general functions: RF: Female reproduction; RM: male reproduction; I: Immunology; RH: Hormonal regulation; BM: General biomarker and Molting).

A wide range of the calibration curve from 50 to 5000 ng/mL was defined. Linearity, intra-run precision and accuracy, limits of detection and quantification, matrix effect were also established to evaluate the method's performance.

Linearity

Proteins were quantified based on the peptide response curves. These curves were generated from the LC-MS-MS analysis of labelled standard peptide samples and required that a given concentration level exhibit precision and accuracy to be qualified. A weighted ($1/x^2$) least-square linear regression of response versus concentration was used for the calibration. The calibration graph of the developed on-line SPE-LC-MS/MS method for the determination of the linearity of peptide was found to be linear, with a coefficient of determination $R^2 > 0.990$ for most of the peptides (40 peptides). Five other peptides have a correlation coefficient R^2 between 0.988 and 0.990, which is acceptable. The peptide ISPLINSPSDLPK for the protein 39606 (Clotting protein precursor function) has a the coefficient of determination (R^2) of 0.981, which is a slightly lower than the other peptides. However, this value stays in the acceptable range. All the results are given in **Table 3**.

Limit of detection and limit of quantification

To determine the LOD and LOQ, the strategy based on the signal-to-noise ratio is chosen here because it is faster. The LOD and LOQ were determined as the analyte concentration that produced a peak signal of three and ten times the background noise from the extracted ion current chromatograms, respectively. Results are shown in **Table 3**. LOD calculated are between 6 ng/mL for peptides HIEIFSPITK-Vitellogenin (RF) – 64; ILTTMWADFAR-JHE-like carboxylesterase n°144144; ILEDFVDVFNR-Cytochrome P450 enzyme, CYP4C39 (Mue)-100255 and 111 ng/mL for the peptide IVIDLLQQSTTVAQLR-Prophenoloxidase (RM/I)-15561. The corresponding LOQ were between 20 ng/mL and 370 ng/mL.

Precision and accuracy

Assay precision (SD/mean concentration x 100) and accuracy (mean determined concentration/nominal concentration x 100) were determined by analysing quality control (QC) samples in triplicate at 250, 625 and 2500 ng/mL, on the same days. Inter-day precision was obtained for the same three concentrations injected for three different days. As reported in **Table 3**, for the low QC level, the intra- and inter-assay mean precision were between 10% and 21% and mainly under 20 %. For the mid and high QC, the precisions were respectively under 20 and 15 %. All the accuracies are satisfactory and mainly between 85 and 115 %.

Evaluation of matrix effect

To assess matrix effects, post-extraction spikes method was chosen. The post-extraction spike method evaluates matrix effects by comparing the signal response of an analyte in neat mobile phase with the signal response in the blank matrix sample spiked post-extraction, for the same amount.

In our case, the matrix effects were determined for two concentrations levels 250 and 2500 ng/mL (i.e. QC1 and QC 3) for all the 46 peptides. All results from the comparison of the relative area of peptides in the pure solvent and that in *G. fossarum* extracted, are reported in **Supplementary Table 1**. At 250 ng/mL, ion signal enhancement ranges from 1.6 to 16.6 % for 26 peptides. The amount of ion signal suppression ranges from -1.3 to -12.2 % for 18 peptides. As expected, the matrix effects are less significant at 2500 ng/mL: an ion signal enhancement ranges between 0.9 and 9.1 % and ion signal suppression between -0.8 and -8.7 %. These results confirm that matrix effects are peptide dependent. In our case, matrix effect is low, staying in an acceptable range but it doesn't suppress the need to spike isotopically labelled peptides at known concentration in sam-

ples, which allow the quantification of peptides despite matrix effects.

Conclusion

In this study, a new on-line SPE-LC-MS/MS method for targeted quantification of protein biomarkers in *G. fossarum* was developed and validated. The developed method provided fast and highly efficient quantitation of 30 protein biomarkers by means of the monitoring of 46 reporter peptides without the need of time-consuming pre-treatment [18]. Due to the column switching system, the proposed method allowed an automated and faster sample preparation step compared to a conventional manually off-line SPE method. With a total duration of 30 min including the on-line SPE step, this method is much faster than previous methods, saving up to 6h per sample [18]. Furthermore, it exhibits higher sensitivity than the off-line SPE. Furthermore, here, less quantities of samples are necessary, and the cost of analysis is drastically reduced by the re-using the same SPE cartridge. Based on these results, this method can be recommended for the routine analysis of targeted quantification of protein biomarkers in *G. fossarum* species, and the principle could be easily applied to the monitoring of novel sets of protein biomarkers from any non-model sentinel species.

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