

# Determination of the neuropeptides arginine vasotocin and isotocin in brains of three-spined sticklebacks (*Gasterosteus aculeatus*) by off-line solid phase extraction-liquid chromatography–electrospray tandem mass spectrometry

Agnieszka Kleszczyńska<sup>a</sup>, Emmie Dumont<sup>b</sup>, Rita Cornelis<sup>b</sup>, Magdalena Gozdowska<sup>a</sup>, Ewa Kulczykowska<sup>a</sup>, Pat Sandra<sup>c,\*</sup>

<sup>a</sup> Department of Genetics and Marine Biotechnology, Institute of Oceanology of Polish Academy of Sciences, Powstańców Warszawy 55 Str, 81-712 Sopot, Poland

<sup>b</sup> Ghent University, Department of Analytical Chemistry, Proeftuinstraat 86, B-9000 Ghent, Belgium

<sup>c</sup> Ghent University, Laboratory of Separation Sciences, Department of Organic Chemistry, Krijgslaan 281 (S4 bis), B-9000 Ghent, Belgium

Available online 25 September 2006

## Abstract

A method based on solid phase extraction (SPE) followed by liquid chromatography–electrospray ionisation tandem mass spectrometry for the determination of the neuropeptides arginine vasotocin (AVT) and isotocin (IT) in brains of three-spined sticklebacks (*Gasterosteus aculeatus*) is described. Separation and detection were optimized using synthetic standards. Limits of detection (LOD) for standard solutions were 160 pg mL<sup>-1</sup> for AVT and 250 pg mL<sup>-1</sup> for IT. The SPE procedure hardly affected the LODs for standard solutions. Mainly because of ion suppression, LODs for AVT and IT in brains were approximately 5 and 25 pg mg<sup>-1</sup>, respectively. The concentrations determined in the brain of several fishes ranged from 10 to 500 pg mg<sup>-1</sup> for AVT and from 400 to 4000 pg mg<sup>-1</sup> for IT.

© 2006 Elsevier B.V. All rights reserved.

**Keywords:** Liquid chromatography; Electrospray ionization mass spectrometry; Arginine-vasotocin; Isotocin; Brain

## 1. Introduction

Arginine vasotocin (AVT) and isotocin (IT) are the evolutionary precursors of the mammalian neurohypophysial hormones, arginine vasopressin and oxytocin. Both neuropeptides (Fig. 1), which are produced in separate hypothalamic neurosecretory neurons located in the preoptic area-anterior hypothalamus (POA-AH), are stored and released in the posterior pituitary [1,2]. Arginine vasotocin is involved in many physiological events, such as osmoregulation and reproduction, and interacts with other endocrine systems in teleostean fish [3–7]. So far, the role of isotocin remains unclear.

Three-spined stickleback, a fish showing specific reproductive behaviour, is a perfect model widely used in many physiological and behavioural studies. Both vasotocin and isotocin seem to influence physiological processes and social behaviour in this species (unpublished observations). There is, however, not yet direct evidence for discrete actions of AVT and IT while present in endogenous concentration. Therefore, the development of sensitive and reliable assays for the measurement of both peptides in biological samples is of special interest.

The most common and sensitive techniques presently used for determination of human neurohypophysial hormones are radioimmunoassay (RIA) [8] and enzyme linked immunosorbent assay (ELISA) [9] with or without preceding purification by solid phase extraction (SPE) or chromatographic separation. To date, however, no commercial RIA or ELISA kits are available for vasotocin and isotocin. In biological samples only low concentrations of AVT and IT are present and,

\* Corresponding author at: UGent—PARC Krijgslaan 281-S4bis, B-9000 Ghent, Belgium. Tel.: +32 0 56204031; fax: +32 0 56204859.

E-mail address: [pat.sandra@richrom.com](mailto:pat.sandra@richrom.com) (P. Sandra).



Fig. 1. Structures of the neurohypophysials arginine vasotocin (AVT) and isotocin (IT).

moreover, matrices are very complex. Both factors hamper the detectability of the compounds. This can be partly overcome by pre-column purification of the samples by SPE.

Chromatographic techniques with fluorescence or mass spectrometric (MS) detection offer unique alternatives for simultaneous detection of both peptides in a single sample. These methods avoid the problem of antibody cross-reactivity associated with RIA. Using fluorescence detection, there is a need to derivatize the peptides prior to chromatographic analysis. Two different derivatization procedures have been described for the determination of AVT alone, namely the use of fluorescamine [9] and of monobromobimane [10]. Only one protocol for simultaneous AVT and IT determination using NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) has been described [11]. Limits of detection (LODs) were 0.8 and 0.5 ng mL<sup>-1</sup> for AVT and IT standards, respectively. This method was used to observe the diurnal and seasonal changes of the AVT and IT concentrations in the brain of several fish species amongst which those of the three-spined stickleback. Concentrations were in the range of 1.5–4 ng mg<sup>-1</sup> for AVT and 2–4 ng mg<sup>-1</sup> for IT [12]. Especially for AVT, concentrations differ from those observed in this study. This can be explained by the fact that the method used here is far more specific and sensitive. The difference in peptide levels may also arise from animals' physiological state (i.e. stress), sampling time and the fact that experiments were carried out on wildlife fish.

Ideally, concentrations of arginine vasotocin and isotocin in body fluids and tissues should be measured down to the femtomole level and robust, sensitive and specific methods are required to reach that goal. Quenzer et al. developed a method based on nano-LC-micro-electrospray ionisation Fourier transform ion cyclotron resonance MS for the determination of peptides in artificial cerebrospinal fluid. The method was used for the detection of AVT, isolated from the pineal gland of rainbow trout [13]. The highest sensitivity obtained with this method was 0.1 ng mL<sup>-1</sup> for AVT in water for a 1 µL injection.

In this contribution, a method based on solid phase extraction followed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) is presented. The assay was tested on extracts of the brain of three-spined sticklebacks (*Gasterosteus aculeatus*).

## 2. Experimental

### 2.1. Chemicals

All chemicals used were of analytical grade purity. Ultrapure Milli Q water was produced by using a Millipore system (Bedford, MA, USA).

Synthetic AVT and IT were obtained from BACHEM (Bubendorf, Switzerland). Formic acid, acetic acid, hydrochloric acid, acetonitrile, methanol and ethanol were purchased from VWR (Leuven, Belgium).

### 2.2. Instrumentation

SPE was carried out on speedisks cartridges (Baker Bond, C18, 20 mg) connected to a Baker SPE 12G column processor (J.T. Baker, Phillipsburg, NJ, USA).

The electrospray ionization tandem mass spectrometer (ESI-MS-MS) was a Quattro Micro system (Micromass, Manchester, UK), equipped with a Z-spray source. Liquid chromatography (LC) experiments were done on a Waters Alliance 2690 (Waters, Milford, MA, USA) using an Agilent ODS column of 10 cm length, 2.1 mm i.d., 3.5 µm particles (Agilent Technologies, Waldbronn, Germany).

### 2.3. Standard solutions

Standards of AVT (1 mg) and IT (1 mg) were dissolved in 1 mL Ultrapure Milli Q water and in 1 mL of a 20% (v/v) acetic acid solution, respectively. Further dilutions were made in an 0.1% (v/v) formic acid solution. To obtain MS spectra of the compounds, standard solutions of AVT and IT were prepared with a concentration of 25 µg mL<sup>-1</sup> in a 0.1% formic acid/methanol mixture (1:1, v/v).

### 2.4. Sample preparation

Adult three-spined sticklebacks (*G. aculeatus*) used in this study were caught in the Vistula river (Northern Poland) 2 h after sunset. Animals were dissected directly after catching. The brains of the fishes were removed, immediately frozen and stored at -70 °C until analysis.

Frozen brains (between 15 and 32 mg) were weighed and subsequently each complete brain was sonicated in a Microson<sup>TM</sup> XL 2000 (Misonix Inc., Farmingdale, NY, USA) in 1 mL 0.25% (v/v) of acetic acid. The sample was then placed in a boiling water bath for 3.5 min according to the procedure described by Pierson et al. [9]. The extracts were cooled in ice before centrifugation (1500 rpm, 30 min, 4 °C). To clean-up the samples, the supernatants (750 µL) were decanted and loaded on conditioned (2 mL methanol, 2 mL water) SPE speedisks cartridges [11]. The cartridges were successively washed with 0.5 mL water and 0.5 mL 4% (v/v) acetic acid. Finally, the peptides were eluted with 0.5 mL of 3 mM HCl in ethanol. The elutes were evaporated to dryness under a gentle stream of helium. The samples were re-dissolved in 0.5 mL of a 0.1% (v/v) formic acid solution for LC-ESI-MS-MS measurements. The recovery of AVT and IT on the SPE speedisks cartridges was >95% as ascertained by comparing the peak areas obtained via LC-MS-MS measurement for a 1 mL aliquot of a solution containing 1 ng mL<sup>-1</sup> of AVT and IT with and without SPE enrichment. A brain sample of 31.85 mg of a male fish caught in July 2005 (L47) was selected to demonstrate the performance of the method. Results for six brain samples, data

Table 1  
ESI-MS–MS settings for measurement of the nonapeptides AVT and IT

	SIR mode		MRM mode	
	AVT singly charged ion	IT singly charged ion	AVT doubly charged ion	IT doubly charged ion
Molecular weight (g/mol)	1050.2	966.2		
<i>m/z</i>	1051.2	967.4	525.6	483.7
Capillary voltage (kV)	3.4	3.4	3.0	3.4
Cone voltage (V)	65	40	10	20
Collision energy (eV)	0	0	2	2
Cone gas flow rate (L h <sup>-1</sup> )	50	50	50	50
Desolvation gas flow rate (L h <sup>-1</sup> )	350	350	350	350

Photomultiplier voltage: 650 V; source temperature: 120 °C, desolvation temperature: 200 °C.

which cover the whole series of samples analysed, are presented.

### 2.5. Separation conditions

AVT and IT were separated from each other and from other interfering compounds under the following optimized conditions. Mobile phase A consisted of 0.1% (v/v) formic acid and mobile phase B was 0.1% formic acid (v/v) in acetonitrile. A gradient programme was used starting with 10% B for 2 min, from 10 to 40% of B in 33 min and finally 100% of B for 5 min. After each analysis, the column was equilibrated for 10 min with 10% of B. The flow rate was 0.2 mL min<sup>-1</sup> and the injection volume 20 µL.

### 2.6. Standard addition

Because of the significant influence of the matrix of the biological samples on the ESI-MS–MS signal, standard addition was used for quantification of AVT and IT in the brains for each individual sample. The concentrations of AVT and IT standard solutions added to the samples were chosen after estimating the total concentration of both peptides from their response in ESI-MS–MS. All samples were divided over five vials all containing 90 µL of the brain extract. A standard addition curve was drawn by the addition of 10 µL of 0, 250, 500,

1000 and 2500 ng mL<sup>-1</sup> solution of AVT and IT to the brain extracts.

## 3. Results and discussion

### 3.1. Optimisation of the ESI-MS-MS detection of AVT and IT

For ESI-MS–MS measurements the positive ion mode was applied. Spectra with singly and doubly charged ions were obtained for both peptides after optimization of the MS parameters (Table 1). No product ion spectra were gathered for AVT and IT, even though high values of collision energy were applied. It seemed that both AVT and IT were too stable to fragment in the collision cell. The doubly charged ions gave the most intense peaks in the spectra and hence it was decided to measure AVT and IT on their doubly charged ions with *m/z* 525.6 and 483.7, respectively. Sensitivity of the method was subsequently enhanced for the analyses of real samples by measuring in multiple reaction monitoring mode (MRM), instead of measuring in the selected ion recording mode (SIR) [14]. Due to the complexity of the matrices several interfering compounds were observed when analysing the real samples in SIR mode. The *m/z* of the doubly charged ion was selected both as the precursor and the product ion. By applying low values of collision energy (2 eV),

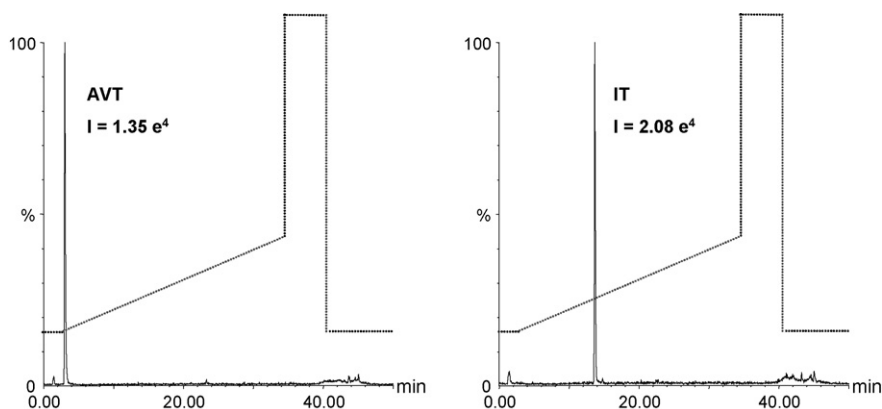


Fig. 2. LC-ESI-MS–MS chromatograms: MRM measurement of AVT (*m/z* 525.6) and IT (*m/z* 483.7) of a standard solution at 5 ng mL<sup>-1</sup>. Gradient elution (%B) is indicated with a dashed line.

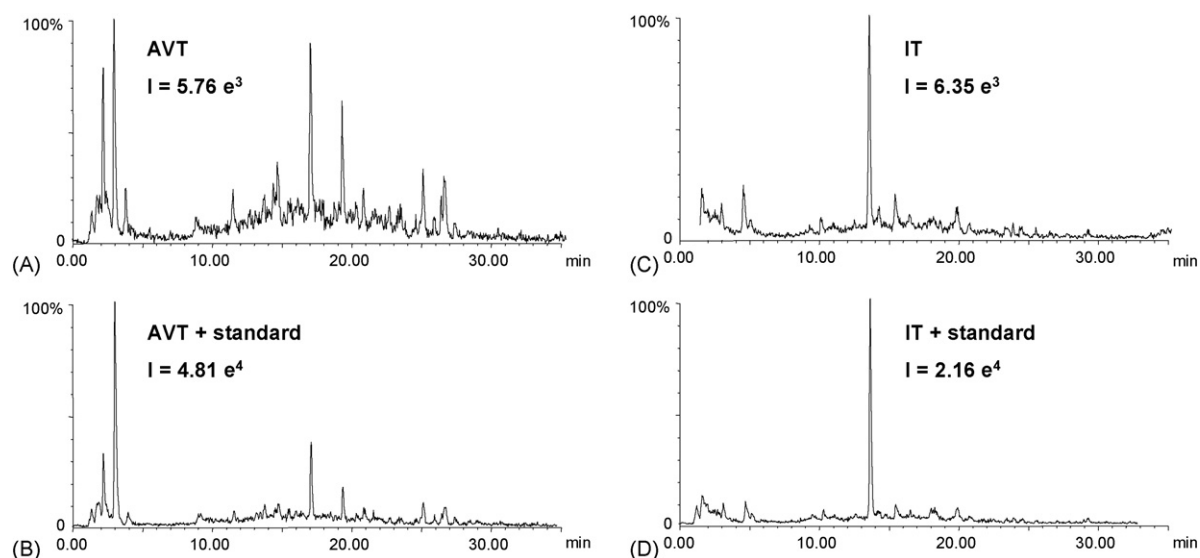


Fig. 3. LC-ESI-MS-MS chromatograms: detection of AVT (A) and IT (C) in the brain of three-spined sticklebacks (L47) and standard addition of AVT (B) and IT (D) at  $25 \text{ ng mL}^{-1}$  to the same brain sample.

interfering compounds were fragmented and lost, resulting in a lower background and subsequently better detection of AVT and IT in brain matrices.

### 3.2. LC-ESI-MS-MS of AVT and IT standard solutions

When hyphenating LC to ESI-MS-MS, the desolvation gas flow rate was raised to  $650 \text{ L h}^{-1}$  and the desolvation temperature was set at  $350^\circ\text{C}$ . Solutions of the compounds of different concentrations (ranging from 1 to  $500 \text{ ng mL}^{-1}$ ) were injected ( $20 \mu\text{L}$ ) on the LC-ESI-MS-MS system. The exemplary chromatograms of AVT and IT obtained for the standards at  $5 \text{ ng mL}^{-1}$  are presented in Fig. 2. Absolute responses are  $1.35 \times 10^4$  for AVT and  $2.08 \times 10^4$  for IT. The retention times ( $t_R$ ) for AVT ( $t_R$  3.02 min) and IT ( $t_R$  13.65 min) standard solutions were repeatable ( $\Delta t_R < 0.1 \text{ min}$  for six consecutive injections and  $< 0.2 \text{ min}$  for five injections at five consecutive days). By using a steeper gradient, it was possible to decrease the analysis time but for real sample analysis, this was accompanied by reduced signals because of matrix effects. Concentrations ranging from 1 to  $500 \text{ ng mL}^{-1}$  were in the linear dynamic range of the instrument.

LODs (three times signal-to-noise) were determined using standard solutions of AVT and IT ( $1 \text{ ng mL}^{-1}$ ) subjected to SPE and analysed using the methodology described in the experimental section. The LODs obtained for AVT and IT were  $0.16$  and  $0.25 \text{ ng mL}^{-1}$ , respectively. Solutions of both peptides were made at that concentration and analysed under the same conditions and both AVT and IT could be detected. LODs for AVT and IT in the brain extracts could not directly be determined, since no blank brain samples are available. Due to the explicit matrix effect of the brain on the ESI-MS-MS performance, LODs will definitely be higher in the brain extracts compared to those obtained in solution (see further).

### 3.3. LC-ESI-MS-MS measurements of AVT and IT in the brain of three-spined sticklebacks

For AVT and IT detection in the biological samples i.e. brains of three-spined sticklebacks,  $20 \mu\text{L}$  of the extracts were injected onto the column. The doubly charged ions were monitored in MRM mode on  $m/z$  525.6 for AVT and  $m/z$  483.7 for IT. The

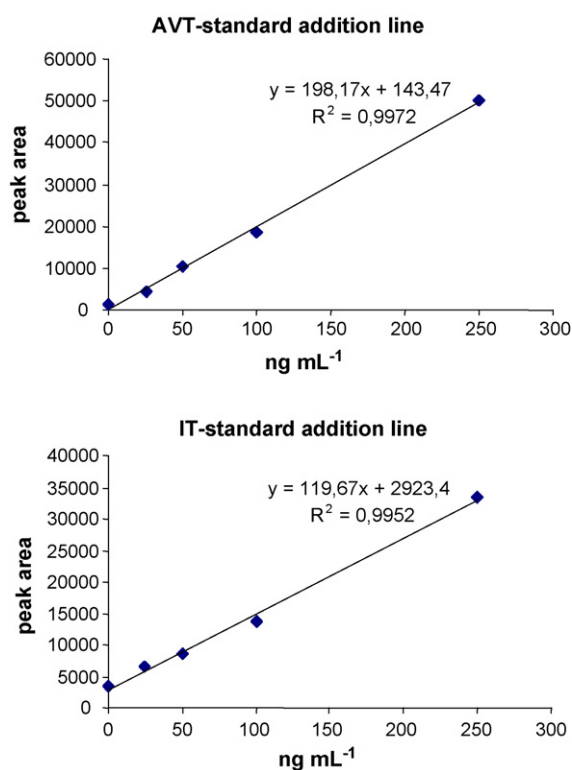


Fig. 4. Standard addition curves for AVT (upper figure) and IT (lower figure) of sample number L47.

Table 2  
AVT and IT concentrations determined in several fish brains

Sample	Mass of the brains (mg)	Gender	Month <sup>a</sup>	C <sub>AVT</sub> (pg mg <sup>-1</sup> )	C <sub>IT</sub> (pg mg <sup>-1</sup> )
L47	31.85	Male	July	12.6	426.1
G52	20.89	Female	December	215.1	1234.5
L49	18.85	Female	July	122.4	420.1
G45	15.35	Female	December	15.6	1197.9
L57	18.96	Male	July	521.7	4346.7
L68	28.70	Female	July	219.5	901.5

<sup>a</sup> December 2004, July 2005.

chromatograms for sample L47 are presented in Fig. 3A for AVT and in Fig. 3C for IT. The retention times of AVT and IT in real samples compared to the standard solutions showed a slight shift of ca. 0.1 min for AVT and of ca. 0.3 min for IT which is due to the sample matrix effect. Some additional peaks can be observed, demonstrating the complexity of this biological sample.

Standard addition of AVT (Fig. 3B) and of IT (Fig. 3D) at 25 ng mL<sup>-1</sup> to the sample (which underwent SPE clean-up before addition of the standards) was done to confirm their co-elution with both peptide signals.

#### 3.4. LC-ESI-MS-MS measurements: quantitative analysis of AVT and IT in the brain of three-spined sticklebacks

As no labelled standards are available for AVT and IT, quantification was done by matrix-matched standard calibration [15–17]. By spiking standard solutions of AVT and IT in the brain extracts a serious signal suppression due to the matrix was noted. For example, comparing the signals for AVT and IT in Fig. 2 (standard solutions) and in Fig. 3 (brain with and without standard addition of AVT and IT) ion suppression is a factor of 1.5 for AVT and a factor of 7 for IT. Quantitative analysis of AVT and IT in the brains was therefore performed for each individual sample by adding of 25, 50, 100 and 250 ng mL<sup>-1</sup> of AVT and IT. The standard addition curves for sample L47 are shown in Fig. 4. All solutions were measured in three-fold and the points represent the average values. The standard addition curves for both nonapeptides showed good linearity. The total concentration of AVT and IT in the extracts of the brain sample L47 were 0.72 ng mL<sup>-1</sup> for AVT and 24.43 ng mL<sup>-1</sup> for IT. Converting the concentrations of AVT and IT in the extracts to the concentrations in the brain, reveals that brain L47 contained 12.63 pg mg<sup>-1</sup> brain (equal to 0.36 pmol mL<sup>-1</sup> extract) of AVT and 426.11 pg mg<sup>-1</sup> brain of IT (equal to 1.2 pmol mL<sup>-1</sup> extract). Several other samples were measured and some representative data on the IT and AVT concentrations are shown in Table 2 together with information on gender and the time the fish got caught. The concentrations of AVT and IT range from 10 to 500 pg mg<sup>-1</sup> and 400 to 4000 pg mg<sup>-1</sup>, respectively.

#### 4. Conclusions

A method for the determination of the neurohypophysial peptides arginine vasotocin and isotocin in fish brains using LC in combination with SPE and ESI-MS-MS detection was

developed. Compared to fluorescence detection, the method offers higher selectivity and sensitivity allowing determinations in the femtomole range. The assay should be applicable with only minor modification to the analysis of other neurohypophysial nonapeptides in vertebrates, like mesotocin, glutitocin, valitocin, aspartocin which are present in biological samples at the same concentration range.

#### Acknowledgements

The authors thank Dr. Jianyu Peng for technical assistance. A. Kleszczyńska was granted a subsistence allowance from the Research Foundation Flandres, Belgium (Fonds voor Wetenschappelijk Onderzoek-Vlaanderen, België) and is a grant holder of the Institute of Oceanology of the Polish Academy of Sciences. E. Dumont is a grant holder of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT).

#### References

- [1] D. Saito, M. Komatsuda, A. Urano, *Neuroscience* 124 (2004) 973.
- [2] H.M. Van den Dungen, R.M. Buijs, C.W. Pool, M. Terlou, *J. Comp. Neurol.* 212 (1982) 146.
- [3] R. Acher, *Regul. Pept.* 45 (1993) 1.
- [4] D.J. Conklin, A. Chavas, D.W. Duff, L. Weaver, Y. Zhang, K.R. Olson, *J. Exp. Biol.* 200 (1997) 2821.
- [5] J.M. Warne, K.E. Harding, R.J. Balment, *Comp. Biochem. Physiol. B* 132 (2002) 231.
- [6] J. Godwin, R. Sawby, R.R. Warner, D. Crews, M.S. Grober, *Brain Behav. Evol.* 55 (2000) 77.
- [7] J.L. Goodson, A.H. Bass, *Nature* 403 (2000) 769.
- [8] B. Liu, J.P.H. Burbach, *Endocrinology* 121 (1987) 1716.
- [9] P.M. Pierson, M.E. Guibolini, N. Mayer-Gostan, B. Lahlou, *Peptides* 16 (1995) 859.
- [10] S.B. Catrina, M. Coculescu, M. Andersson, *J. Cell. Mol. Med.* 5 (2001) 195.
- [11] M. Gozdowska, E. Kulczykowska, *J. Chromatogr. B* 807 (2004) 229.
- [12] M. Gozdowska, A. Kleszczyńska, E. Sokolowska, E. Kulczykowska, *Comp. Biochem. Physiol. B* 143 (2006) 332.
- [13] T.L. Quenzer, M.R. Emmet, C.L. Hendrickson, P.H. Kelly, A.G. Marshall, *Anal. Chem.* 73 (2001) 1721.
- [14] M.J. Gomez, M. Petrovic, A.R. Fernandez-Alba, D. Barcelo, *J. Chromatogr. A* 1114 (2006) 224.
- [15] C. Jansson, T. Pichelstrom, B.G. Osterdahl, K.E. Markides, *J. Chromatogr. A* 1023 (2004) 93.
- [16] A. Sannino, L. Bolzoni, M. Bandini, *J. Chromatogr. A* 1036 (2004) 161.
- [17] A. Aguera, S. Lopez, R. Fernandez-Alba, M. Contreras, J. Crespo, L. Piedra, *J. Chromatogr. A* 1045 (2004) 125.