Review Paper

Analytical techniques for serratiopeptidase: A review

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ABSTRACT

A review is presented on different analytical techniques used for qualitative and quantitative analysis of serratiopeptidase, a proteolytic enzyme, which has recently gained importance as an anti-inflammatory agent. Efforts have been made to collate all the relevant references to the extent possible. The review discusses the advantages and disadvantages of the cited analytical techniques, which will help to give insights into the methods used for estimation of serratiopeptidase as such, from clinical isolates and from its dosage forms. The review highlights the basic as well as advanced techniques performed for estimating serratiopeptidase. The techniques illustrated here have been demonstrated to be useful for qualitative and quantitative determination of serratiopeptidase and may find application in analyzing other related proteases.

1. Introduction

Proteolytic enzymes are specific enzymes that metabolize protein. These enzymes are naturally produced by human body and other living organisms and help carry out essential functions. Supplements with these enzymes show a favorable anti-inflammatory effect on the tissues of the body.

A unique enzyme named 'serratiopeptidase' (Serratia E15 protease) has emerged as one of the most potent anti-inflammatory supplements and has EC number 3.4.24.40. Serratiopeptidase having molecular weight of about 52 kDa was first isolated from enterobacterium *Serratia sp*. This microorganism was originally isolated in the late 1960s from silk worm *Bombyx mori* L. Serratiopeptidase is presented in the silk worm intestine and allows the emerging moth to dissolve its cocoon. It is produced by purification mainly from fermentation of *Serratia marcescens* or *Serratia sp. E 15*. The enzyme belongs to Serralysin group of enzymes and is known to cleave the peptides with linkages of Asn-Gln, CysSO$_3$H-Gly, Arg-Gly, and Tyr-tyr as well as the bond between His-Leu, Gly-Ala, Ala-Leu, Tyr-leu, Gly-Gly, Phen-Tyr, and Tyr-Thr, showing broad substrate specificity [1].

Serratiopeptidase has been used in Europe and Asia for over 30 years, but is relatively new in the United States and Canada [2]. It has powerful anti-inflammatory properties. Clinical studies have shown that it is effective in reducing swelling and edema and metabolizing scar tissues in the body [3,4] and particularly useful for post-traumatic swelling, fibrocystic breast disease and bronchitis [5–8]. It can digest dead tissue, blood clots, cysts, and arterial plaques [9]. The anti-inflammatory properties of serratiopeptidase was first studied in Japan in 1967. Later during the 1970s these parenteral enzyme formulations were replaced by their enteric coated successors. During the 1980s and 1990s it was proposed by separate research conducted in Europe and Japan that serratiopeptidase is the most effective agent in reducing inflammation among all enzyme preparations.

Serratiopeptidase is used either alone or in combination with other drugs to treat inflammation. It is proved to be a superior alternative to traditional NSAIDS like diclofenac sodium and ketoprofen which have pronounced side effects. Serratiopeptidase is also referred as serra-peptase which has been used to treat chronic sinusitis, carpal tunnel syndrome, sprains, torn ligaments, and postoperative inflammation [10–12]. Thus serratiopeptidase is proved to be a stronger caseinolytic agent than any other known alkaline or neutral proteases.

Most of the studies published so far are either on clinical efficacy of serratiopeptidase, or on its production by fermentation and purification. A review on serralysin was published in 2004 by Baumann [13] who described the functional class of serratiopeptidase, its occurrence, biological function, amino acid sequence, its production, purification and molecular characterization.

The major goal of this review was to summarize the analytical techniques currently used for the determination of serratiopeptidase as well as provide critical insights into this field. Primary attention is given to emphasizing different methods which are employed for determination of serratiopeptidase, which may be from serum, plasma which are clinical isolates, fermentation or different dosage forms. Many of these advanced techniques may be seen an increased application in the analysis of serratiopeptidase or related enzymes from different sample matrices in the future.

2. Qualitative and quantitative analytical techniques for serratiopeptidase

2.1. Qualitative techniques

Qualitative analysis can be accomplished by simple classical chemical tests, measurement of physical constants such as melting point/boiling point, refractive index, or chromatographic techniques using a reference standard.

2.1.1. Thin layer chromatography (TLC)

TLC is a simple, quick, inexpensive chromatographic separation technique used to identify a compound wherein the R_f can be compared against that of the standard, and is routinely used in synthetic laboratories to monitor the completion of reactions. It was successfully applied by Kyoko et al. [14] for quality testing of different high molecular weight biomolecules. They used thin layer gel filtration chromatography in which phosphate buffer was used as solvent and nigrosine as the detection reagent. Biomolecules separated by this technique include serratiopeptidase, lysozyme, urokinase, cytochrome C, trypsin, alphachymotrypsin, streptokinase and insulin. This preliminary analytical technique, which is age old, simple but mostly applied for small molecules for process monitoring, has been extrapolated to large molecules like serratiopeptidase. This technique, though easy to use and fast, can be utilized only for qualitative purpose and identification of serratiopeptidase, and has disadvantage like long plate running time and laboriousness. This technique, though modern, can be used as an analytical tool but the preferred and commonly used technique for identification of proteins is sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), rather than thin layer gel filtration chromatography.

2.1.2. Electrophoretic techniques

Electrophoretic techniques are specifically applied to large molecules such as serratiopeptidase. Serratiopeptidase was qualitatively analyzed by SDS-PAGE [15] and its apparent molecular weight was determined in its characterization carried out by Ananthakrishnan et al. [16]. Its activity determination was done by enzymatic assay. The serratiopeptidase was obtained from UV-mutated strain of *Serratia marcescens*. Different sources of carbon and nitrogen were evaluated to select the best media composition to achieve the maximum activity. The best source of nitrogen was tryptone and the best source of carbon was maltose, as per the study. The activity determination was done by caseinolytic assay as cited in the Indian pharmacopeial monograph of serratiopeptidase. As stated earlier, SDS-PAGE is the preferred and the most common routine technique utilized for protein identification.

2.1.3. X-ray powder diffractometry (XRPD)

XRPD was used to identify qualitatively the peaks of the main active ingredient serratiopeptidase and its excipients by Kazuyuki et al. [17]. This technique was utilized during preformulation studies as well as for identification of serratiopeptidase by X-ray profile in final dosage forms such as tablets and capsules. X-ray diffractometry was routinely used for determination of authenticity of serratiopeptidase and for dispensing of dosage forms in hospital pharmacies. XRPD was found to be a very useful technique as complete analysis of ingredients in mixed powder or codeless tablets or capsules was near to impossible and hence it served as a fast and reliable technique which could segregate fake tablets from the authentic ones based on the X-ray diffractogram as per the authors of this paper.

X-ray studies were performed on the serratiopeptidase obtained from *Serratia sp. E15* by crystallography and small angle light scattering techniques as presented by Yoshio et al. [18]. Serratiopeptidase was crystallized in three different forms. Form I had orthorhombic space groups C2221 while other 2 forms had P212121. The radius of gyration and maximum dimension of serra-

tiopeptidase, as determined by small angle scattering, were 26.6 and 95.4ºA. The molecular weight of serratiopeptidase was also determined to be between 45000 and 48000 Da by the known methods. These advanced techniques have been advantageously applied by these researchers for serratiopeptidase. But as this technique is very expensive, the cost involved in purchase of the instrument and its upkeep may prevent its use for estimation of serratiopeptidase as a simple routine tool. Hence serratiopeptidase has been estimated using simple techniques such as TLC, SDS-PAGE to modern techniques like XRPD.

2.2. Quantitative techniques

Analytical techniques for quantitative analysis help an analyst to accurately determine the concentration of individual component in the test sample. The separation of analyte is often performed before analysis by classical methods or during analysis by instrumental methods. Wet chemistry methods such as titrations, precipitations, and extractions are used to quantitate a drug or an analyte. Classical methods use weight or volume as the quantitation parameter.

Quantitative instrumental methods use chromatography, field flow fractionation, electrophoresis, etc. for separation, and physical properties such as absorption, fluorescence, conductivity, and light scattering are utilized to measure the analyte accurately and precisely.

The first and the most commonly used quantitative analytical technique for serratiopeptidase is the enzymatic activity determination. Serratiopeptidase as a protease catalyses the cleavage of protein or peptide to yield soluble products which can be estimated quantitatively using different methodologies. Caseinolytic assay is still a widely used analytical method to estimate serratiopeptidase using casein as the substrate. The detailed assay procedure is published in FCC and pharmacopeias. There are innumerable publications based on this simple basic technique but a few were selected here.

2.2.1. Caseinolytic assay

The studies published by Tariq et al. [19] discussed about purification and characterization of serratiopeptidase of molecular weight 56 kDa which was isolated from the *Serratia marcescens* by ammonium sulphate precipitation and subsequently by 80% saturated acetone; further the serratiopeptidase was fractioned on DEAE column to obtain the pure form. The pI and optimum pH at which the enzyme shows maximum activity were found to be 6.4 and 8, respectively. The activity of the serratiopeptidase was determined by caseinolytic assay similar to the Japanese pharmacopeial method. This technique is used widely as it is reliable and reproducible when the enzyme is in its pure form. This technique suffers from the interference from components similar in nature to serratiopeptidase or from serine proteases or other proteases which have proteolytic property and may be present in the sample. These enzymes can easily cleave casein to tyrosine, which is estimated by this method.

The same technique was used in the purification and characterization of protease produced by *Pseudomonas aeruginosa* which was carried out by El-Hawa et al. [20]. The extracellular protease was extracted and purified from culture broths of the microbe by ammonium sulphate precipitation and ethanol precipitation. The enzyme was fractionated using sephadex G100 column. The optimum pH was found to be pH 9 and pH stability was determined in the range of 8–10. The optimum temperature at which the enzyme showed maximum activity was 60 ºC. As a comprehensive study, inhibitors and activators of serratiopeptidase were also studied.

The studies carried out by Salamone et al. [21] also utilized the proteolytic assay to analyze isoform of serratiopeptidase produced by soil isolates of *Serratia marcescens NRRL B 23112* using casein and bovine serum albumin as substrates. The enzyme was purified and characterized. Purification was carried out by ammonium sulphate precipitation and further by acetone. Preparative iso-electric focusing was used to separate and purify serratiopeptidase. The molecular

weight of the enzyme was determined by SDS-PAGE and was 50.9 kDa. The optimum parameters of the enzyme such as pH, temperature, its inducers and inhibitors were determined in this study. The serratiopeptidase obtained as presented in this paper differed from that produced by *Serratia marcescens ATCC 27117* in terms of pI, peptide mapping and neumatolytic properties.

Zeng et al. [22]described the same caseinolytic assay method in detail which was utilized for assaying serratiopeptidase in enteric coated tablets. The enzymatic assay was performed with casein as the substrate and tyrosine as the analytical standard at 1.6 μg/mL. Trifluroacetic acid was used as a precipitating agent. The hydrolytic reaction was performed at 37 °C+50 °C with reaction time of 20 min. The main features of this method are simple, accurate and precise when enzyme is in pure form. This assay procedure mentioned exactly matched the one presented in Indian Pharmacopeia and Japanese pharmacopeia.

Exactly the same method was used in the optimization experiments conducted by Pansuriya et al. [23] in their EVOP experiments. In this study, EVOP, a powerful statistical design, was used to optimize the media composition for production of serratiopeptidase through fermentation by them. Serratiopeptidase encapsulation was carried out by Mali et al. [24] with different concentrations of chitosan (CS) and tripolyphosphate (TPP) in which TPP acted as a cross-linking and condensing agent to obtain nanoparticles. Particle size could be modulated by changing concentrations of CS and TPP. This led to sustained release of serratiopeptidase for more pronounced and long-term anti-inflammatory effect. The loading capacity of nanoparticles was determined by caseinolytic assay of serratiopeptidase. Bio-adhesive periodontal gel of tetracycline and serratiopeptidase was prepared using Aerosil 200 by Maheshwari et al. [25]. The drug release of serratiopeptidase during formulation and stability study were performed using caseinolytic assay method. The gel was used in preliminary clinical study to evaluate its clinical efficiency. Similarly, Singh et al. [26] prepared a dual controlled release thermo reversible periodontal sol of serratiopeptidase and ciprofloxacin. Serratiopeptidase was used as an anti-inflammatory agent for periodontal anaerobic infections. The content of serratiopeptidase in the sols was determined by caseinolytic assay [26]. The study presented by Nirale et al. [27] investigated the feasibility of topical formulations of serratiopeptidase in treatment of local infections. The activity of serratiopeptidase was performed using caseinolytic assay during formulation development and stability study of the formulations. The clinical efficacy of the topical formulation was tested in mice by oxazone-induced ear edema method. The same concept of topical formulations was presented by Shinde et al. [28]. They formulated topical niosomal gel with serratiopeptidase. The clinical efficacy was comparable with that of diclofenac gel. The content of serratiopeptidase in the topical gel was analyzed using the universal caseinolytic assay.

The researchers from the above cited papers advantageously used enzyme catalyzed cleavage of casein while azocasein instead of casein was the preferred substrate in the studies demonstrated by Doerr et al. [29]. Extracellular protease from two strains of *Serratia marcescens* were separated and purified by these researchers using ammonium sulphate precipitation followed by gel adsorption chromatography with sephadex G100 and ion exchange chromatography. The bands at pH 5.4 and 5.5 were separated and analyzed for enzyme activity successfully using azocasein. Though the two methods were not compared, it was found that azocasein could be used as a substrate in place of casein. The similar idea was demonstrated by Dominique et al. [30] when they used dipeptide derivative, "N carbobenzyl oxy arginyl arginyl-para-nitroanilide" (z-arg-arg-pNA), as substrate instead of casein in assaying serratiopeptidase by enzymatic hydrolysis method. The method was successfully applied for quantifying serratiopeptidase in the culture supernatant of clinical strain. Hence previously emphasized caseinolytic assay can be precisely utilized only when serratiopeptidase is in pure form and is free from other proteases or interfering components.

2.2.2. Spectroscopy

It is impossible to envision any literature reference not taking advantage of this powerful technique in assaying serratiopeptidase. Many studies have focused on this technique and have performed quantitative estimation of serratiopeptidase as an active pharmaceutical agent or in its dosage form, either when formulated alone or with other active components.

Spectrophotometric method used for the analysis of tablets of diclofenac sodium, paracetamol and serratiopeptidase was cited by Pandya et al. [31]. The method used mixture of methanol and water as the extraction solvent with a ratio of 40:60 (v/v). The samples were extracted, scanned, and analyzed at 252, 276 and 330 nm wavelengths. The method was found to be accurate, precise and linear over the concentration ranges of 2–15 μg/mL, 2–30 μg/mL, and 2–80 μg/mL for serratiopeptidase, diclofenac sodium and paracetamol, respectively. Similarly, Patel et al. [32] published a study on simultaneous estimation of diclofenac sodium and serratiopeptidase in tablet formulation by UV spectrophotometric method. Diclofenac sodium was estimated at 272.4 nm while serratiopeptidase at 275 nm. The linearity of the method was established for both the actives at concentrations of 7–35 μg/mL and 100–500 μg/mL, respectively. The quantification was performed by absorption ratio and area under the curve (AUC). The method mentioned was rapid, specific, precise and accurate in the established concentration range and can be applied to both bulk and formulations of the actives.

UV spectrophotometric method for determination of tablets containing aceclofenac and serratiopeptidase was presented by Parmar et al. [33]. The extraction medium used was ethanol and water mixture, and the wavelengths used for assaying the two components, namely, aceclofenac and serratiopeptidase, were 316 and 375 nm, respectively. The method was showed to be linear in the range of 100–300 μg/mL for serratiopeptidase, and 30–70 μg/mL for aceclofenac. The limit of detection and limit of quantitation estimated were 12.5 and 37.88 μg/mL for serratiopeptidase while 2.33 μg/mL and 7.07 μg/mL for aceclofenac, respectively. The method was found to be precise, accurate, robust, simple and economical. In all the above cited methods, none of the excipients used in formulating the tablets interfered at the wavelengths used for analyzing serratiopeptidase and other actives.

Serratiopeptidase and metronidazole were estimated in the dosage form by Singh et al. [34] by UV derivative spectroscopy at 229 and 346.5 nm, respectively. The method did not require the separation of serratiopeptidase and metronidazole prior to assaying and was found to be precise, accurate, simple and rapid. It was used routinely in quality control laboratory for estimation of the said dosage form. Similar UV spectroscopic estimation of serratiopeptidase was also carried out by Rakibe et al. [35] in which rapid estimation of serratiopeptidase and diclofenac sodium was performed by absorption ratio method. The absorption wavelengths were 255 and 276 nm. Linearity and accuracy were observed between 140–240 μg/mL and 14–24 μg/mL for serratiopeptidase and diclofenac sodium, respectively [35]. Limbasiya et al. [36] presented similar UV-spectroscopic method for simultaneous estimation of serratiopeptidase and diclofenac potassium, at 230 and 276 nm. Both the drugs obeyed Beer's law in the concentration ranges of 2–40 μg/mL and 1–70 μg/mL for serratiopeptidase and diclofenac potassium, respectively.

The derivative spectroscopic method for assaying nimesulide and serratiopeptidase in tablets was developed by Daharwal et al. [37]. The first derivative spectrum of nimesulide was determined and showed two absorption wavelengths. Estimation of nimesulide was performed at 360.5 nm, where serratiopeptidase did not show any absorption while serratiopeptidase was quantitated at 375 nm. The linearity of the method was found to be well within acceptable norms. The first derivative spectroscopic technique was also used by Rawat et al. [38] for estimation of serratiopeptidase from eudragit microspheres.

Thus it was demonstrated that serratiopeptidase can be easily quantitated by using simple absorption property of protein at the

specific wavelength or by using the first derivative or by ratio method. This simple technique has wide and easy application, but again if interfering protein components are present, it can lead to it being non specific.

2.2.3. Fluorescence spectrometry and fluorescence polarimeter

Comprehensive studies published by Hiroshi and Kazuyuki [1] on Serralysin and related proteinases in 1995 discussed enzymes specifically from serralysin family. The distinguishing feature was that the alkaline proteases from *Pseudomonas fragi, Serratia marcescens, Proteus mirabilis* and *Serratia E 15*, belong to serralysin sub-family MI 12. There were about four different types of proteinases produced by these microbes, out of which the serratiopeptidase having E.C. no: 3.4.24.40 was the one, produced in abundance. This enzyme was extensively studied. Different metal activators and inhibitors were discussed in the study. The serratiopeptidase was assayed by casein digestion method. The proteolytic assay was also monitored by fluorescence spectropolarimeter. The substrate such as gelatin was labeled with fluorescein isothiocyanate and hydrolysis was conducted at 30 °C with precise temperature control. The change in the polarization potential measured was directly proportional to change in the molecular volume after hydrolytic cleavage of the substrate by serratiopeptidase. The researchers also presented fluorometric assay using peptidyl methyl coumarylamide as a substrate, which released 7-amino- 4-methyl coumarin when cleaved by serratiopeptidase, which fluoresced at 440 nm when excited at 380 nm. This could be linked directly to the determination of serratiopeptidase in samples.

This analytical technique is widely used today during the development of biological drugs and has its own prominent advantages in quantitative estimation at microgram and sometimes even at nanogram levels. It has found wide application in conducting bioequivalence and bioavailability studies of macromolecules.

2.2.4. Enzyme-linked immunosorbent assay (ELISA)

Another bio-application technique used was ELISA, by which the concentration of serratiopeptidase was determined at nanogram levels precisely. Sensitive enzyme immunoassay was performed by Yoshiyuki et al. [39] wherein the reaction of serrapeptase was carried out with 2, 4-dinitro phenyl bovine albumin as the substrate for hydrolytic cleavage of serratiopeptidase. The method was found to be reliable, precise and sensitive for assessing the bioavailability of serratiopeptidase from serum samples of animals and humans. The method performed by ELISA was found to be linear between 0.1 and 30 ng/mL, and the lower limit of quantitation was 0.1 ng/mL.

Dominique et al. [40] isolated alkaline protease from *Pseudomonas aeruginosa* (EC 3.4.24). The alkaline protease concentration and activity were determined in culture supernatants of pseudomonas from patients with cystic fibrosis. The techniques used for serratiopeptidase activity determination were ELISA and specific activity testing. The enzymatic hydrolytic activity testing was the only technique which allowed quantification of the proteolytic enzyme.

ELISA assay was developed by Norihiko et al. [41] for activity determination of serratiopeptidase obtained from *Serratia* sp. *E 15*. The method was established using anti-rabbit IgG and Fab fragment conjugated with horseradish peroxidase as its first and second antibodies respectively. Serratiopeptidase was administered in rats and its concentration was determined in rat blood plasma. Simultaneously, the serratiopeptidase concentration was also determined by fluorometric proteinase assay. Both the methods were compared and found to be correlating. The results obtained indicated that both the methods could be applied for determination of serratiopeptidase concentration in rat plasma samples.

Though this technique appears to be simple, the availability of antibodies against serratiopeptidase is a constraint. The cost involved in performing a single analysis is a prohibiting factor for the technique being used universally.

2.2.5. Radioimmunoassay (RIA)

Another related and sensitive technique which can be applied at nano levels is RIA, which has demonstrated to be applicable in quantifying serratiopeptidase. RIA was developed by Kouichi et al. [42] for detection of serratiopeptidase, in the range as low as 1–10 ng at which enzyme activity could not be determined using enzymatic hydrolysis method. The method has drawbacks like being unable to be directly applied to human blood samples, but with acetone as the extraction solvent the technique could be performed. This method could have its own application if the detection range required for other related macromolecules was at the nanogram level. This technique is normally of academic interest and has very little application in routine analysis.

2.2.6. UV microplate method

This is another offbeat analytical tool widely used in the analysis of biomolecules and gaining acceptance. Serratiopeptidase being a biomolecule, this technique was successfully demonstrated by Sandhya et al. [43]. Serratiopeptidase was isolated from *Serratia* sp. *HY6*. The assay for various formulations was carried out using UV microplate reader by vertical photometry. The method was validated and robustness of the method was checked on various formulations such as liposomes and tablets from the market. The method was found to be linear from 1 to 4 μg/mL at 230 nm. Recovery obtained was 97%–98% and there was no interference from the excipients of tablets. Thus the method cited was novel, fast, robust and simple to use. This method can be applied for estimating serratiopeptidase as such or from dosage forms.

2.2.7. HPLC

The versatile technique of HPLC dates back to the 1990s and when adopted it changed the working of pharmaceutical industry totally. Today not a single molecule is analyzed without the use of this powerful analytical tool. Pharmaceutical preparations, mainly tablets of serratiopeptidase, were analyzed using gel filtration column for degradation studies during compression of serratiopeptidase tablets. The method was compared against the enzymatic method and correlation established was presented by Hiroyoshi et al. [44].

The method used for quantitation of serratiopeptidase from clinical blood samples from humans using HPLC was presented. The method used methanol and sodium acetate mixture as mobile phase in the ratio of 4:6 (v/v) using Lichrocart cyano, Merck column. The determination was performed by UV–vis detector and t_{max} observed was 22.64 μg/mL after 5 h. Serratiopeptidase was detected after 3 h while it remained in the blood stream for more than 6 h as presented by Dallas et al. [45]. The method did not show any interference from plasma components and recoveries were within the defined limits.

2.2.8. Liquid chromatography mass spectrometry

Louis et al. [46] used liquid chromatography mass spectrometry (LC–MS) to monitor the hydrolysis of a peptide by serratiopeptidase produced by *Pseudomonas aeruginosa*. The peptide was identified by LC–MS as well as the hydrolytic products formed from peptide were identified. This also led to identification of the cleavage sites of serratiopeptidase. The method by the hyphenated technique was not only efficient and sensitive but also precise.

3. Conclusions

Serratiopeptidase is a 'super enzyme' which has a huge list of health benefits. In this review, we summarized the analytical detection techniques used in identification and quantification of serrapeptase and discussed the advantages and disadvantages of the techniques through their application. The enzyme catalytic assays are widely applied for activity determination of serratiopeptidase as well as for estimating serratiopeptidase as an active pharmaceutical ingredient or

its estimation in dosage forms. The substrates used for the proteolytic assay of serratiopeptidase vary in nature, and have been demonstrated to be used successfully. This technique can be precisely applied only when serratiopeptidase is in pure form and is free of interfering serine proteases. Other than this important analytical method, other unique analytical techniques like ELISA and RIA have also found their application for estimating serratiopeptidase. XRPD analysis, one of the latest and advanced analytical techniques, has been remarkably and successfully utilized for authenticating tablet formulations of serratiopeptidase.

Most of the techniques like SDS-PAGE and reverse phase HPLC have been applied when serratiopeptidase is in pure form. As far as we know there is not a single research paper which refers to the quantification of serratiopeptidase from fermentation broth, or in the presence of other related enzymes. Hence there is still scope for the development of new selective and specific analytical techniques for quantitation of this molecule.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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