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Quantitative profiling of the pathological prion protein allotypes in bank voles by liquid chromatography–mass spectrometry ☆

C. Cartoni [a,*], M.E. Schininà [b], B. Maras [b], R. Nonno [a], G. Vaccari [a], M. Di Bari [a], M. Conte [a], A. De Pascalis [c], S. Principe [c], F. Cardone [c], M. Pocchiari [c], U. Agrimi [a]

[a] *Istituto Superiore di Sanità, Department of Food Safety and Veterinary Public Health, Viale Regina Elena 299, 00161 Rome, Italy*
[b] *Department of Biochemical Sciences, University "La Sapienza", Rome, Italy*
[c] *Istituto Superiore di Sanità, Department of Cell Biology and Neurosciences, Rome, Italy*

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Abstract

The conversion of the cellular prion protein (PrP^{C}) into a misfolded isoform (PrP^{TSE}) that accumulates in the brain of affected individuals is the key feature of transmissible spongiform encephalopaties (TSEs). Susceptibility to TSEs is influenced by polymorphisms of the prion gene suggesting that the presence of certain amino acid residues may facilitate the pathological conversion. In this work, we describe a quantitative, fast and reliable HPLC–MS method that allowed to demonstrate that in the brain of $109^{Met/Ile}$ heterozygous bank voles infected with the mouse adapted scrapie strain 139A, there are comparable amounts of PrP^{TSE} with methionine or isoleucine in position 109, suggesting that in this TSE model the two allotypes have similar rates of accumulation. This method can be easily adapted for the quantitative determination of PrP allotypes in the brain of other natural or experimental TSE models.

Keywords: Prion protein; Quantitative method; Mass spectrometry

1. Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal neurodegenerative disorders that includes Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep [1].

The key event in TSEs pathogenesis is represented by the conversion of a membrane-linked glycoprotein – the cellular prion protein (PrP^{C}) – into an abnormally-folded isoform (PrP^{TSE}) that accumulates in the central nervous system of infected individuals [2,3]. The two proteins share the same amino acidic sequence and post-translational modifications [4], but they fold into distinct conformations: PrP^{C} is rich in α-helices while PrP^{TSE} has a higher content of β-sheet [5]. As a consequence, each conformer displays specific biochemical properties: PrP^{C} is a soluble protein that is easily digested by proteolytic treatments, whereas PrP^{TSE} is highly insoluble with a strong tendency to aggregate into amyloid fibrils that are partially resistant to proteases [2,6,7]. The mechanism of the pathological conversion of the prion protein is not fully understood, but it is widely accepted that it may occur through a seeded polymerization mechanism where small PrP^{TSE} nuclei (either spontaneously formed in the host organism or introduced from the outside) interact with PrP^{C} and facilitate the acquisition of the pathological conformation.

The presence of specific polymorphisms in the primary structure of PrP influences the susceptibility and the pathogenesis of natural [8–11] and experimental TSEs [12,13]. For example, in mice the PrP gene has two alleles, *prnp a* (encoding 108^{Leu} and 189^{Thr}) and *prnp b* (encoding 108^{Phe} and 189^{Val}) [14]: with some mouse-adapted scrapie strains, the incubation period of *prnp a/a* homozygous animals is shorter than in *prnp b/b* mice; with other strains the reverse situation occurs. The incubation period of heterozygous mice ($prnp^{a/b}$)

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* Corresponding author. Tel.: +39 06 49902854; fax: +39 06 49387077.
E-mail address: cartoni@iss.it (C. Cartoni).

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may lie between, or be even longer, than those of homozygous individuals [15]. A similar phenomenon may occur in sporadic CJD, where heterozygous $129^{Met/Val}$ individuals show longer mean survival time than homozygous $129^{Met/Met}$ or $129^{Val/Val}$ [16].

In bank vole (*Clethrionomys glareolus*), a wild rodent species highly susceptible to TSE from different species [17], the genotype composition at the polymorphic position 109 of PrP (encoding isoleucine or methionine) has a clear influence on the incubation times after infection with natural scrapie isolates, with $109^{Met/Met}$ voles showing shorter incubation period than heterozygous $109^{Met/Ile}$ animals [18].

The mechanism by which variations in the primary structure of PrP are critical for the development of TSE infection is only partially understood. Recent data suggest that in humans, the presence of methionine at the polymorphic position 129 may favor the acquisition of a beta-sheet-rich conformation, thus explaining the excess of $129^{Met/Met}$ genotype among sCJD, in comparison with the normal population [19]. In mice, residue 189 is believed to regulate the interaction and binding between PrP^{C} and PrP^{TSE}, while residue 108 is thought to influence the rate of conversion of PrP^{C} to PrP^{TSE} [20]. A similar phenomenon may occur in scrapie infected heterozygous bank voles where both PrP allotypes are involved in the formation of PrP^{TSE} [18].

The investigation of this hypothesis at the molecular level depends on the availability of analytical methods able to quantify the relative amounts of the two PrP^{TSE} allotypes accumulating in the CNS of diseased animals.

We have previously developed an HPLC ESI-MS (electrospray ion trap mass spectrometry) protocol that allowed to characterize the allotypes of PrP^{TSE} in the brain of infected heterozygous bank-voles [18]. The protocol relies on the identification of "reporter" peptides encompassing the polymorphic residue 109 that are produced after digestion of purified PrP^{TSE} and can be directly used for quantitative determinations with reference to a dedicated standard calibration curve built with serial dilutions of synthetic reporter peptides. The analysis of amyloid proteins extracted from biological tissues should resolve the possible effect of co-accumulating components that interfere with the solubility and chromatographic behavior of each reporter peptide. Thus, the quantitative protocol developed in this study was optimized on scrapie-infected rodents before it was used to quantify the relative amount of PrP^{TSE} allotypes that accumulates in brains of heterozygous bank voles infected with the 139A strain of scrapie.

2. Experimental

2.1. Chemicals

The synthetic peptides Thr-Asn-Met-Lys (TNMK), Thr-Asn-Ile-Lys (TNIK) were purchased from Inbios (Napoli, Italy). Trifluoroacetic acid and acetonitrile were purchased from Fluka (St. Louis, MO, USA) and were chromatography grade. All other reagents were of analytical grade available from Sigma–Aldrich (St. Louis, MO, USA).

Water was either purified in the laboratory using a Millipore Milli-Q water purification system (Volketswil, Switzerland) or was HPLC grade (Fluka).

2.2. Animals and Western-blot analysis

Two different lines of voles, carrying either the $109^{Met/Met}$ (Cg109MM) or the $109^{Ile/Ile}$ (Cg109II) genotype were selected. Groups of Cg109MM ($n=19$), Cg109II ($n=5$) and of heterozygous $109^{Met/Ile}$ bank voles ($n=12$) obtained by crossing Cg109MM × Cg109II were inoculated by the intracerebral route with 20 μl of a 10% (w/v) brain homogenate from mice affected by the 139A scrapie strain. Golden Syrian hamster ($n=5$) were inoculated intracerebrally with 50 μl of a 10% brain homogenate from hamsters affected by the 263K scrapie strain. Animals were housed in the animal facility of the Istituto Superiore di Sanità under the supervision of the Service for Biotechnology and Animal Welfare of the ISS who warrants the adherence to national and international regulations on animal welfare. Beginning one month after inoculation, the animals were examined weekly until the appearance of neurological signs, and then were examined daily until they reached the terminal stage of the disease when they were sacrificed with carbon dioxide. Brains were collected and immediately frozen. Survival time was calculated as the interval between inoculation and sacrifice.

The diagnosis of transmissible spongiform encephalopathy was confirmed by Western blot detection of PrP^{TSE} in brain tissues according to published protocols [17].

2.3. Sample preparation for HPLC–MS analysis

The protease resistant core of PrP^{TSE} (PrP27–30) was purified from diseased brains according to the protocol by Silvestrini et al. [21] with some modifications. Briefly, 3 g of cerebral tissue were homogenised in 9 ml of 10% sarcosyl (*N*-laurylsarcosine sodium salt) and kept 30 min at room temperature. The solutions were clarified in a Beckman rotor 100.3 at 22,000 × *g* for 20 min at 10 °C. The supernatants were ultracentrifuged at 200,000 × *g* for 90 min at 10 °C. Each pellet was resuspended by sonication in 1 ml of 10 mM Tris/HCl (pH 7.4) 10% NaCl, and 1% sarcosyl (TNS). The suspensions were pooled in two tubes (each containing 3 ml of suspension) and centrifuged at 200,000 × *g* for 90 min at 20 °C. The two pellets were resuspended in 1.5 ml of TNS and incubated overnight at 4 °C. The suspensions were sonicated and ultracentrifuged at 200,000 × *g* for 90 min at 20 °C. The resulting pellets were sonicated in 300 μl of TNS containing 10 μg/ml of proteinase K (Sigma, St. Louis, MO, USA) (10 U/mg) and incubated for 2 h at 37 °C, and the reaction was stopped by incubation in 0.2 mM of PMSF (Sigma) for 15 min at 4 °C. The suspensions were diluted with 500 μl of TNS and sonicated. The samples were ultracentrifuged at 200,000 × *g* for 90 min at 20 °C. The two pellets were sonicated in a solution of 800 μl of 0.1% sarcosyl and stirred for 2 h at 37 °C. Finally, the two suspensions were pooled and centrifuged at 200,000 × *g* for 90 min. Purified PrP27-30 samples were inactivated with 80% formic acid for

3 h at 4 °C and centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was neutralized with 0.5 M ammonium bicarbonate (pH 7.8). PrP27-30 suspension was desiccated in speed vacuum (Speed Vac Sc 110; Savant), re-suspended in 50 μl of digestion buffer (1 mM EDTA, 25 mM Tris/HCl pH 8.25 and 1% octyl-β-glucopyranoside), and incubated with 2.5 μg of bovine trypsin (Roche, Basel, Switzerland) at 37 °C for 24 h. Enzymatic digestion was stopped by adding 50 μl of 20% trifluoroacetic acid.

2.4. PrP27-30 allotypes profiling by HPLC–MS

Tryptic peptide samples were injected onto a reversed-phase C18 mass spec column (220 mm × 2.1 mm I.D., 5 μm particle size, 300 Å pore size, Vydac, Hesperia, CA, USA) and fractionated by HPLC on a LabService Analytica apparatus (model LabFlow 4000, Bologna, Italy), which was connected to an ion trap mass spectrometer equipped with an electrospray ion source (ES-IT, mod. LCQ, ThermoElectron, San Jose, CA, USA). Elution was performed at a flow rate of 150 μl/min with a linear gradient from 1% to 45% acetonitrile containing 0.05% trifluoroacetic acid in 90 min. Detection of the PrP^{TSE} peptides was achieved on-line by sequentially monitoring ion currents of the parent ions at *m/z* 493.2 ($^{107}TNMK^{110}$) and 475.3 ($^{107}TNIK^{110}$). Both the signal from the parent ions and their fragmentation patterns were acquired in the mass spectrometric cycle to increase the accuracy of peptide detection within the amyloid matrix. Source conditions have been optimised at an electrospray voltage of 5.15 kV and capillary temperature of 260 °C. SIM traces building, peak detection and peak integration were automatically achieved by the Excalibur software provided with the instrument.

2.5. Quantification procedure

For quantification purposes, stock solutions of synthetic peptides TNMK and TNIK were made in 10% trifluoroacetic acid (TFA). Identical volumes of these solutions were mixed and serially diluted to a range of concentrations of 2–16 μM in 0.05% TFA. To assess linearity in the ionization efficiency, mass spectra were acquired on five microliters (10–80 pmol) of each diluted solution by loop injection in 150 μl/ml flow rate of 1% acetonitrile in 0.05% acqueous TFA, and selectively monitoring of parent ions (expected MH^+ at 493.2 and 475.3, respectively) in the range of *m/z* 491.2–495.2 and 473.3–477.3. To evaluate the chromatographic yield of each synthetic peptide, identical aliquots were loaded on the C_{18} mass spec column and eluted following the conditions reported above. Finally, to evaluate the effect of PrP27-30 amyloid on peptide ionization efficiency, quantitative data were also achieved during the HPLC–MS analysis of the PrP^{TSE} allotypes profiling: immediately after the elution of the endogenous peptides $^{107}TNMK^{110}$ and $^{107}TNIK^{110}$, 5 μl of each calibration solution were loaded in the HPLC column at 5 min intervals. Quantifications were carried out by measuring Peak Area from SIM traces. Calibration curves were obtained from triplicate analyses by plotting Peak Area values versus peptide amounts.

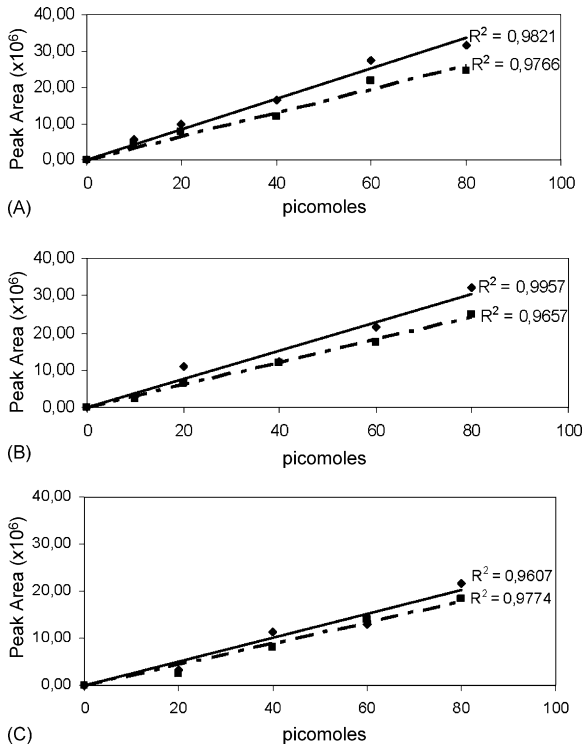

Fig. 1. Calibration curves of synthetic peptides TNMK and TNIK. Quantitative mass spectrometric data were achieved from 5 μl of serial dilution samples (2–16 μM) in 0.05% TFA, by sequentially monitoring ion currents of the parent ions at *m/z* 475.3 ± 2 (TNIK peptide; continuos line) and 493.2 ± 2 (TNMK peptide; dotted line), respectively. Peptide responses have been evaluated by loop injecting calibrating samples on mass spectrometer source (panel A), by elution from a C_{18} column (panel B) or by loading increasing amount of peptides on the same C_{18} column immediately after elution of the hamster prion endogenous $^{107}TNMK^{110}$ peptide (panel C). All calibration curves were obtained from triplicate analyses.

3. Results and discussion

3.1. Quantitative HPLC–MS method

Three different experiments were performed to build up calibration curves for the quantitative measurements of PrP27-30 $^{107}TNMK^{110}$ and $^{107}TNIK^{110}$ peptides.

In the first one, we only used increasing amounts (10–80 pmol) of synthetic peptides and measured their total ion current HPLC–MS response. Each peak was then plotted against the amount of analyte loaded in the HPLC–MS system. In Fig. 1A is drawn the linear response curve obtained after direct injections of peptide into the loop ($R^2 = 0.9766$ for peptide TNMK and $R^2 = 0.9821$ for peptide TNIK). These data show that it is possible to achieve reliable calibration curves with both synthetic reporter peptides by mass spectrometric setting. In the second experiment, we showed that similar linear response curves (Fig. 1B; $R^2 = 0.9657$ for peptide TNMK and $R^2 = 0.9957$ for peptide TNIK) were also obtained when both synthetic reporter peptides were eluted through an HPLC–MS system used for the analyses of purified PrP^{TSE} from scrapie infected brains.

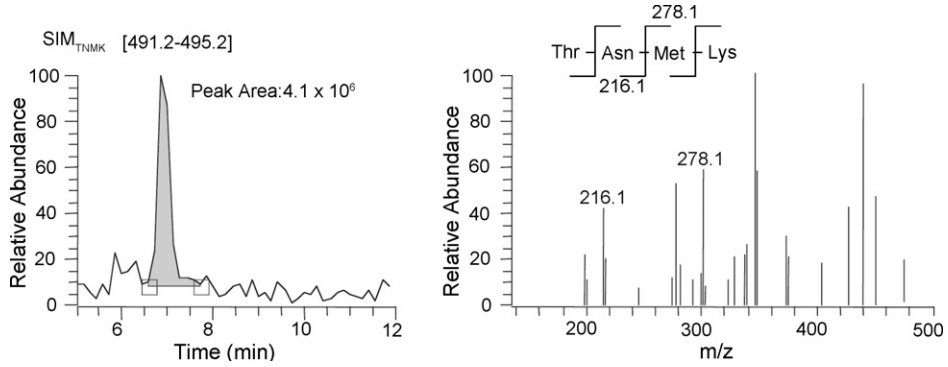

Fig. 2. Mass spectrometric detection of the hamster PrP27-30. Detection of peptide ^{107}TNMK^{110} was achieved by selectively monitoring parent ion currents (SIM mode) at *m/z* 491.2–495.2 (left panel). Assignment of the peptide was also achieved from its fragmentation pattern (right panel). Quantitative data are also reported, as peak area.

In the third experiment, we investigate the possibility that trypsinized PrP27-30 amyloid matrix may cause non-linear responses or alter peptide yields. This experiment was optimized with PrP27-30 extracted from brains of golden Syrian hamster infected with the 263K strain of scrapie and then confirmed with PrP27-30 from scrapie-infected bank voles. The 263K strain was selected because it produces large amounts of PrP^{TSE}, which after trypsin digestion produces a 107–110 peptide identical to the 109^{Met} in voles. Trypsin digested PrP27-30 was loaded in the HPLC system and as soon as the ^{107}TNMK^{110} peptide was eluted from the column (as revealed by monitoring the parent ion current and its fragmentation pattern, Fig. 2), the gradient was stopped and increasing amounts of synthetic reporter peptides were injected into the system and their peak areas recorded. This procedure was performed to maintain the possible matrix effect on the HPLC–MS system and calibrating samples. Quantitative MS data reveal that linear response curves were maintained (Fig. 1C, $R^2 = 0.9774$ for peptide TNMK and $R^2 = 0.9607$ for peptide TNIK). A similar negligible effect on the dose response curves of synthetic peptide was observed with the amyloid matrix of PrP27-30 isolated from Cg109MM and Cg109II bank voles (data not shown).

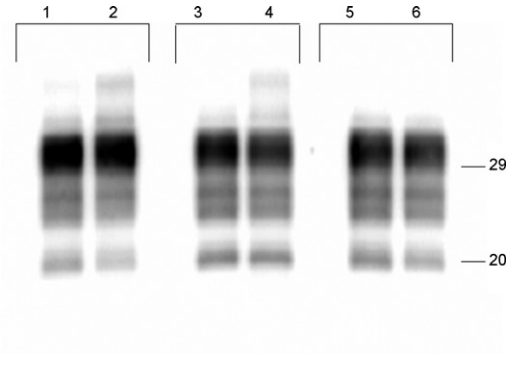

Fig. 3. Western blot analysis of PrP27-30 levels from brains of voles inoculated with the 139A strain of scrapie. Samples from two animals for each genotype (109$^{Met/Met}$, 109$^{Met/Ile}$, and 109$^{Ile/Ile}$) were loaded. Lanes 1–2, homozygous 109$^{Met/Met}$; lanes 3–4, heterozygous 109$^{Met/Ile}$; lanes 5–6, homozygous 109$^{Ile/Ile}$. All samples were treated with PK at 50 μg/ml for 1 h at 37 °C. PrP27-30 was detected by the monoclonal antibody SAF 84. The molecular markers are shown in kilodaltons on the right.

These data indicate that the HPLC–MS approach that we have previously developed for the identification of PrP^{TSE} allotypes [22–24] may also be used for the quantitative measurement of PrP^{TSE} allotypes with the required reliability and linearity [25].

3.2. *Quantitative determination of PrP^{TSE} allotypes in 109$^{Met/Ile}$ heterozygous bank voles*

After intracerebral inoculation of the 139A strain of scrapie into bank voles, 109$^{Met/Ile}$ heterozygous animals showed survival times (140 ± 25 days post inoculation, mean ± S.D.) similar to 109 homozygous voles (109$^{Met/Met}$, 135 ± 14 and 109$^{Ile/Ile}$, 139 ± 10).

Immunoblotting of PrP27-30 extracted from brains of bank voles at late stage of clinical disease showed similar amounts

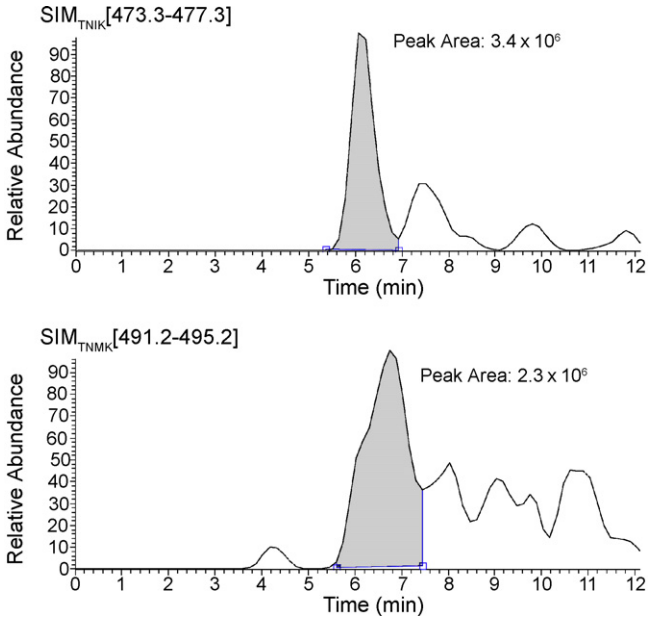

Fig. 4. Mass spectrometric detection of PrP27-30 allotypes from heterozygous voles inoculated with the 139A strain of scrapie. Simultaneous detection of ^{107}TNIK^{110} and ^{107}TNMK^{110} peptides was achieved both by monitoring parent ion currents (SIM mode) at *m/z* 473.3–477.3 (upper panel) and 491.2–495.2 (lower panel), respectively, and by fragmentation patterns (data not shown). Quantitative data are also reported, as peak area.

of PrP27-30 in the three different genotypes (Fig. 3). The amount of the two PrP^{TSE} allotypes was inferred by the measurement of their respective reporter peptides (^{107}TNMK^{110} and ^{107}TNIK^{110}) by the optimized quantitative HPLC–MS method.

Both 109^{Met} and 109^{Ile} allotypes were detected in the tryptic digest of PrP27-30. The amount of the reporter peptides, measured by the ratio between the areas under the peaks (Fig. 4), was similar for the two allotypes (about 10 pmol).

4. Conclusions

Apart from a single report on the identification of PrP^{TSE} allotypes by specific antibodies in a patient affected by E200K genetic CJD [26], no allotype-specific immunological reagents have ever been described in the literature or put on the market. Thus, in the absence of such reagents the application of any immunological method for the quantitative measurement of PrP^{TSE} allotypes is presently unfeasible.

The method described in this paper allows the quantification of amounts of PrP^{TSE} allotypes with analytical sensitivity at picomolar levels. This was achieved by a quantitative implementation of a proteomic strategy based on coupling liquid chromatography with ESI-MS analysis for the identification of proteolytic PrP fragments encompassing polymorphic residues [18].

Quantitative HPLC–MS analysis of different peptides in a mixture is expected to suffer from heterogeneities in ionization efficiencies and different responsiveness related to the amount of peptides. Two alternative procedures have been developed for peptide quantification. One is to add labelled synthetic peptides into the protein mixture as standards for relative or absolute quantification [27]. Alternatively, quantification of peptides can be performed without internal standards when uniform ESI-MS responses are obtained under specific experimental conditions [28].

This approach was applied to measure the amount of two PrP^{TSE} allotypes, which accumulate in the brain of heterozygous $109^{Met/Ile}$ voles affected by experimental scrapie (139A strain). The finding that similar amounts of the two PrP27-30 allotypes accumulate in the brain of heterozygous voles suggests that in this experimental TSE model the presence of 109^{Met} or 109^{Ile} PrP does not influence the pathogenesis of the disease. This is further confirmed by the similar incubation periods observed in 139A scrapie-infected heterozygous and homozygous voles and by the comparable amount of PrP27-30 observed at western blotting.

This fast and reliable quantitative HPLC–MS method might provide an important tool for the investigation of the preferential accumulation of PrP^{TSE} allotypes in the brain of human or animal TSEs where the PrP polymorphism plays an important role in the phenotypic manifestation of the disease [16].

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