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Quantification of Gluten Exorphin A5 in cerebrospinal fluid by liquid chromatography–mass spectrometry

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Abstract

In the present work, for the first time, a method for the quantification of the alimentary opioid peptide Gluten Exorphin A5 (GE-A5; Gly-Tyr-Tyr-Pro-Thr) in cerebrospinal fluid (CSF) was developed. Aliquots (5 μL) of CSF were injected into a liquid chromatography–mass spectrometry (LC–MS) instrument equipped with a reversed-phase C18 column at a flow-rate of 0.4 mL/min. The mobile phase consisted of Eluent A water with 0.6% acetic acid as an ion-pairing reagent and Eluent B acetonitrile/methanol (75:25, v/v). The LC–MS system was programmed to divert column flow to waste for 4 min after injection, after which time flow was directed into the mass spectrometer that operated in positive ion mode. No significant interfering peaks were detected at the retention times of GE-A5 in CSF blanks. The lower limit of detection and the lower limit of quantitation values for GE-A5 in CSF were established at 0.60 and 1.50 ng/mL, respectively. The intra- and inter-day precision values were <5% relative standard deviation. The intra- and inter-day accuracy were 99.6–102.8% and 100.0–101.9%, respectively. The reported assay employs extremely small volumes of CSF, thus allowing the analysis of GE-A5 from both small and large animal models.
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Keywords: Gluten Exorphin A5; Cerebrospinal fluid; Liquid chromatography–mass spectrometry

1. Introduction

Literature data suggest that the digestion of food proteins may produce peptides having opiate effects [1]. These substances are generally known as "exorphins".

Gluten Exorphin A5 (GE-A5; Gly-Tyr-Tyr-Pro-Thr, Fig. 1) is an exorphin identified in enzymatic digests of wheat gluten [2,3]. GE-A5, when administered orally, seems to facilitate the acquiring/consolidation process of learning and memory and to suppress the endogenous pain-inhibitory system in animal models [4]. These effects on the central nervous system (CNS) strongly suggest that GE-A5 is able to cross the blood brain barrier; however, identification and quantification of this alimentary opioid peptide in cerebrospinal fluid (CSF) has not been reported.

Liquid chromatography–mass spectrometry (LC–MS) has proven to be an economical and effective tool in detecting opioid peptides in CSF [5]. We have therefore developed a quantitative assay for GE-A5 in CSF, using bench top single stage quadrupole LC–MS.

The synthetic peptide DADLE (Tyr-D-Ala-Gly-Phe-D-Leu), an opioid peptide with high stability in biological fluids in the absence of enzymatic inhibitors [6], was chosen as the internal standard (IS).

2. Experimental

2.1. Samples

Five samples of sheep CSF (20 mL each) from a tissue bank at the National Research Council (CNR) of Sassari, Italy, were used. The samples were pooled and then stored at −20 °C.

2.2. Materials

GE-A5 (peptide free base, MW 599.3) and the IS DADLE (peptide free base, MW 569.3) were obtained from Bachem

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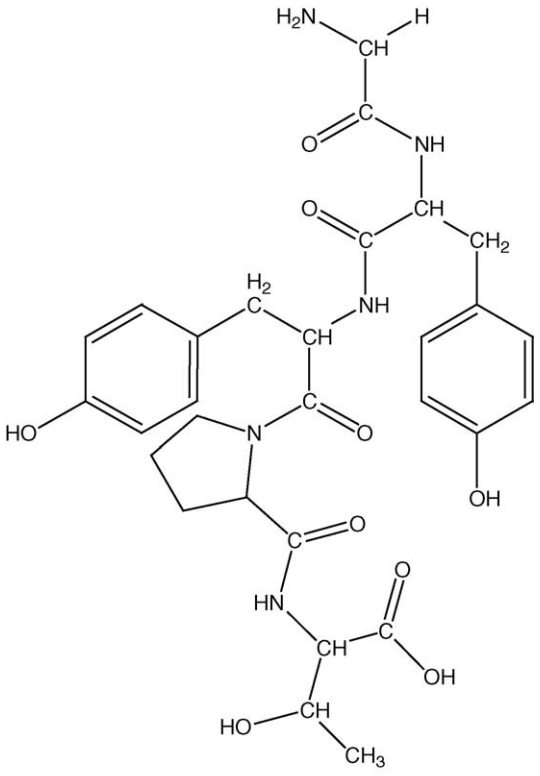

Fig. 1. Chemical structure of GE-A5.

Chimica (Milan, Italy). Both peptides were >99% pure. LC-grade methanol, acetonitrile, trifluoracetic acid (TFA), formic acid and acetic acid were procured from Mallinckrodt J.T. Baker (Deventer, Holland). Water was purified by a Milli-Q Academic System from Millipore (Bedford, MA, USA). Syringe filters (surfactant free cellulose acetate 0.2 μm 0.13 mm) were obtained from Nalgene Company (Rochester, NY, USA). The protease inhibitor aprotinin was obtained from Boehringer-Mannheim (Mannheim, Germany).

2.3. LC system

Separations in LC were performed using an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC/MSD equipped with a Diode-Array Detector and Rheodyne injector 5 μL loop. A ChemStation HP A.09.01 was used for data analysis. In our experimental conditions, GE-A5 displayed at 270 nm reasonable absorbance and no interfering peaks. Therefore, 270 nm was chosen as UV monitoring wavelength. Chromatographic separation was achieved using a Luna C18-(2) (150 mm × 4.6 mm, 3 μm, 100 Å) (Phenomenex, Torrance, CA, USA) fitted with a 3 μm Luna C18 security guard cartridge (4 mm × 2 mm). The column temperature was maintained at 25 °C. The mobile phase consisted of Eluent A water with 0.6% acetic acid and Eluent B acetonitrile/methanol (75:25, v/v). The mixtures were filtered through a 0.45 μm membrane prior to use. The separation was under gradient conditions with a flow rate of 0.4 mL/min and a run time of 20 min, and was followed by clean-up and equilibration stage (Table 1). The injection volume was 5 μL. The guard column was replaced after 100 injections, and the LC system was rinsed daily with 10 mL 100% methanol.

Table 1
Chromatographic gradient program

Time (min)	Eluent A (%)	Eluent B (%)	Flow (mL/min)
0	100	0	0.4
20	45	55	0.4
21	30	70	0.5
26	30	70	0.5
28	75	25	0.4

2.4. MS set-up

Mass spectrometric detection was performed by using an Agilent G1946 (MSD 1100) single stage quadrupole instrument equipped with an electrospray atmospheric pressure ionization (ES-API) source. The system was calibrated with the procedures provided by Agilent; the mass spectrometer was optimized with an infusion of 0.24 μg/mL GE-A5 at a flow rate of 100 μL/min. The LC–MS system was programmed to divert column flow to waste for 4 min after injection, after which time flow was directed into the mass spectrometer that operated in positive ion mode. For quantitative measurement of GE-A5, selected ion monitoring (SIM) was employed. The mass spectrometer was programmed to admit protonated molecules at the mass-to-charge ratios (*m/z*) of 600.3 (GE-A5) and 570.3 (DADLE) from 4 to 20 min after injection. The following ES-API conditions were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 9.0 L/min; nebulizer gas (nitrogen) at a pressure of 40 psi; capillary voltage in positive mode at 3350 V; fragmentor voltage at 90 V; dwell time was 460 ms.

2.5. Standard solutions

Stock solutions of GE-A5 were prepared by dissolving 4.8 mg of GE-A5 in 10 mL of methanol (final concentration 480 μg/mL). Stock solutions of DADLE were prepared by dissolving 4.9 mg of DADLE in 10 mL of methanol (final concentration 490 μg/mL). Stock solutions of GE-A5 and DADLE were stored at −20 °C in high-density polypropylene cryovials.

Working solutions of GE-A5 were prepared daily at the concentration of 0.24 μg/mL by diluting (1:2000) aliquots of the stock solutions with the solvent system (Eluent A) and were used to spike samples. Working solutions of DADLE were prepared daily at the concentration of 0.49 μg/mL by diluting (1:1000) aliquots of the stock solutions with the solvent system and were used to spike samples.

2.6. Standard curves

Six different concentrations of GE-A5 (0.96, 1.92, 9.60, 48.00, 67.20, and 96.00 ng/mL) were obtained by adding appropriate concentrations of working solutions in CSF and sol-

vent system. These solutions were used to construct standard curves.

2.7. Quality control (QC) samples

QC samples were prepared by fortifying CSF blanks with know quantities of the GE-A5 to obtain final concentrations of GE-A5 representative of the standard curve range (1.92, 19.20, and 67.20 ng/mL), and were stored at −20 °C.

2.8. Sample preparation

To a 25 μL aliquot of CSF standards, blank and QC samples, in a 0.5 mL high-density polyethylene tube, 5 μL of IS working solution were added and the resultant solution was vortexed for 5 s. A 5 μL aliquot of the solution was then injected directly into the LC–MS system.

2.9. Assay calibration

Calibration curves were produced by plotting peak area ratio of the analyte to the internal standard from the single ion chromatogram against the analyte's concentration ratio. The linear regression was fitted to the concentration range 0.96–96.00 ng/mL.

2.10. Sensitivity

The lower limit of detection (LLOD) and the lower limit of quantitation (LLOQ) were determined by analyzing ten replicates of CSF blank samples; LLOD and LLOQ were, respectively, defined as three and ten times the standard deviation (S.D.) of the LC–MS peak-areas detected at the retention time of the GE-A5.

2.11. Assay precision and accuracy

Intra- and inter-day precision and accuracy of the assay were assessed by performing replicate analyses of QC samples at the nominal concentrations of 1.92, 19.20, and 67.20 ng/mL. Intra-day repeatability was determined by analyzing spiked samples in replicate ($n = 5$) the same day. The procedure was repeated on different days ($n = 5$) to determine inter-day repeatability. The precision was expressed as relative standard deviation of the samples replicates over their mean values at each concentration. The accuracy was evaluated as:

$$\frac{\text{mean found concentration}}{\text{nominal concentration}} \times 100.$$

2.12. Matrix effect

A recovery study was conducted to investigate possible matrix effect on this assay. Two sets of standards, within the concentration range of 0.96–96.00 ng/mL, were prepared in CSF and in solvent system. Recovery was calculated by the following equation:

$$\%\text{Recovery} = \frac{\text{peak area slope of CSF standard curve}}{\text{peak area slope of solvent system standard curve}}.$$

2.13. Stability

The stability tests were designed to cover the conditions that the samples may experience during storage and freeze–thaw. Stability of GE-A5 in CSF, CSF plus aprotinin (10 μg of aprotinin/mL of CSF), and solvent system was evaluated in samples spiked with concentrations of 3.80 and 24.00 ng/mL. After thawing, samples were kept at room temperature and analyzed at regular intervals (60, 120, 240, and 480 min). Stability of GE-A5 in CSF during 3 freeze–thaw cycles was also evaluated.

3. Results

3.1. Specificity

The nominal retention times for GE-A5 and DADLE were 11.2 and 16.4 min, respectively. Throughout this study no significant interfering peaks were detected at the retention times of GE-A5 and IS in ten different CSF blanks. Fig. 2 illustrates a baseline signal typical of analysis near the method's LLOQ. A single column (with the above noted guard column replacements and daily methanol rinses) was employed for the duration of this study (12 months).

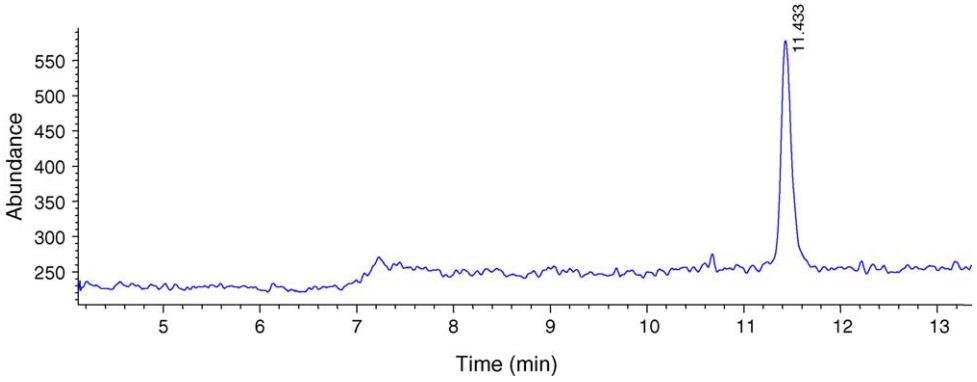

Fig. 2. Typical SIM chromatogram obtained from CSF fortified with GE-A5 at the concentration of 1.92 ng/mL.

Table 2
Intra- and inter-day precision and accuracy results for GE-A5 ($n = 5$)

Nominal concentrations of GE-A5 (ng/mL)	Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
1.92	2.65	4.99	102.10	101.04
19.20	0.61	0.66	99.60	100.01
67.20	0.21	2.10	102.82	101.88

Table 3
Recovery of GE-A5 in CSF

Nominal concentrations of GE-A5 (ng/mL)	Recovery (%) mean ± S.D.
1.92	88.54 ± 0.085
19.20	88.07 ± 0.112
67.20	83.05 ± 1.400

3.2. Linearity and sensitivity

Regression was linear over the tested concentration range (0.96–96.00 ng/mL), with an average correlation coefficient, R^2, of 0.998 (±S.D. 0.001), calculated from five calibration curves. The average slope and intercept were 0.989 ± S.D. 0.009 and −0.396 ± S.D. 0.089, respectively. The LLOD and LLOQ values for GE-A5 in CSF were established at 0.60 and 1.50 ng/mL, respectively.

3.3. Assay precision and accuracy

Intra- and inter-day precision and accuracy results are presented in Table 2. Intra- and inter-day precision values were <5%. Intra- and inter-day accuracy deviated by <103% from the corresponding nominal concentrations.

3.4. Matrix effect

Results of the comparison of solvent system standards versus CSF standards indicated that the recovery of GE-A5 in CSF samples was greater than 83% (Table 3).

3.5. Stability

In CSF samples without aprotinin, GE-A5 concentrations markedly decreased over time (−27.6 and −58.7% at times 60 and 480 min, respectively, in samples spiked with 3.80 ng/mL). In CSF samples added with aprotinin, GE-A5 appeared to be stable (−3.16% at time 480, in samples spiked with 3.80 ng/mL) (Table 4). Finally, after three freeze–thaw cycles GE-A5 concentrations were 3.75 ± S.D. 0.110 (starting from nominal concentrations of 3.80 ng/mL), and 23.16 ± S.D. 0.236 (starting from nominal concentrations of 24.00 ng/mL).

4. Discussion

Evidence to support that the digestion of food proteins may produce substances having opiate properties (exorphins) has been reported in the literature for the last two decades [1]. In humans, it has been hypothesized that these substances can act on the brain, and possibly play a pathogenetic role in some mental disturbances [7–9]. In sheep, where opioid peptides are involved in the regulation of the food intake [10,11], the role of exorphins on intake regulation and even the possibility to use exorphins to promote animal productivity are under study [12].

Gluten Exorphins are a family of small opioid peptides (tetra and pentapeptides) identified in enzymatic digests of wheat gluten [2,3]. It has been hypothesized that Gluten Exorphins reach the alimentary tract protected by the gluten proteins in which they are encrypted, and that they are released at the intestinal level by the combined action of gastrointestinal proteases [2].

GE-A5 appears to be one of the most interesting peptides among this family, due to its effects on CNS in animal models: pharmacological studies, in fact, indicate that GE-A5 administration influences pain inhibitory systems, emotionality, learning and memory processes in mice [4].

To date, there are no data on the minimum concentration of GE-A5 in CSF able to exert an effect on CNS. Although experimental data have suggested that intracerebroventricular administration of GE-A5 at the dosage of 30 μg/mouse produces antinociception [4], the possible effect of lower doses of GE-A5 has not been evaluated.

Therefore, we chose our analytical range (0.96–96.00 ng/mL) in order to allow both the quantification of GE-A5 levels compa-

Table 4
Stability of GE-A5 ($n = 4$)

Nominal concentrations of GE-A5	Concentration at time 60	Concentration at time 120	Concentration at time 240	Concentration at time 480
Stability in CSF				
I Level (3.80 ng/mL)	2.75 ± 0.103	2.50 ± 0.120	2.20 ± 0.183	1.57 ± 0.237
II Level (24.00 ng/mL)	23.18 ± 0.975	22.11 ± 0.864	20.61 ± 0.848	19.78 ± 0.919
Stability in CSF (with aprotinin)				
I Level (3.80 ng/mL)	3.88 ± 0.122	3.70 ± 0.130	3.67 ± 0.053	3.68 ± 0.110
II Level (24.00 ng/mL)	23.79 ± 0.783	23.46 ± 0.470	23.19 ± 0.577	23.35 ± 0.605
Stability in solvents				
I Level (3.80 ng/mL)	3.84 ± 0.047	3.67 ± 0.090	3.64 ± 0.064	3.73 ± 0.071
II Level (24.00 ng/mL)	23.78 ± 0.203	23.38 ± 0.139	23.37 ± 0.304	23.22 ± 0.258

Concentrations at the different times are expressed as mean ± S.D.

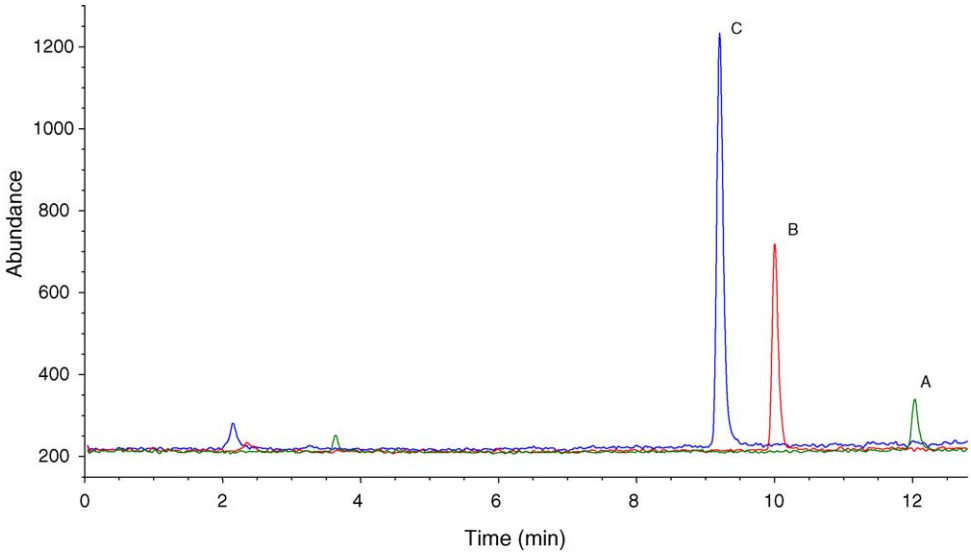

Fig. 3. GE-A5 peak height obtained with different organic acids: (A) TFA 0.2%; (B) formic acid 0.2%; and (C) acetic acid 0.6%.

rable to those observed for other (endogenous) opioid peptides in mammalian CSF [13], and the evaluations of higher concentrations, expected in pharmacokinetic and toxicity studies.

In our study, we did not detect GE-A5 in naive CSF fluid. This can be due to several factors, depending on the samples used and/or methodological aspects. Firstly, GE-A5 could be detectable only in few animals, and pooling the samples could bring the concentration of GE-A5 below the LLOD of our method. Secondly, GE-A5 could be detectable only in certain conditions, for example after feeding.

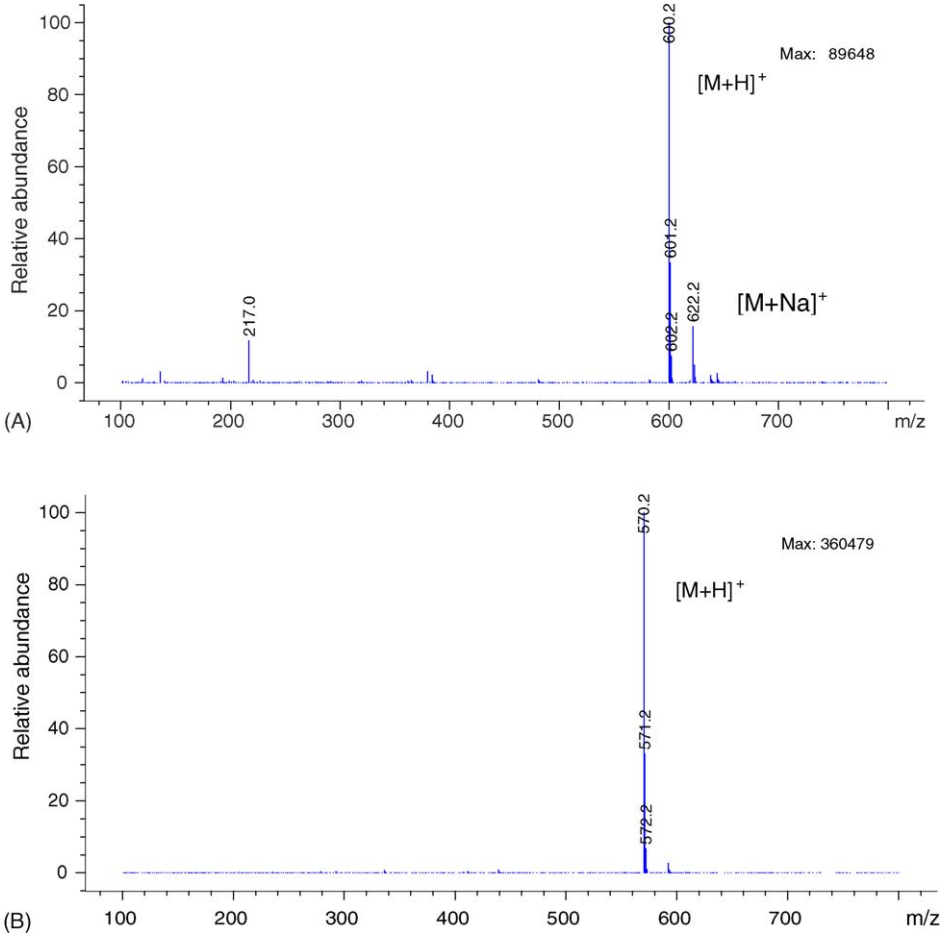

Fig. 4. Full scan mass spectra of: GE-A5 (A) and DADLE (B).

Moreover, conditions such as storage of the sample should be taken into consideration.

Our results indicate that 60 min after thawing (occurring at room temperature), GE-A5 concentrations in CSF are strongly reduced; this reduction is even more dramatic at 480 min. Under the same experimental conditions, the protease inhibitor aprotinin almost completely prevents the degradation of GE-A5. Therefore, use of protease inhibitors seems to be crucial in in vivo studies on the concentrations of GE-A5, especially when exposure to room temperature is expected or cannot be ruled out.

In our study, the selection of chromatographic column and aqueous-organic mobile phase for quantitative analysis of GE-A5 was based on the previous experience in analyzing other peptides and proteins [14]. In the course of method development, attempts were made in order to improve LC separation and to enhance MS sensitivity. Modifiers such as TFA, formic acid and acetic acid were added to mobile phases consisting of water–methanol or water–acetonitrile solutions. Mobile phases containing TFA solutions are routinely employed for this class of molecules, even though TFA was reported to suppress electrospray signals due to its ion pairing in the gas phase with the analyte ions [15]. To improve spraying performance at the LC–MS interface, in order to achieve adequate sensitivity, acetic acid 0.6% was chosen over TFA (tested at the concentration of 0.01, 0.05, 0.1, and 0.2%) and formic acid (tested at the concentration of 0.1, 0.2, and 0.6%). As shown in Fig. 3, by using acetic acid a better peak height was obtained than with the other organic acids. In the ES-API ion source, GE-A5 formed predominantly the protonated molecule $[M + H]^+$ at m/z 600.3. DADLE was also efficiently ionized to form its $[M + H]^+$ ion to m/z 570.3 (Fig. 4).

During the development of our method we did not detect any significant interfering peaks producing a signal at 600 m/z at the retention time of GE-A5 (no false positives). Indeed, GE-A5 behaves differently from other Gluten Exorphins, such as Gluten Exorphin B5 (Tyr-Gly-Gly-Trp-Leu), which shows the presence of interfering components of identical m/z (our unpublished data), thus requiring LC–MS/MS to structurally identify which component is Gluten Exorphin B5.

5. Conclusions

A quantitative method was for the first time developed for determination of the alimentary opioid peptide GE-A5 in CSF. The procedure, performed with bench top LC–MS instrumentation, was shown to be sensitive, precise and accurate. Moreover, the reported method employs extremely small sample volumes, so allowing the analysis of CSF from both small and large animal models.

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