

Research Article

INSIGHTS INTO STOICHIOMETRY OF ARGININE MODIFICATION BY PHENYLGlyOXAL AND 1,2-CYCLOHEXANEDIONE PROBED BY LC-ESI-MS

P. Boomathi Pandeswari, V. Sabareesh* and M.A. Vijayalakshmi*Advanced Centre for Bio Separation Technology (CBST), Vellore Institute of Technology (VIT) University, Vellore, Tamil Nadu - 632014, India*

Abstract: Several arginine modification studies on enzymes have mostly been done by phenylglyoxal (PG) or 1,2-cyclohexanedione (CHD) as the modifying reagents. It has been found that one molecule or two molecules of PG or one CHD molecule covalently modify sidechain guanidine of arginine of the enzymes. To seek clearer insights into stoichiometry of this reaction, herein, we decided to investigate some model amino acids that include L-arginine (L-Arg) and two model peptides (an octapeptide and a 30 amino acids long peptide). Reactions were conducted at room temperature (RT: ~25°C), involving 'equimolar concentrations' of reactants in seven different mediums: five buffers (all at basic pH, 7.4 - 8.4) and two solvents. The two solvents are: (1) water (H_2O) and (2) mixture of acetonitrile and water (ACN: H_2O , 1:1, v/v). Progress of every reaction was monitored as a function of time by liquid chromatography - electrospray ionization mass spectrometry (LC-ESI-MS). With PG, L-Arg forms 1:1 adduct ([L-Arg+PG]; m/z 309), 1:2 adduct ([L-Arg+2PG]; m/z 443) and water condensed products of respective 1:1 and 1:2 adducts (m/z 291 & m/z 425) in all six mediums, except in borate, where only uncondensed 1:1 adduct ([L-Arg+PG]; m/z 309) was observed. However, with CHD, L-Arg yielded 1:1 adduct (m/z 287), 1:2 adduct (m/z 399) and corresponding water condensed products (m/z 269 & m/z 381) in borate. Interestingly, in H_2O and in ACN: H_2O (1:1) too, L-Arg undergoes modification. This is the first LC-ESI-MS study illustrating modification of L-Arg by phenylglyoxal and 1,2-cyclohexanedione. Molecular structures are proposed for every observed modified product, depicting stoichiometry and mode of binding by PG or CHD onto guanidine moiety of L-Arg.

Keywords: Arginine Modification; Electrospray Ionization-Mass Spectrometry (ESI-MS); Liquid Chromatography-MS (LC-MS); L-Arginine; Peptides and Proteins; Arginyl Enzymes

Note : Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

1,2-dicarbonyl compounds are known to have higher specificity to modify guanidine group of arginine's sidechain, among which phenylglyoxal (PG) has been utilized in numerous investigations, especially in relation to enzymatic activity studies (Takahashi, 1968; Kitson and Knowles, 1971; Kazarinoff and Snell, 1976; Weng

et al., 1978; Vensel and Kantrowitz, 1980; Pullan and Noltmann, 1985; Jackson and Hersh, 1986; Vanoni et al., 1987; Sancho et al., 1990; Gadda et al., 1994; Dong et al., 1991; Adak et al., 1996; Corbalan-Garcia et al., 1996; Yamamoto and Kawakita, 1999). PG has also been proven to be useful in knowing the significance of arginine in the context of anion transport across red cell membrane and in the case of regulation of mitochondrial permeability transition pore (Wieth et al., 1982; Eriksson et al., 1998). Another compound, 1,2-cyclohexane dione (CHD) too has been used extensively to realize the role of arginine for various biochemical and biological

Corresponding Author: V. Sabareesh
E-mail: v.sabareesh@vit.ac.in/sabareesh6@gmail.com

Received: October 6, 2016

Accepted: November 25, 2016

Published: December 14, 2016

purposes (Patthy and Smith, 1975a and b; Mahley *et al.*, 1977; Fleer *et al.*, 1981; Ferti *et al.*, 1981; Traub and Vorgias, 1984; Ni and Beevers, 1990; Suckau *et al.*, 1992; Zappacosta *et al.*, 1997; Masuda *et al.*, 2005; Wang *et al.*, 2009; Mendoza and Vachet, 2009). Steric hindrance due to the modified arginine residue by PG or CHD may hamper binding of the substrate and/or may cause conformational changes, which might be responsible for affecting the catalytic activity of enzymes (Takahashi, 1968; Jackson and Hersh, 1986; Corbalan-Garcia *et al.*, 1996; Patthy and Smith, 1975b). These modifying reagents become essential, particularly, when functional arginine(s) need(s) to be identified in enzymes, especially in those enzymes that recognize anionic substrates (Riordan, 1979).

Most of such investigations have been carried out by radioactively labeled PG (e.g. ^{14}C -labeled PG) and the enzyme's activity would be assessed by monitoring the levels of radioactivity incorporated into the enzyme (Takahashi, 1968; Kazarinoff and Snell, 1976; Weng *et al.*, 1978; Vensel and Kantrowitz, 1980; Pullan and Noltmann, 1985; Jackson and Hersh, 1986; Vanoni *et al.*, 1987; Sancho *et al.*, 1990; Gadda *et al.*, 1994; Dong *et al.*, 1991; Adak *et al.*, 1996; Corbalan-Garcia *et al.*, 1996; Yamamoto and Kawakita, 1999). Takahashi was the first to report on the use of PG for selective modification of guanidine of arginine (Takahashi, 1968). Based on ^{14}C -labeled PG, Takahashi noted that 2 molecules of PG modified sidechain guanidine group of 1 molecule of arginine, i.e., 1:2 adduct. However, both 1:1 (Pullan and Noltmann, 1985; Vanoni *et al.*, 1987; Sancho *et al.*, 1990; Gadda *et al.*, 1994), and 1:2 stoichiometric adducts of Enzyme:PG have been detected, utilizing ^{14}C -labeled PG (Takahashi, 1968; Dong *et al.*, 1991; Adak *et al.*, 1996; Corbalan-Garcia *et al.*, 1996; Yamamoto and Kawakita, 1999). Additionally, data in supportive of 1:1 as well as 1:2 stoichiometries for Enzyme:PG have been observed from electrospray ionization (ESI) - mass spectrometry (MS) (Krell *et al.*, 1995; Krell *et al.*, 1998; Wood *et al.*, 1998). In the case of modification by CHD, Suckau *et al.* found 1:1 adduct (protein:CHD) with hen egg white lysozyme based on plasma desorption MS (Suckau *et al.*, 1992); and 1:1 adducts of CHD modified tryptic peptides were noted by means of ESI-MS (Zappacosta *et al.*, 1997).

Observation of different stoichiometric adducts of PG- or CHD- modified arginine in enzymes could be ascribed to two factors: degree of surface-accessibility of arginine residue(s) on the protein, which would depend on the conformational or folding state of the protein and secondly, relative concentrations of the protein and the modifying reagent. In fact, many studies report addition of molar excess of PG or CHD with reference to the concentration of proteins or enzymes and hence, it may be obvious to anticipate 1:2 stoichiometric adducts under such conditions (Takahashi, 1968; Kitson and Knowles, 1971; Patthy and Smith, 1975a; Fleer *et al.*, 1981; Ferti *et al.*, 1981; Suckau *et al.*, 1992; Zappacosta *et al.*, 1997; Wood *et al.*, 1998; Watanabe and Funatsu, 1986; Gould and Norton, 1995). Consequently, in this study, we decided to carry out experiments on the amino acid, L-arginine (L-Arg), which is a simpler system than protein and peptide, in that conformational constraint can not impact the progress of the reaction and hence, may enable to obtain better insights and understanding into the stoichiometry of arginine modification. Further, we chose to use only 'equimolar concentrations' of the reactants in this study, for which we have selected different working concentrations. Thus, a major aim of this investigation is to probe and compare the structural modifications occurring to guanidine of L-Arg due to PG and CHD, using liquid chromatography (LC) coupled to ESI-MS (LC-ESI-MS). Indeed, Takahashi's initial experiments were done on L-Arg, but by using ^{14}C -PG. Likewise, modification by CHD was also first studied on L-Arg, yet Toi *et al.* (1965 and 1967) identified the modified structure with the help of chemical synthesis, while Patthy and Smith (1975a and b) applied nuclear magnetic resonance spectroscopy and chemical methods to elucidate the modified structure. However, through the virtue of LC-ESI-MS, it is possible to know the number of multiple products formed (if any) during the course of the chemical reaction, which could offer clearer understanding about various reaction events. Hence, to the best of our knowledge, this is the first study on the application of LC-ESI-MS to investigate modifications of sidechain guanidine of the amino acid L-Arg.

Chemical nature and pH of the medium, viz. buffer could influence the protein folding state, thereby altering the degree of surface-accessibility of arginine(s) of the protein; also, it is well known that the ionization state of the sidechain guanidine group depends on the pH of the reaction medium. Therefore, another major objective of this study is to examine the influence of reaction medium, wherein we have experimented different buffers and solvents to monitor the extent of arginine modification by LC-ESI-MS. Additionally, reactions on two model peptides, human angiotensin II (8 amino acids long) and B-chain of insulin from bovine pancreas (30 amino acids long) have been probed; each of these two peptides has one arginine. A few observations noted on arginine modification of bovine pancreatic ribonuclease A probed by LC-ESI-MS are also presented.

Materials and methods

Materials

L-alanine (L-Ala), L-arginine (L-Arg), L-lysine (L-Lys), N-acetyl arginine, insulin (Bovine pancreas), dithiothreitol (DTT), iodoacetamide (IAM), phenylglyoxal (PG), 1,2-cyclohexane dione (CHD), ammonium bicarbonate, acetonitrile (LC/MS-grade), formic acid, ribonuclease A (RNase A from Bovine pancreas) and sodium bicarbonate were purchased from Sigma-Aldrich (Bengaluru, Karnataka, India). Borax, boric acid, Tris-(hydroxymethyl) aminomethane (Tris), sodium phosphate (monobasic and dibasic) were from Sisco Research Laboratories (Mumbai, Maharashtra, India). Glycine was from Fischer Scientific. Angiotensin II (an octapeptide) was from USV Private Limited (Mumbai, Maharashtra, India). Water was from the Milli-Q system (Millipore) available in-house. Aeris Peptide 2.6 μ m XB C-18 (100 \times 2.1 mm) column was procured from Phenomenex India Private Limited, Hyderabad, Telangana, India. Acetic anhydride (Avra Synthesis Pvt. Ltd.) was obtained as a gift.

Methods

Reactions with L-Arg

Modification of L-Arg (0.1 M) with PG (0.1 M) was carried out (1:1 mole ratio; equimolar) in each

one of these buffers: **1.** sodium borate ($\text{Na}_3\text{BO}_3 \cdot \text{H}_3\text{BO}_3$) (0.1 M, pH 8.4); **2.** sodium phosphate ($\text{NaH}_2\text{PO}_4 - \text{Na}_2\text{HPO}_4$) (0.1 M, pH 7.4); **3.** Tris.HCl (0.1 M, pH 7.4); **4.** sodium bicarbonate (NaHCO_3) (0.1 M, pH 8.4) and **5.** ammonium bicarbonate (NH_4HCO_3) (0.1 M, pH 8). Reactions were also carried out separately in: **6.** water (H_2O) and **7.** 1:1 (v/v) mixture of acetonitrile:water (ACN: H_2O). All reactions were done at room temperature (RT; $\sim 25^\circ\text{C}$) and final volume of every reaction mixture was 100 μl . In the same manner, equimolar quantities of L-Arg (0.1 M) and CHD (0.1 M) were allowed to react in each of the above mentioned reaction medium, including H_2O and ACN: H_2O (1:1). Aliquots of every reaction mixture was analyzed at regular time intervals, starting from about 5 mins., after 30 mins., after 1 hour and up to 24 hours, by LC-ESI-MS. Further, reactions were also conducted using equimolar concentrations of 10 mM and 50 mM of the reactants, whose progress was monitored as a function of time by LC-ESI-MS.

Reactions with angiotensin II (Human), B-chain of Insulin (Bovine pancreas) & ribonuclease A (RNase A, bovine pancreas)

Angiotensin II: Arginine modification of angiotensin II (382 nM, 50 μl) was done by adding equimolar as well as 50 fold molar excess of PG or CHD, in all the seven mediums. The progress of every reaction was monitored after 3 hours, 5 hours and after overnight incubation (viz., ~ 16 hours) at RT, by LC-ESI-MS.

Insulin: Modification of intact insulin was carried out in borate (buffer **1**) and phosphate (buffer **2**) at RT, using ~ 50 , ~ 100 and ~ 200 molar excess concentrations of PG or CHD.

Reduction & Alkylation reaction: Intact insulin (174 nM, 50 μl) was first reduced by DTT (50 mM) upon incubation for one hour at 60°C . Subsequently, the reduced peptide was subjected to alkylation using IAM (100 mM) by incubating in dark at room temperature for an hour. To this reduced-alkylated insulin, equimolar or two fold molar excess of PG or CHD was added. The progress of this reaction was checked, subsequent to 5 hours incubation and also after overnight (~ 16 hours) incubation at RT by LC-ESI-MS. Further, the modification of (reduced-alkylated) B-chain of insulin was performed at 60°C as well, under

equimolar conditions and this reaction was allowed to proceed for about 30 - 60 minutes and examined by LC-ESI-MS.

RNase A: Three different concentrations of PG or CHD: equimolar, ~38 molar excess and ~100 molar excess were used, with respect to the concentration of intact RNase A. The progress of every reaction was monitored after ~16 hours incubation at RT. Only borate (buffer 1) was used for this. The LC-ESI-MS data of modified RNase A were acquired on a high-resolution mass spectrometer (see LC-ESI-MS section).

Acetylation reaction of PG and CHD modified L-Arg
 Acetylation was carried out by using acetic anhydride (Ac_2O). 1 μl of Ac_2O was added to the reaction mixture containing PG or CHD modified L-Arg. Progress of this reaction was monitored after 30 minutes and after 1 hour by LC-ESI-MS. Increment of mass by 42 Da would indicate that the molecule is acetylated.

LC-ESI-MS

Model amino acids and peptides: All LC-ESI-MS data acquisitions on the model amino acids and peptides were done on Acquity UPLC coupled to Quattro Premier XE (Waters), which is a triple quadrupole ESI mass spectrometer. LC was carried out on Aeris Peptide 2.6 μm XB C-18 (100 \times 2.1 mm; Phenomenex) column employing water (H_2O , solvent A) and acetonitrile (ACN, solvent B), each containing 0.1% formic acid. A linear gradient, 5% - 95% solvent B over 20 minutes was followed at a flow rate, 0.1 ml/min. The sample manager (Acquity) was kept in partial loop mode and either 3 or 5 μl sample was injected onto the column. Prior to detection by MS, the LC eluents are monitored by photo diode array (PDA) detector (Acquity), which was set to measure absorbance at wavelengths 214 nm, 254 nm and 280 nm. All MS data were acquired in positive ion polarity of ESI. For characterizing amino acids and angiotensin II, range of detection of mass spectrometer was set to m/z 100-1500, while for experiments on insulin, the m/z range of detection was 300-3000. The optimized source parameters of ESI-MS are: Capillary voltage: 3.5 kilo Volts (kV); Cone: 25 V; Extractor: 5 V; Source Temperature: 100°C; Desolvation Temperature:

300°C; Desolvation Gas (nitrogen; N_2) Flow: 700 L/hr; Cone Gas (nitrogen; N_2) Flow: 75 L/hr. The analyzer parameters were set to the following: LM 1, HM 1, LM 2, HM 2 Resolution: 15; Entrance and Exit: 50; Multiplier: 550. For daughter scan (MS/MS) experiments, the collision gas (argon; Ar) flow was optimized to 0.04 ml/min. and the collision energy was varied in the range 10-40 eV. The data were processed and analyzed in MassLynx.

Model protein: For RNase A samples, LC-ESI-MS data were recorded on 6540 Ultra High Definition Accurate-Mass Q-TOF LC/MS attached to 1290 Infinity LC (Agilent Technologies). Agilent Jet Stream (AJS) ESI mode was used and the source parameters were: Gas (N_2) Temperature: 300°C; Gas Flow: 8 L/min.; Nebulizer Pressure: 35 psi; Sheath Gas (N_2) Temperature: 350°C; Sheath Gas Flow: 11 L/min.; VCap: 3.5 kV; Nozzle Voltage: 1000 V and Fragmentor: 175 V. The m/z range of detection was set to 300 - 1700. LC was carried out on Aeris Peptide 2.6 μm XB C-18 (100 \times 2.1 mm; Phenomenex) column using H_2O (solvent A) and ACN (solvent B), each containing 0.1% formic acid. A linear gradient, 5% - 60% solvent B over 15 minutes was followed at a flow rate, 0.1 ml/min. The data were processed and analyzed in Agilent MassHunter Workstation Software, Qualitative Analysis Version B.07.00.

Results

Modification of L-Arg by PG

Reaction medium: Sodium borate, pH 8.4 (buffer 1)
 LC-ESI-MS analysis of L-Arg modification by PG carried out in sodium borate buffer gave rise to an intense peak at m/z 309 (see Figure 1a). Interestingly, after one day also, LC-ESI-MS of the same reaction mixture (incubated at RT) yielded the peak at m/z 309 with good intensity. Strikingly, even after about six or seven days, LC-ESI-MS of the same reaction mixture (incubated at RT) again yielded the same peak at m/z 309 of good intensity (see Figure S1, Supplementary Material). This signal can be assigned as protonated adduct ($[\text{M}+\text{H}]^+$) of the product, whose molecular mass (M) = 308 Da. This product can result from the addition reaction between the

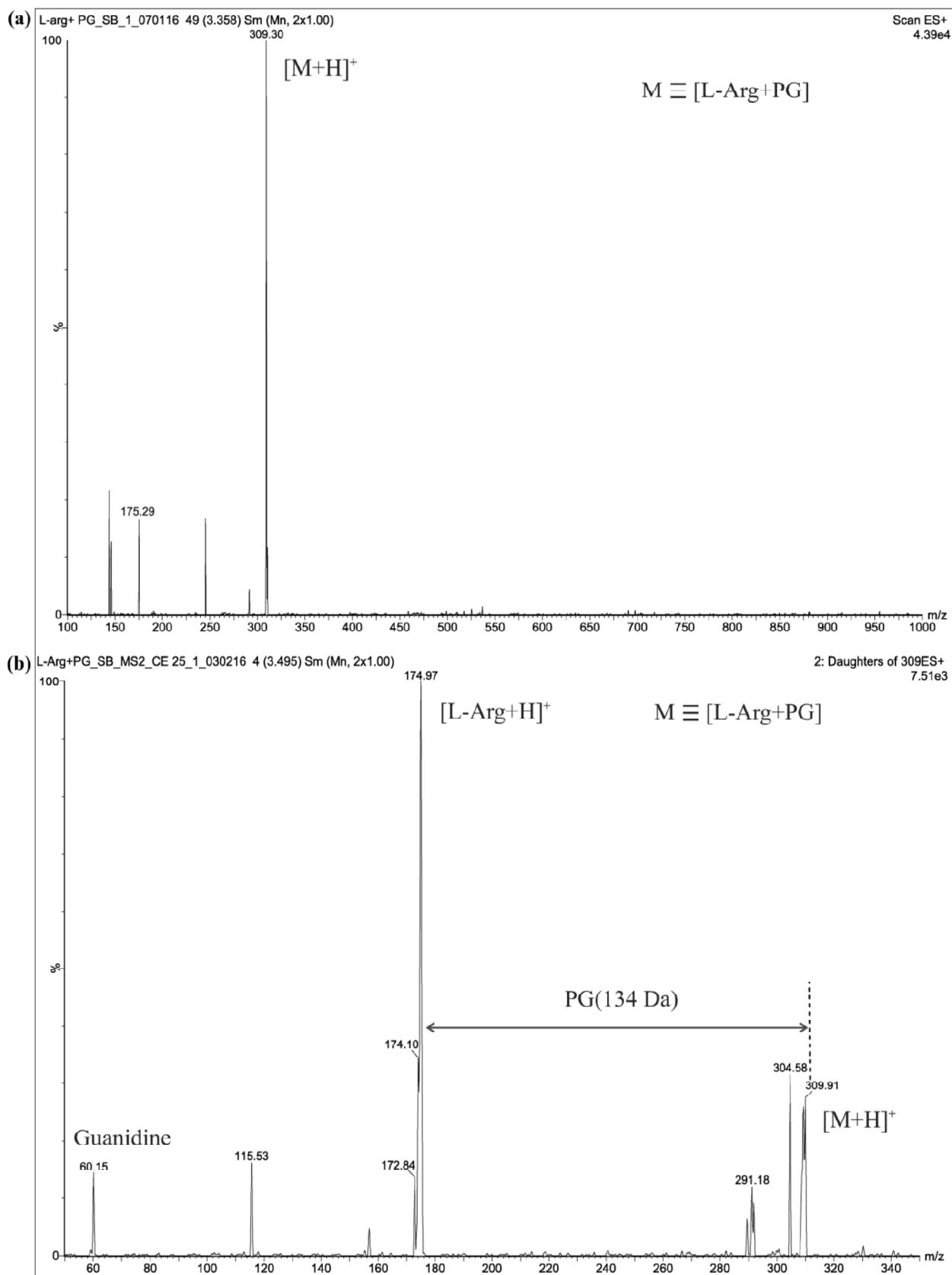


Figure 1: (a) LC-ESI mass spectrum of PG-modified L-Arg in borate, buffer 1 (equimolar working conc. ~ 5 μ mole); (b) LC-ESI-MS/MS spectrum of PG modified product, precursor ion m/z 309.30, acquired at collision energy 25 eV

two carbonyl groups of PG and the guanidine moiety of L-Arg, giving rise to structures **I-A** or **I-B**, as depicted in Scheme 1. It can be noted that the molecular structures, **I-A** and **I-B** contain 1,2-diol group. Borate has been shown to interact well with 1,2-diol group (London and Gabel, 2002; Transue *et al.*, 2004; Zhang *et al.*, 2007; Gabel and London, 2008). Thus, the structures **I-A** and **I-B**, respectively can exist in the form of structures **II-A** and **II-B**, and this could be the reason for the long time stability of the product ($M = 308$ Da) in borate buffer, when compared to other buffers/mediums (*vide infra*). Figure 1b shows daughter scan (MS/MS) spectrum of precursor ion m/z 309, which contains an intense peak at m/z 174.97, showing that the product of molecular mass 308 Da is indeed due to modification of arginine by PG. Furthermore, acetylation reaction of the product **I-A** or **I-B** ($M: 308$ Da) was performed in borate buffer and its LC-ESI-MS analysis clearly yielded a peak at m/z 351 (see Figure S2a, Supplementary Material), suggesting that the α -amino (-NH₂) group in PG-modified-L-Arg has been free, which was able to undergo acetylation. In other words, PG has not reacted with α -NH₂ group of L-Arg, suggesting that PG has selectively modified the sidechain guanidine only of L-Arg. As supportive evidence, LC-ESI-MS/MS (daughter scan) of precursor ion m/z 351 was done, which gave rise to a peak at m/z 217.02, indicative of acetylated L-Arg; the mass difference between the precursor ion m/z 351 and m/z 217 corresponds to the molecular mass of PG, suggesting that m/z 351 loses PG upon CID (see Figure S2b, Supplementary Material).

Reaction medium: Sodium phosphate, pH 7.4 (buffer 2) and Tris. HCl, pH 7.4 (buffer 3): The peak at m/z 309 was also observed in the mass spectrum recorded from the reactions carried out in phosphate and Tris.HCl (buffers 2 and 3), but this signal is not detected after about 30 minutes. In the case of these two buffers, the signal at m/z 309 may be ascribed to the structures **III** or **IV** (each of whose molecular mass is also 308 Da), which is formed by the reaction between any one of the two carbonyl groups and an amino group of guanidine (see Scheme 2). Absence of peak at m/z 309 after about 30 mins and consistent detection of signal at m/z 291, even after 24 hours, could be due to loss of a water (H₂O) molecule from **III** or

IV, thereby yielding structures **III-A** or **IV-A**, respectively. These observations prompted that this reaction could proceed in a way similar to the formation of Schiff-base product, in buffers 2 and 3. And thus, it was decided to depict **III-A** and **IV-A** as conventional Schiff-base products, which are known to be formed via a typical, unstable intermediate aminoalcohol or carbinolamine, **III** or **IV** that lack the 1,2-diol group. This can also be understood in a manner that phosphate and Tris.HCl (buffers 2 & 3) are not able to stabilize 1,2-diol moiety containing product of mass 308 Da, unlike borate (see **I-A** or **I-B**, Scheme 1). Subsequent to PG-modification reaction, acetylation reaction was performed in each of phosphate and Tris.HCl buffers, whose LC-ESI-MS analysis gave rise to good intense peaks at m/z 333 (291 + 42) and m/z 351 (309 + 42) (data not shown), which indicate freely available α -NH₂ group in **III** or **IV** and **III-A** or **IV-A**. Thus, PG selectively modifies sidechain guanidine moiety only of L-Arg.

Reaction medium: NaHCO₃, pH 8.4 and NH₄HCO₃, pH 8 (buffers 4 and 5); H₂O and ACN/H₂O (1:1) (solvents 6 and 7)

The intensity of the product of m/z 309 was very less, when the reaction was allowed to proceed in NaHCO₃, NH₄HCO₃, H₂O and ACN:H₂O (1:1). This peak at m/z 309 was found only during the initial ~ 5 min, from the onset of the reaction.

Reaction medium: Buffers 2 - 5 and Solvents 6 & 7: A major signal in LC-ESI-MS data acquired from each of these reaction medium was at m/z 291.34 (see Figure 2), which can be assigned to the water condensed or Schiff-base product (**III-A** or **IV-A**; Scheme 2). As a representative example, Figure 2a shows total ion chromatogram (TIC) of the reaction mixture having 'H₂O' as the medium, recorded following a reaction period of 1 hour. The TICs of the reaction mixture performed in other buffers (2 - 5 and solvent 7) too were very similar to that shown in Figure 2a (data not shown). The mass spectrum at retention time (t_R): 3.97 min. contains a peak at m/z 291.34 (Figure 2b), which can be ascribed to Schiff-base product, **III-A** or **IV-A**. Additionally, two more products were detected from the mass spectrum at t_R : 5.00 min. and 7.06 min. (Figure 2a), which had prominent peaks at m/z 443.31 and m/z 425.29,

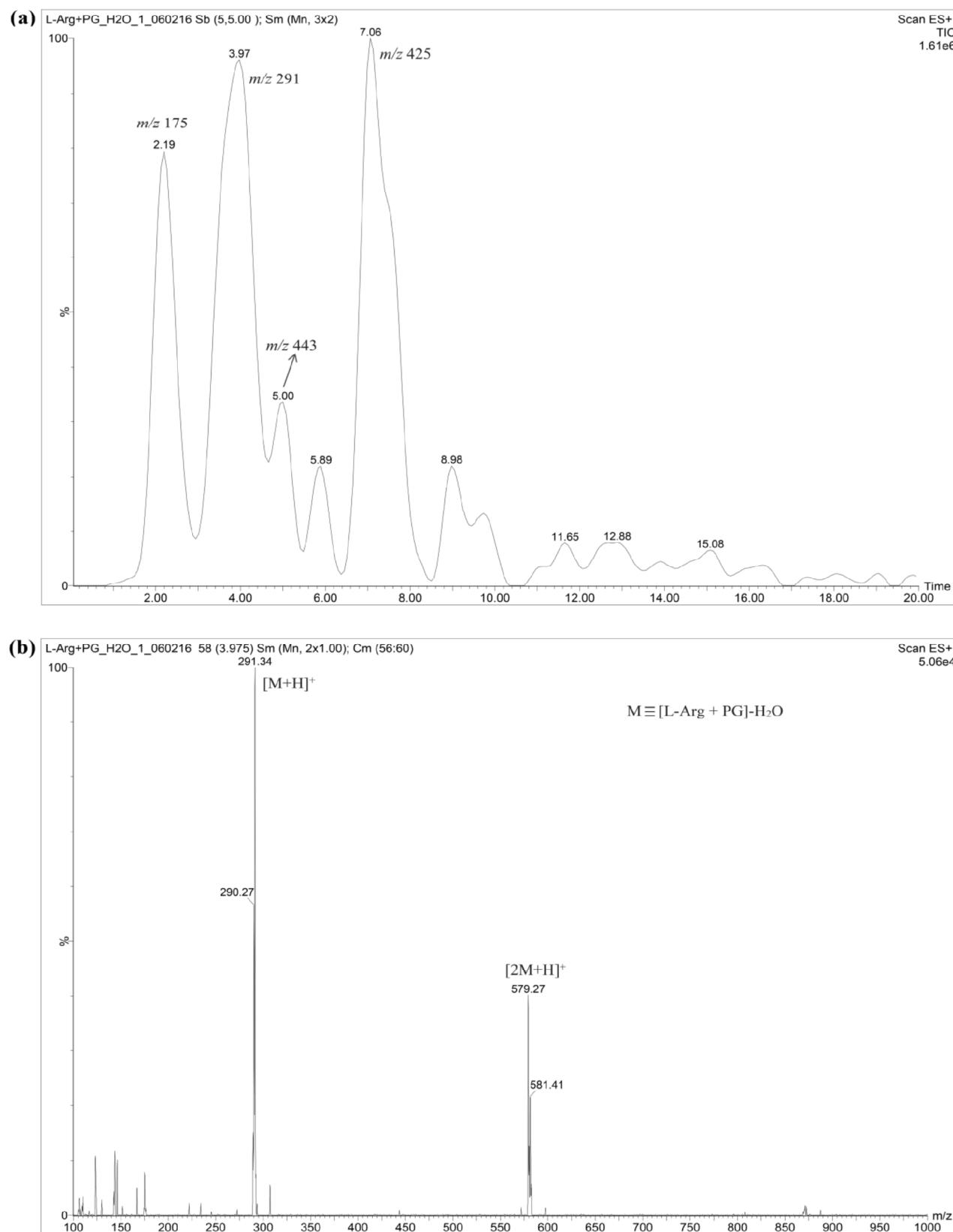
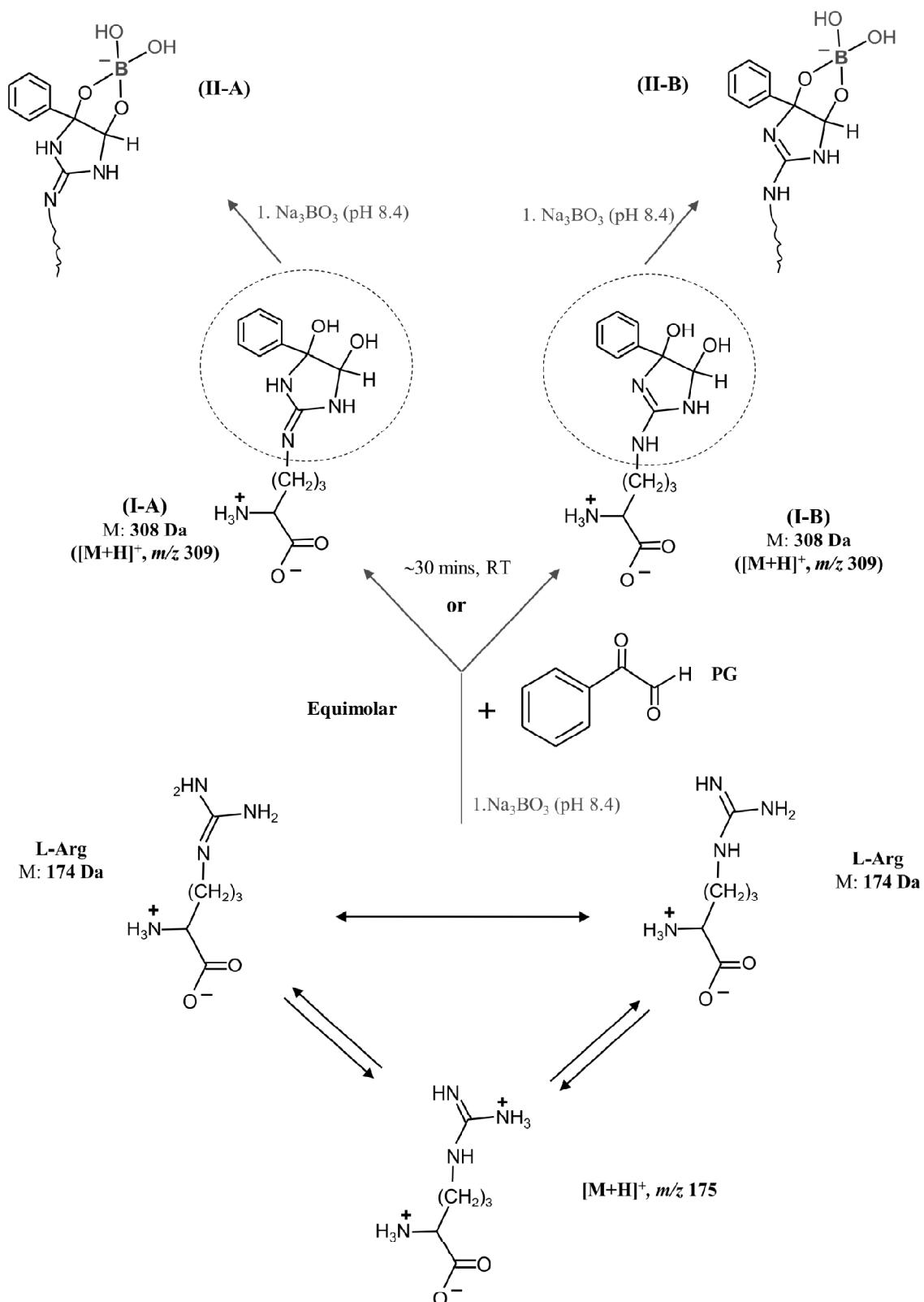
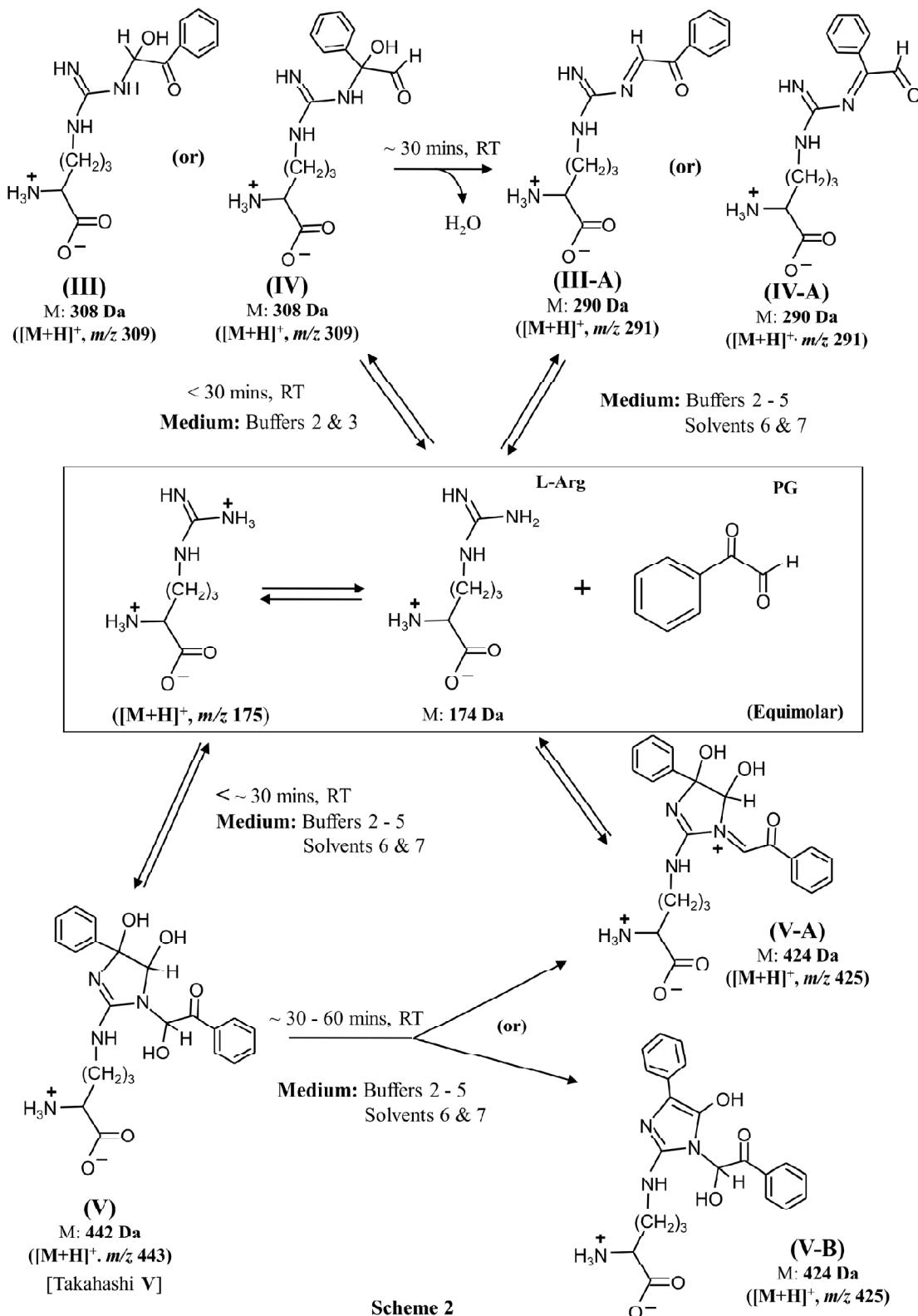


Figure 2: (a) Total Ion Chromatogram (TIC) of [L-Arg + PG] reaction mix in H₂O, solvent 6 (equimolar working conc. ~ 5 μmole), recorded after a reaction period of 1 hour; (b) LC-ESI mass spectrum at t_r: 3.97 min. (see above), m/z 291.34 corresponds to 'water condensed 1:1 adduct'



Scheme 1: Modification of L-Arg by PG in borate, buffer 1 (equimolar working conc. $\sim 5 \mu\text{mole}$): Plausible molecular structures depicting different modes of binding of PG onto guanidine sidechain of L-Arg rationalizing for the observed peak at m/z 309 (see Figure 1a). This product at m/z 309 was observed to be stable up to 24 hours and even up to about 7 days, in borate. And hence, it was proposed to depict the structures I-A or I-B in the form of II-A or II-B, where borate is shown to stabilize the 1,2-diol moiety of the PG modified L-Arg.



Scheme 2

Scheme 2: Modification of L-Arg by PG in buffers 2 - 5 and solvents 6 & 7 (equimolar working conc. $\sim 5 \mu\text{mole}$): Plausible molecular structures of different products interpreted from LC-ESI-MS data, showing various kinds of structural modifications occurring to guanidine sidechain of L-Arg. Approximate duration for the formation of each product is also indicated, suggesting the relative stability of every product. The products at $m/z 291$ & $m/z 425$ were observed to be stable up to about 24 hours also.

respectively (Figure 3). The peak at m/z 443 (Figure 3a) can be attributed to the product (structure **V** in Scheme 2) originally proposed by Takahashi (1968), wherein two molecules of PG modify a guanidine moiety of L-Arg. However, Takahashi's product (**V**) was found to be not quite stable. Subsequent to about 30 mins, from the LC-ESI-MS runs, the peak intensity of m/z 443.31 was found to be gradually decreasing and after 60 mins., the peak at m/z 443.31 was completely absent. The disappearance of the peak at m/z 443.31 was concomitant with the detection of intense peak at m/z 425.29 (Figure 3b), which can be rationalized as to loss of a H_2O molecule from Takahashi's adduct (**V**), leading to formation of structures, **V-A** and **V-B** (Scheme 2).

Further, LC-ESI-MS analysis of acetylation reaction of PG-modified L-Arg obtained in H_2O yielded a peak at m/z 333 (see Figure S3a, Supplementary Material), implying that **III-A** or **IV-A** (290 Da) has undergone acetylation, which confirms that PG indeed has specifically reacted with sidechain guanidine and not the $\alpha\text{-NH}_2$ in H_2O . Likewise, acetylation reactions were done in other buffers and solvents, whose outcome also confirmed that $\alpha\text{-NH}_2$ group had not undergone modification by PG (data not shown). Moreover, both condensed and uncondensed 1:2 adducts were also found to undergo acetylation (m/z 485.14 & m/z 467.25), suggesting that the both the molecules of PG are added on the sidechain guanidine only (see Figure S4, Supplementary Material).

It was striking to note two peaks at m/z 290.27 and at m/z 289.39 that were consistently detected along with the Schiff-base product signal at m/z 291.34, but the intensities of those two peaks were considerably lower than the intensity of m/z 291.34 (see Figure S5a, Supplementary Material). Plausible pathways reasoning for the formation of products that may contribute for the signals at m/z 290.27 and m/z 289.39 are illustrated in Figure S5 (Supplementary Material). It was possible to think of only a 'radical' containing molecular structure (**IV-C^A**, Figure S5d), for interpreting the peak at m/z 290.27 (Figure S5a, Supplementary Material). As of now, it is not clear to us, as to, why these peaks are reproducibly detected at m/z 290.27 and m/z 289.39 and how a radical (**IV-**

C^A; m/z 290) could form in these reaction media (i.e., buffers **2 - 5** and solvents **6 & 7**). This requires further probing, which may be pursued in future.

Modification of L-Arg by CHD

Modification of L-Arg by 1,2-cyclohexane dione (CHD) yielded contrasting results, when compared to the reactions carried out between L-Arg and PG. Especially, the case of sodium borate (buffer **1**) was intriguing, in that, both 1:1 and 1:2 (L-Arg:CHD) stoichiometric adducts were detected by LC-ESI-MS (see Figure 4 and structures **VI**, **VII**, **VIII & IX** in Scheme 3), unlike the case of PG modified L-Arg in sodium borate (see Scheme 1). Another striking observation is that even water condensed products, i.e., Schiff-base products (Figure 4 and structures **VII-A**, **VIII-A** & **IX-A** in Scheme 3) were detected in sodium borate medium due to CHD modification of L-Arg. However, in sodium borate, PG did not yield Schiff-base/water condensed product at all with L-Arg (*vide supra*). Figure 4 shows mass spectrum of CHD modified L-Arg revealing formation of both 1:1 (m/z 287.44, Figure 4a) and 1:2 (m/z 399.33, Figure 4b) adducts in borate. Also peaks corresponding to water-condensed/Schiff-base products yielding from both 1:1 and 1:2 adducts were detected (m/z 269.23, Figure 4b and m/z 381.44, Figure 4c). In the case of condensed/Schiff-base product from 1:1 adduct (**VII-A**, m/z 269.23), no peaks were found at one or two mass units lower than m/z 269. But, in the case of PG modified L-Arg, excepting the sodium borate (buffer **1**), peaks at m/z 289 and m/z 290 along with m/z 291 were very well observed in all buffers (**2 - 5**) and solvents (**6** and **7**) (Figure S5a, Supplementary Material). Moreover, the CHD modified L-Arg product yielded in borate and in H_2O , further underwent acetylation, giving rise to a peak at m/z 329 (Figure S6, Supplementary Material), which confirms that CHD modifies sidechain guanidine and not $\alpha\text{-NH}_2$ group. Additionally, both condensed and uncondensed 1:2 adducts underwent acetylation indicating that the 2 molecules of CHD have indeed added onto guanidine sidechain of L-Arg (see Figure S7, Supplementary Material). Furthermore, L-Arg did not seem to undergo modification by CHD in Tris. HCl (buffer **3**); rather it was evident that Tris itself reacted with CHD as observed from the

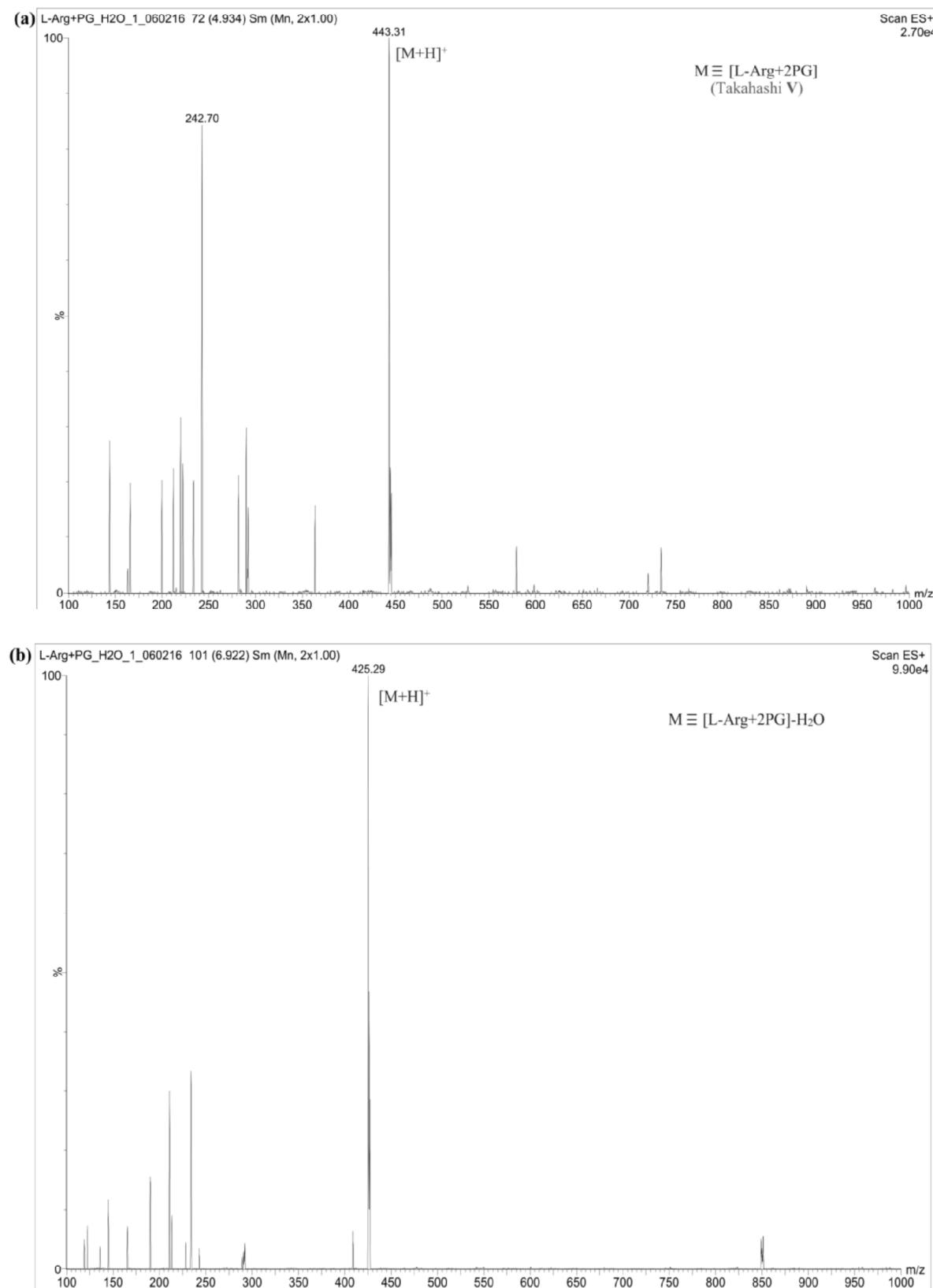


Figure 3: L-Arg modification by PG in H₂O, solvent 6 (equimolar working conc. ~ 5 μmole): (a) LC-ESI mass spectrum at t_r: 4.93 min. (see Figure 2a) showing evidence for 'uncondensed 1:2 adduct', m/z 443.31, i.e., Takahashi V (Scheme 2); (b) LC-ESI mass spectrum at t_r: 6.92 min. (see Figure 2a), indicating formation of 'water condensed 1:2 adduct', m/z 425.29.

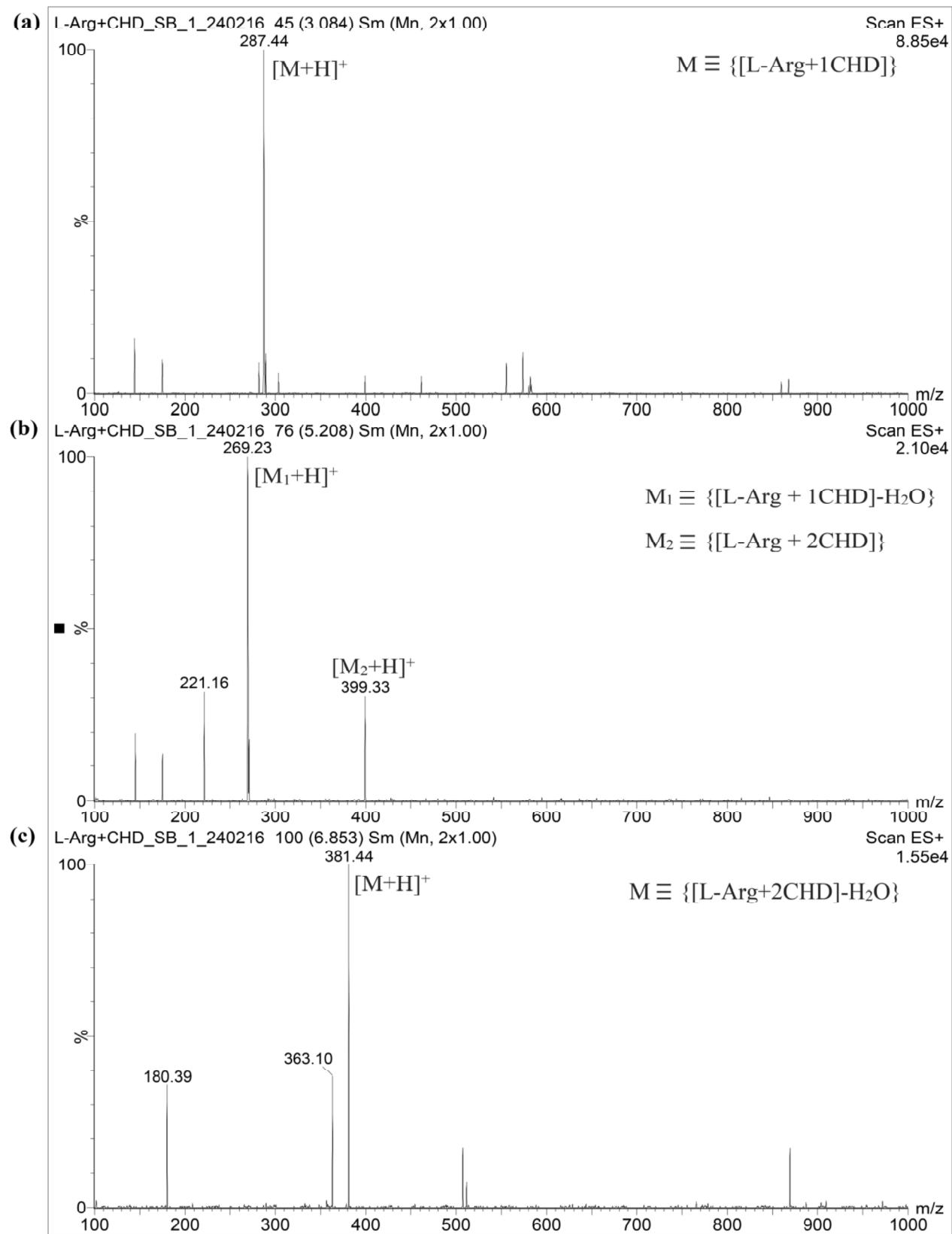
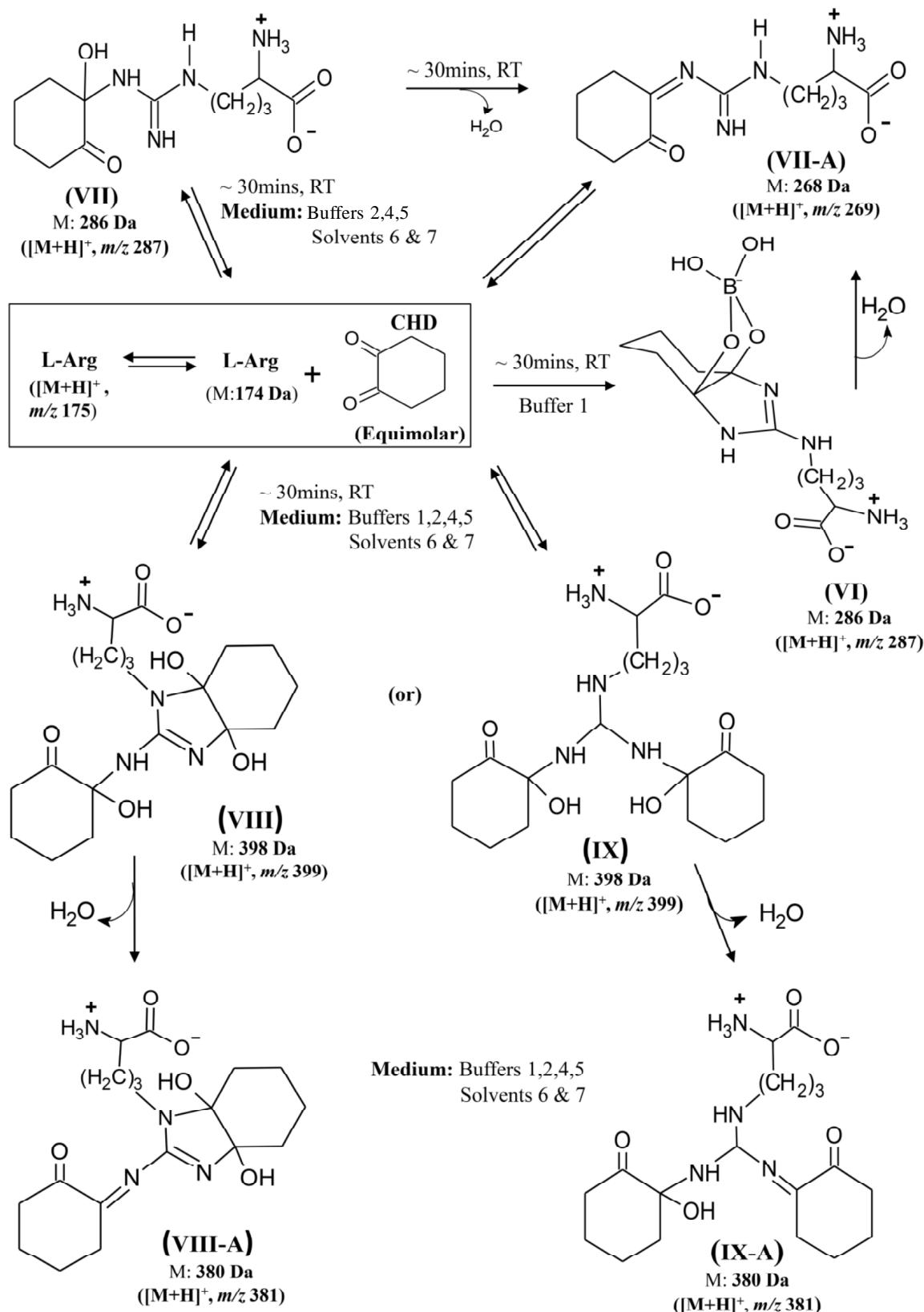


Figure 4: LC-ESI mass spectrum of CHD-modified L-Arg in borate, buffer 1 (equimolar working conc. ~ 5 μ mole): (a) {[L-Arg + 1CHD]}, m/z 287.44; (b) M_1 ; {[L-Arg + 1CHD] - H_2O }, m/z 269.23 and M_2 ; {[L-Arg + 2CHD]}, m/z 399.33; (c) {[L-Arg + 2CHD] - H_2O }, m/z 381.44.



Scheme 3: Modification of L-Arg by CHD in buffers 1 - 5 and solvents 6 & 7: Probable molecular structures representing various forms of structural modifications that could most likely take place on the guanidine sidechain of L-Arg. Most of these products were observed to be stable up to about 24 hours in every reaction medium.

LC-ESI-MS data (Figure S8, Supplementary Material).

Impact of concentration of the reactants on the progress of the reaction

It is important to note that the data presented thus far on modification of L-Arg (Table 1) were all from the equimolar working concentration of ~ 5 μ mole of reactants. Reactions on L-Arg were also carried out at lower equimolar working concentrations of 2.5 μ mole and 0.5 μ mole. At those lower concentrations, it was intriguing to note that L-Arg formed only 1:1 adducts with CHD in all the reaction mediums, whereas with PG, both 1:1 and 1:2 adducts and their respective condensed products were observed. Absolutely no signals were detected in the mass spectral data that indicated the formation of any type of 1:2 adducts with CHD. Again, in borate, L-Arg and PG formed only 'uncondensed 1:1 adduct (*m/z* 309)', as observed in the case of higher concentration (5 μ mole).

Modification of model peptides, angiotensin II (human), B-chain of insulin (bovine pancreas) and a model protein, ribonuclease A (RNase A, bovine pancreas)

The outcomes obtained from the modification of L-Arg prompted us to probe the nature of stoichiometry of arginine modification on peptides. For this, we chose two peptides: an octapeptide and another, 30 amino acids long peptide.

Angiotensin II (human) is an octapeptide, whose sequence isDRVYIHPF (monoisotopic mol. mass 1045.534 Da). At RT, in all the seven mediums, modification of angiotensin II by PG or CHD yielded only uncondensed 1:1 adduct, which was observed after 3 hours, 5 hours and also after 16 hours, when equimolar concentrations of the reactants were used. Water condensed products of angiotensin II with PG or CHD were not at all observed (see Figure 5). Up on adding 50 fold molar excess of PG or CHD, in addition to uncondensed 1:1 adduct, there was evidence of some population of condensed 1:1 adduct with this peptide, which were observed after 3 hours, 5 hours and after 16 hours. There were no signals indicative of 1:2 adducts at all

with this peptide, even after 16 hours of incubation under this molar excess condition of PG or CHD.

In the case of B-chain of insulin, when reactions were conducted at RT, two fold molar excess of PG or CHD was needed (see Materials and methods), so as to observe the modification, which corresponded to the formation of 1:1 adduct after about 5 hours. Overnight (viz., ~16 hours) incubation at RT with two fold molar excess of reagents, resulted in detection of better signals of the modified products, which was again found to be 1:1 adduct only (data not shown). However at 60°C, the modifications were detected with equimolar concentrations of the reactants itself. Upon incubating at 60°C, the modifications rather began to take place from about 30 minutes and better peak intensities yet again due to 1:1 adduct were observed in the mass spectrum acquired after 60 minutes incubation at 60°C (Figure 6). No water condensed products and no 1:2 adduct were observed, irrespective of incubation at RT (5 hours and overnight) and at 60°C (30 - 60 minutes).

From the outcomes noted on L-Arg and model peptides, it was decided to carry out modification reactions on intact RNase A in borate buffer only. Further, the formation of products was observed after overnight (~16 hours) incubation at RT (see Materials and methods). Under equimolar conditions, a significantly higher population of intact RNase A remained unmodified, while the protein was observed to be modified by 1 molecule of PG or CHD, whose population was considerably very small as compared to native RNase A (data not shown). However, use of molar excess of PG or CHD on intact RNase A (see Materials and methods), gave rise to multiple modified products corresponding to addition of 1 and 2 and 3 molecules of PG or CHD onto the protein; the abundance of modified protein having 3 molecules of PG or CHD was quite less (see Figure 7). A very little population of modified RNase A containing 4 molecules of CHD too was noted (Figure 7b). It was interesting to note a relatively higher population of intact RNase A to be unmodified, even upon adding molar excess of PG or CHD and that too after incubating for ~16 hours (Figure 7).

Table 1
Overview of products obtained from PG or CHD modification of L-Arg in various reaction mediums under equimolar conditions (working conc. ~ 5 μ mole)^{^a, #}

S.No.	Buffers/Solvents	Phenylglyoxal (PG) modified L-Arg			
		1:1 adduct	1:1 adduct- H_2O	1:2 adduct [§]	1:2 adduct- H_2O
		m/z 309	m/z 291	m/z 443	m/z 425
1	$Na_3BO_3 - H_3BO_3$	✓	✗	✗	✗
2	$NaH_2PO_4 - Na_2HPO_4$	✓ [§]	✓	✓	✓
3	Tris.HCl	✓ [§]	✓	✓	✓
4	$NaHCO_3$	✗	✓	✓	✓
5	$(NH_4)HCO_3$	✗	✓	✓	✓
6	H_2O	✗	✓	✓	✓
7	ACN: H_2O	✗	✓	✓	✓

S.No.	Buffers/Solvents	1,2 cyclohexanedione (CHD) modified L-Arg			
		1:1 adduct	1:1 adduct - H_2O	1:2 adduct	1:2 adduct- H_2O
		m/z 287	m/z 269	m/z 399	m/z 381
1	Na_3BO_3	✓	✓	✓	✓
2	Na_3PO_4	✓	✓	✓	✓
3	Tris.HCl	✗	✗	✗	✗
4	$NaHCO_3$	✓	✓	✓	✓
5	$(NH_4)HCO_3$	✓	✓	✓	✓
6	H_2O	✓	✓	✓	✓
7	ACN: H_2O	✓	✓	✗	✓

^a Ionic intensities higher than 1000 (1e3) only were considered to arrive at this summary.

Products observed up to a period of 1 hour are only shown here.

§ These products are noted only during the initial ~ 30 mins. (see Scheme 2).

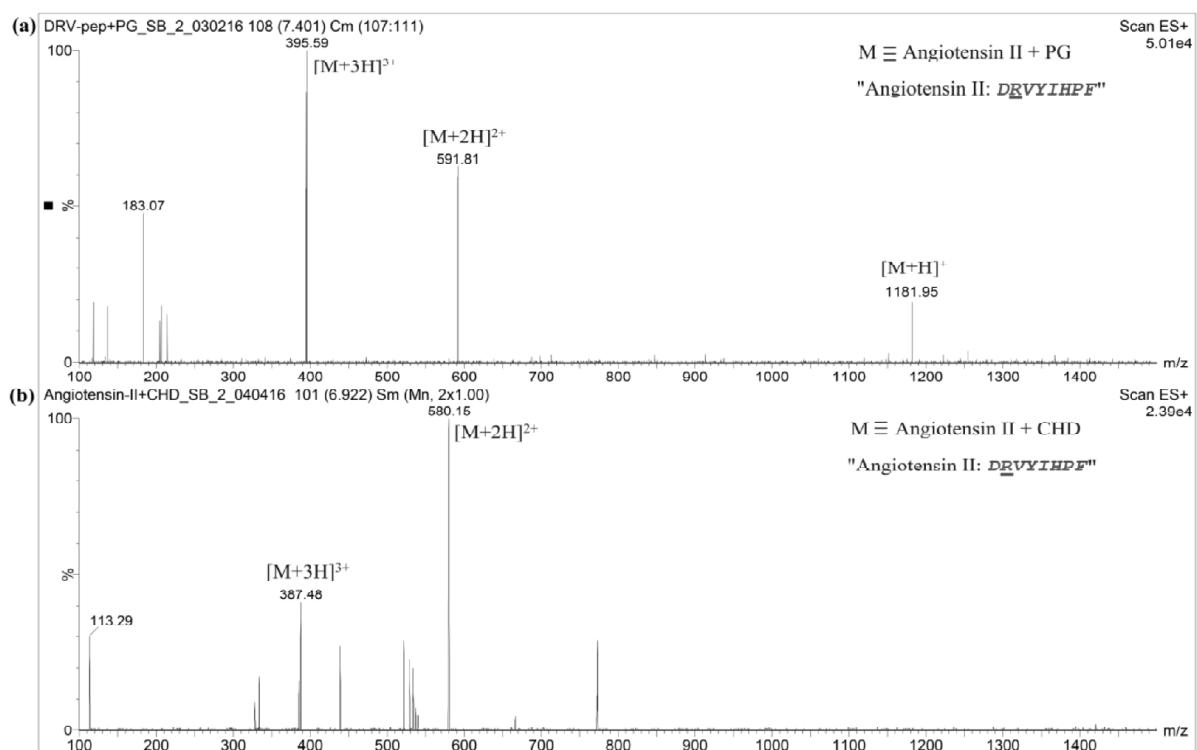


Figure 5: LC-ESI mass spectrum of (a) PG-modified and (b) CHD-modified, angiotensin II in borate buffer (equimolar working conc. of reactants in nanomole range). The charge states of the modified products are indicated.

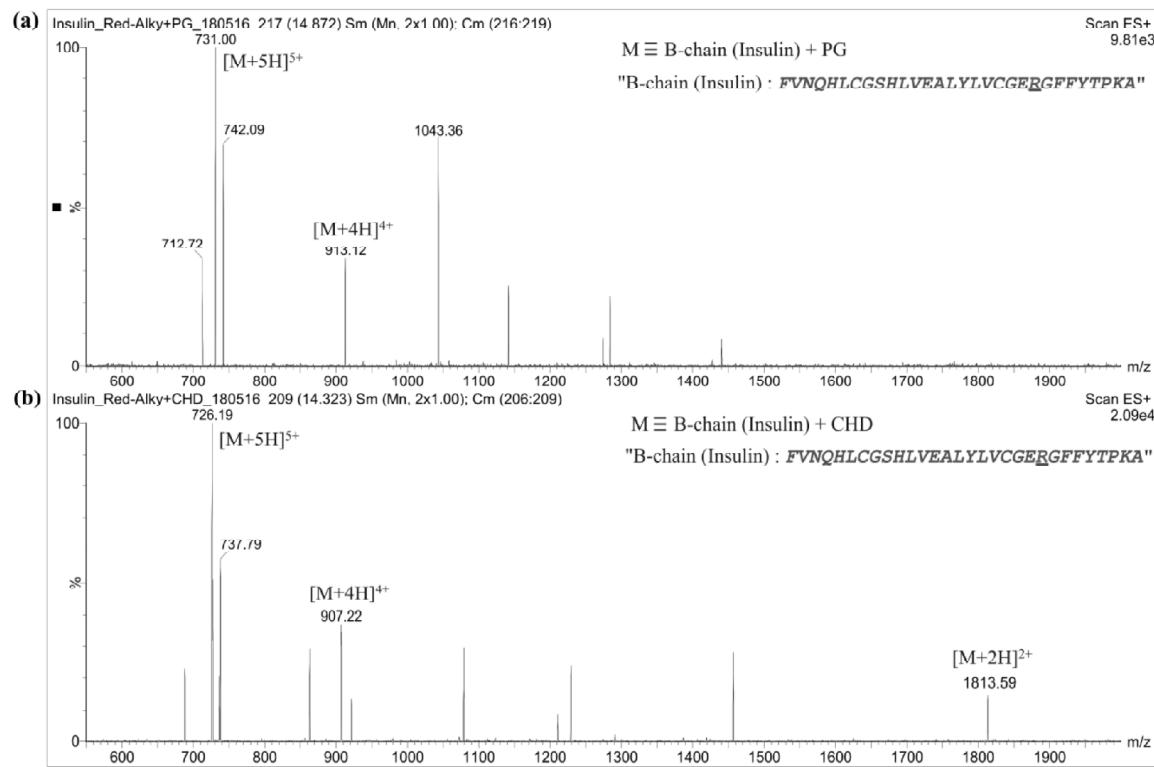


Figure 6: LC-ESI mass spectrum of (a) PG-modified and (b) CHD-modified, B-chain of insulin (reduced & alkylated) in borate buffer. The charge states of the modified products are indicated.

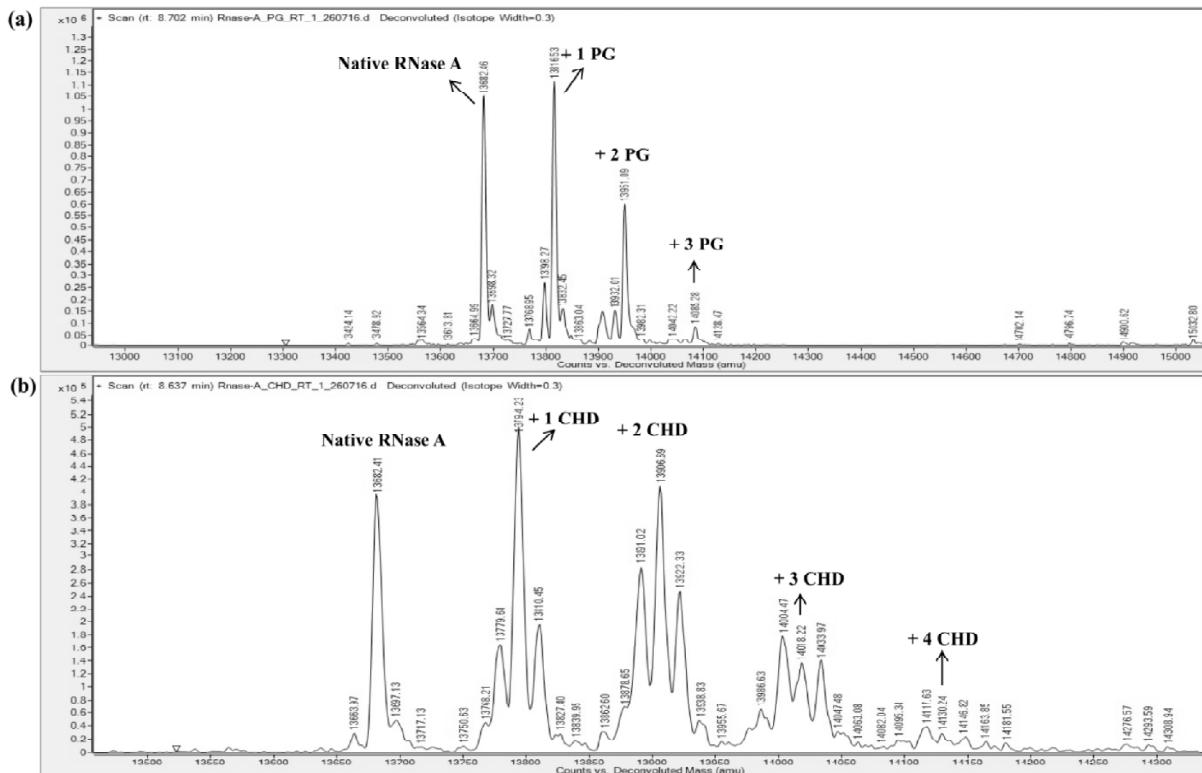


Figure 7: Deconvoluted mass spectrum obtained from LC-ESI-MS of (a) PG-modified and (b) CHD-modified RNase A. Reactions performed using molar excess of PG or CHD, with reference to protein's concentration (see Materials and methods section for further details)

Discussion

Arginine is critical for certain class of enzymes to carry out their respective function normally, particularly in those cases, where the enzymes need to recognize or bind to anionic substrates, for instance, phosphate containing substrate molecules (Riordan, 1979). To identify essential arginine residues for enzymatic activity, 1,2-dicarbonyl compounds have been used, since they show higher specificity to modify guanidine sidechain. For example, in the case of creatine kinase, PG was used to deduce necessary arginine residues, wherein the modification reaction was monitored by high-resolution ESI-MS (Wood *et al.*, 1998). Likewise, phospholipase, phosphatase site of carbonic anhydrase III and ATPase are a few more examples of enzymes that recognize negatively charged substrates, wherein the significance of functional arginine was comprehended by use of PG and CHD (Vensel and Kantrowitz, 1980; Pullan and Noltmann, 1985; Corbalan-Garcia *et al.*, 1996; Yamamoto and Kawakita, 1999; Fleer *et al.*, 1981; Viale and Vallejos, 1985). Several studies have found chemical nature and pH of the buffer significantly impacting the process of arginine modification reactions on enzymes or proteins. Especially, basic pH condition (pH 3.8) has been found to be favorable for the progress of this reaction, which is owing to the fact that pKa of guanidine sidechain of arginine is ~11 - 12. Among various buffers that provide basic pH environment, Tris.HCl has been found to be not suitable to perform arginine-modification, since Tris itself has been noted to react well with PG and CHD (Takahashi, 1968; Patthy and Smith, 1975a; Vlahos *et al.*, 1985). Borate buffer has been used extensively, particularly in cases of modification of arginine by CHD (Patthy and Smith, 1975b; Mahley *et al.*, 1977; Fleer *et al.*, 1981; Ferti *et al.*, 1981; Traub and Vorgias, 1984; Suckau *et al.*, 1992; Zappacosta *et al.*, 1997; Masuda *et al.*, 2005; Watanabe and Funatsu, 1986; Gould and Norton, 1995). This has been attributed to the ability of borate in stabilizing the vicinal diol group that is formed when CHD modifies the guanidine moiety of arginine (Patthy and Smith, 1975a). In the case of arginine-modification by PG, various buffer types have been employed such as phosphate (Kazarinoff and Snell, 1976; Vanoni *et*

al., 1987; Sancho *et al.*, 1990; Gadda *et al.*, 1994; Adak *et al.*, 1996), bicarbonate (Vensel and Kantrowitz, 1980; Krell *et al.*, 1995; Krell *et al.*, 1998; Watanabe and Funatsu, 1986), HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) (Yamamoto and Kawakita, 1999; Eriksson *et al.*, 1998), MOPS (3-(N-morpholino) propane sulfonic acid) (Corbalan-Garcia *et al.*, 1996), bicine (Pullan and Noltmann, 1985; Gardner *et al.*, 1983) as well as borate (Kazarinoff and Snell, 1976; Jackson and Hersh, 1986; Gadda *et al.*, 1994; Eriksson *et al.*, 1998). Sodium succinate (pH 5.5 and 6.0) and sodium diethylmalonate (pH 7.0 and 7.5) have also been attempted for arginine modification by PG in carbonic anhydrase III (Pullan and Noltmann, 1985). In certain investigations, even two buffers have been used in combination, e.g., bicine and sodium bicarbonate (pH 8.7) (Wood *et al.*, 1998).

Consequently, in this study, we decided to carry out modification of the amino acid L-Arg and a model peptide, angiotensin II (sequence: DRVYIHPF) in five different buffer types: (1) sodium borate (pH 8.4), (2) sodium phosphate (pH 7.4), (3) Tris.HCl (pH 7.4), (4) sodium bicarbonate (pH 8.4) and (5) ammonium bicarbonate (pH 8.0). The choice of these buffers is based on the past reports, however little has been studied about the role of NH_4HCO_3 (buffer 5) in the context of arginine modification reactions. Furthermore, we were curious to know the fate of the L-Arg modification in two very widely used solvents: (6) water (H_2O) and (7) acetonitrile:water (ACN: H_2O , 1:1), despite knowing the fact that solvents 6 and 7 cannot bring about basic pH condition, so as to keep guanidine in deprotonated state, which is an important prerequisite for the modification reaction to occur. The reason to choose ACN: H_2O (1:1) (solvent 7) as a reaction medium was that it is an often used solvent system in reverse phase chromatography and also for LC-ESI-MS experiments. If reactions are found to proceed in each of these two solvents, 6 and 7, it was decided to monitor the stability of the product(s) formed as well, in each of these two media. Another rationale to select the solvents 6 and 7 was based on the solubility of L-Arg in these two solvents. Moreover, pH of L-Arg solution in H_2O was found to be ~12 and therefore, we decided to

perform L-Arg modification reaction in H₂O medium. After 4 hours, the pH of this reaction mixture in H₂O (**6**) was found to be about 7 and it further decreased to ~6 in about 24 hours from the onset of the reaction. A similar trend of pH change during the course of the reaction in ACN:H₂O (**7**) too was observed. However, in the case of buffers **1 - 5**, the pH of the L-Arg containing reaction mix remained almost constant over a period of 24 hours of the reaction. Modification of angiotensin II also was examined in solvents **6** and **7**.

Based on the appearance of new peaks in the total ion (mass-detected) chromatogram (TIC) and according to the change or increase in the mass, as observed from LC-ESI-MS data, the nature of products formed due to reaction between L-Arg and PG or CHD, was inferred. Accordingly, different structural modifications were proposed that are depicted in Schemes 1-3. It was intriguing that TICs acquired from all the reaction mixtures (for both PG and CHD modification reactions), contained an early eluting peak of good intensity that corresponded to unreacted L-Arg (*m/z* 175), which was observed for a period of more than one day. This clearly suggests that there exists equilibrium between reactants and products; accordingly this is indicated in Schemes 1-3. This implies the reversible nature of these reactions, which has been demonstrated in many of the past studies. The illustrations in Schemes 1-3 have been summarized in Table 1, which offers a quick look at different types of stoichiometric adducts formed due to reaction between L-Arg and PG or CHD, in different mediums. Products interpreted from the data recorded up to a period of 1 hour only are presented in Table 1. Some interesting observations emanate from the outcome of all these experiments.

Comparison of PG modified L-Arg with CHD modified L-Arg

Reaction medium, sodium borate (buffer 1): [1:1 adduct-H₂O] (structure **VII-A**, Scheme 3) is detected with good intensity (*m/z* 269.23, Figure 4b) in the case of L-Arg's reaction with CHD in borate buffer, although borate is known to stabilize the 1,2-diol group (structure **VI**, Scheme 3) (Suckau *et al.*, 1992; Riordan, 1979); whereas

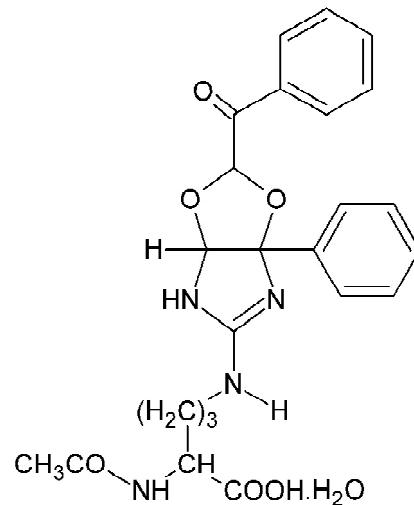
water condensation does not take place from 1:1 adduct of L-Arg and PG in borate buffer (Table 1), (structures **II-A/II-B**, Scheme 1). [1:1 adduct - H₂O] with CHD has been detected by matrix assisted laser desorption/ionization (MALDI) - MS, when arginine modification was carried out on an antifreeze protein in borate buffer (0.1 M, pH 9.0), wherein 10-fold molar excess of CHD was used (Wang *et al.*, 2009). Another interesting observation is that 2 molecules of CHD add on to 1 molecule of L-Arg in borate, but only 1:1 adduct is formed between L-Arg and PG in borate (Table 1). Two molecules of CHD adding on to sidechain guanidine of one arginine residue was not noted while modifying proteins (Suckau *et al.*, 1992; Zappacosta *et al.*, 1997). When hen egg white lysozyme was subjected to modification by CHD in borate buffer (pH 8.5 - 9), Suckau *et al.* (1992) found that one CHD molecule modified one arginine residue on the protein, which was detected by plasma desorption MS. By means of ESI-MS, Zappacosta *et al.* (1997) identified a tryptic peptide (from a *de novo* designed protein) that carried one molecule of CHD, where they had used 70-fold molar excess of CHD over the protein that was incubated for 30 minutes in sodium borate (50 mM, pH 8.5). The results obtained herein therefore hint that 1:1 adduct of L-Arg and PG is better stabilized by borate than 1:1 adduct of L-Arg and CHD. Therefore, not more than 1 molecule of PG adds onto guanidine in borate buffer, whereas 2 molecules of CHD modify guanidine sidechain in borate buffer and also condensation from the 1:1 adduct of L-Arg and CHD takes place in borate. Further support to this inference came from an interesting observation, wherein L-Arg with PG gave rise to all products (viz., both uncondensed and condensed forms of 1:1 & 1:2 adducts) in about 30 minutes itself, when this reaction was conducted at 60°C in borate (data not shown). This suggests that at a higher temperature than RT, the interaction between borate and 1,2-diol (structures **II-A** and **II-B**, Scheme 1) could get disrupted in a certain population of uncondensed 1:1 adduct and this could pave way for the formation of other products. This also indicates that there exists some population of uncondensed 1:1 adducts, where this interaction (structures **II-A** and **II-B**, Scheme 1) persists, even at 60°C.

Formation of 1:1 adduct with ^{14}C -PG in the absence of borate buffer also has been reported, upon modifying enzymes such as muscle carbonic anhydrase, D-amino acid oxidases and ferredoxin-NADP $^+$ reductase, but it is not possible to differentiate the amino-alcohol containing modified guanidine adduct (viz., 1:1 adduct) from the water condensed adduct/Schiff-base product ([1:1 adduct - H_2O]) by using ^{14}C -PG (Pullan and Noltmann, 1985; Vanoni *et al.*, 1987; Sancho *et al.*, 1990; Gadda *et al.*, 1994). Furthermore, ESI-MS characterization of PG modified dehydroquinases and creatine kinase revealed evidences for water condensed 1:1 adduct ($\Delta M = +116$ Da), whereby the modification was not carried out in borate buffer (Krell *et al.*, 1995; Krell *et al.*, 1998; Wood *et al.*, 1998). Moreover, reports thus far on arginine modification of certain enzymes by PG conducted in borate buffer have involved the use of ^{14}C -PG only and not mass spectrometry (Kazarinoff and Snell, 1976; Gadda *et al.*, 1994). Therefore, it could be interesting to carry out mass spectrometric investigation on PG modification of enzymes in borate buffer so as to look for the formation of 'uncondensed 1:1 adduct ($\Delta M = +134$ Da)', which has not been reported thus far. Correspondingly, the activity of the modified enzyme in borate buffer, where the enzyme's molecular mass would have increased by integral multiple of 134 Da, also needs to be assessed and compared with the catalytic properties of the modified enzyme in another buffer; such an investigation is not known yet.

Reaction medium, Tris.HCl (buffer 3): No products were conspicuous from the reaction between L-Arg and CHD in Tris.HCl buffer (Table 1b), as Tris itself was found to react with CHD (Figure S8, Supplementary Material). However, L-Arg underwent modification by PG in Tris.HCl (Table 1a), though signals were noted in the LC-ESI-MS data due to reaction between Tris and PG also (data not shown). Thus, CHD reacts with Tris more vigorously than PG reacting with Tris. This finding is in concurrence with the report of Patthy and Smith (1975a), who also observed Tris to be not suitable for carrying out arginine modification by CHD. Takahashi (1968) too reported the unsuitability of Tris buffer to modify arginine by PG.

First mass spectrometric evidence for a Takahashi's adduct (L-Arg+2PG: Uncondensed)

Formation of 1:2 adducts from equimolar concentrations of reactants is indeed a subject of intrigue. 1:2 adduct involving L-Arg and PG was first reported by Takahashi (1968), using ^{14}C -PG. For this, Takahashi had proposed two different structures: one structure depicting direct addition of 2 molecules of PG on guanidine of L-Arg (structure V, Scheme 2) and another representing 'water condensed 1:2 adduct', i.e., [1:2 adduct - H_2O]; herein, we call this as structure 'Takahashi V-C'. Based on qualitative periodate test, Takahashi surmised the structure shown below (viz. 'Takahashi V-C') as the most likely product for the 'water condensed 1:2 adduct'.



(Takahashi V-C)
(water condensed 1:2 adduct; viz. [1:2 adduct - H_2O])

Subsequently, several research groups too detected 1:2 adducts using ^{14}C -PG in enzymes, particularly in the studies that were focused to understand the functional role of arginine, however, these investigations did not describe about the nature of structural modification of sidechain guanidine (Dong *et al.*, 1991; Adak *et al.*, 1996; Corbalan-Garcia *et al.*, 1996; Yamamoto and Kawakita, 1999). Indeed, there were a few studies reporting detection of 1:2 adduct by ESI-MS, but all those mass spectrometric studies showed evidence for formation of [1:1 adduct - H_2O] and [1:2 adduct - H_2O] only, as interpreted

from the increment of intact molecular mass of the enzyme or proteolytic peptide by 116 Da ($\{1 \times 134 \text{ Da}\} - 18 \text{ Da}$) and 250 Da ($\{2 \times 134 \text{ Da}\} - 18 \text{ Da}$), respectively (Krell *et al.*, 1995; Krell *et al.*, 1998; Wood *et al.*, 1998). Herein too, [1:2 adduct - H₂O] is observed upon reaction of L-Arg with PG, as noted from the peak at *m/z* 425.29 (Figure 3b), which was found to be stable up to a period of about 24 hours. However, besides the structural modification depicted in 'Takahashi V-C', we have proposed structures **V-A** and **V-B** (see Scheme 2), to rationalize for *m/z* 425.29 ([1:2 adduct - H₂O]).

Additionally, a peak at *m/z* 443.31 was detected consistently in the LC-ESI-MS data acquired on the reaction mix of L-Arg and PG (Figure 3a). *m/z* 443.31 ([M+H]⁺), viz., M: 442 Da corresponds to the product resulting from the direct addition of 2 molecules of PG onto 1 molecule of L-Arg, i.e., 'uncondensed 1:2 adduct' (see structure **V** in Scheme 2). Thus, it is apparent that this is the first study demonstrating the mass spectrometric evidence for a Takahashi's adduct. It may be realized that the nature of structural modification represented on sidechain guanidine in structure **V** (Scheme 2) is the same that is proposed by Takahashi (1968) and we now refer it as "Takahashi **V**". Another notable feature is that the 'uncondensed 1:2 adduct' (structure Takahashi **V**) is noted in all six reaction mediums (four buffers and two solvents), excepting the borate buffer. Albeit, the peak at *m/z* 443.31 was noted to be stable for the initial 30 minutes only from the start of the reaction (see Scheme 2).

Water condensed 1:1 adduct (*m/z* 291) and 1:2 adduct (*m/z* 425) of PG modified L-Arg

As already explained previously, excepting the medium of borate buffer, L-Arg modification by PG predominantly resulted in 3 products: (1) [1:1 adduct - H₂O] (*m/z* 291), (2) 1:2 adduct (*m/z* 443) and (3) [1:2 adduct - H₂O] (*m/z* 425) (see Scheme 2 and Table 1a). The 1:1 adduct, *m/z* 309 remains to be stable for only 30 minutes or so in phosphate (buffer **2**) and tris (buffer **3**) and it is not at all detected in other media (buffers **4 & 5** and solvents **6 & 7**) (Scheme 2). The uncondensed 1:2 adduct (*m/z* 443, Takahashi **V**, Scheme 2) also seems to be stable for about 30 minutes. Hence,

[1:1 adduct - H₂O] (*m/z* 291) and [1:2 adduct - H₂O] (*m/z* 425) are the two products that are quite stable for a duration of about one hour in all the six mediums, but for borate. Further, we anticipated that the population of [1:2 adduct - H₂O] could be less than that of [1:1 adduct - H₂O], since the reactions are performed using only equimolar concentrations of the reactants. Therefore, using the peak intensities of *m/z* 291 and *m/z* 425 from the respective mass spectrum, we estimated the relative population distribution of these two products in buffers **2 - 5** and solvents **6 & 7** (see Figure S9, Supplementary Material). From Figure S9, quite contrary to our expectation, it is somewhat striking to see that the population of [1:2 adduct - H₂O] (*m/z* 425) is higher than the population of [1:1 adduct - H₂O] (*m/z* 291), excepting the buffer **5**, NH₄HCO₃ (pH 8). This suggests that the formation of [1:2 adduct - H₂O] (*m/z* 425) is more favored in buffers **2 - 4** and solvents **6 & 7**. Further, these two products 'condensed 1:1 and 1:2 adducts' of L-Arg and PG (*m/z* 291 and *m/z* 425) were found to be stable up to about 24 hours from the onset of the reaction, except in the borate medium.

Formation of condensed products of 1:1 and 1:2 stoichiometric adducts involving arginine and PG has been noticed with enzymes as well. For example, Krell *et al.* found [1:1 adduct - H₂O] resulting in $\Delta M = +116 \text{ Da}$ and [1:2 adduct - H₂O] giving rise to $\Delta M = +250 \text{ Da}$ from ESI-MS data, while modifying arginines in type II dehydroquinases by PG in sodium bicarbonate buffer (pH 9.4) (Krell *et al.*, 1995; Krell *et al.*, 1998). Likewise, McLafferty and co-workers too detected water condensed 1:1 ($\Delta M = +116 \text{ Da}$) and 1:2 ($\Delta M = +250 \text{ Da}$) adducts by ESI - Fourier Transform MS, when arginine modification was carried out on creatine kinase by PG in a buffer containing a mixture of sodium bicarbonate and bicine; however, the abundance of [1:1 adduct - H₂O] ($\Delta M = +116 \text{ Da}$) was noted to be greater than that of [1:2 adduct - H₂O] ($\Delta M = +250 \text{ Da}$) with creatine kinase (Wood *et al.*, 1992). In a very recently published report, Wanigasekara and Chowdhury (2016) too have observed 'condensed 1:1 and 1:2 adducts' of few model peptides with PG in potassium bicarbonate buffer; as detected from MALDI - quadrupole ion trap (QIT) - time of flight (TOF) MS.

Arginine modification of peptides

In the case of arginine modification of human angiotensin II, DRVYIHPF by PG or CHD, LC-ESI-MS data did not show any evidences for the formation of water condensed adducts under equimolar condition. Also, no signals corresponding to 1:2 stoichiometric adducts were observed. Peaks with very good intensity clearly corresponding to 1:1 adduct alone were detected in the mass spectra (Figure 5). As already mentioned, the reactions on angiotensin II were done in all mediums at RT, utilizing equimolar conditions, but in the nanomole range (sections Materials and methods and Results). Another notable feature is that the ESI charge state distribution of PG modified- as well as CHD modified peptides were not significantly different from that of unmodified peptide. Initially, it was expected that the basicity of guanidine would decrease upon modification, thereby rendering the peptide to take up less number of protons under ESI conditions. Therefore, we hypothesized that peaks corresponding to lower protonation states could have higher intensity than the peaks of higher protonation states in the ESI mass spectrum of the modified peptide. Quite contrary to the expectation, both modified and unmodified peptides exhibited same protonation states under ESI conditions, viz., the relative intensity distributions of $[M+3H]^{3+}$ and $[M+2H]^{2+}$ ions of unmodified peptide did not alter drastically subsequent to modification by PG or CHD. This implies that even after modifying sidechain guanidine of arginine, the modified peptide adopts same number of protons as that of unmodified peptide. Indeed, a very similar observation was reported under nanospray ionization conditions for CHD modified angiotensin II and in the case of CHD modified hen egg white lysozyme by ESI-MS (Suckau *et al.*, 1992; Fligge *et al.*, 1999). Further, with reference to ESI charge states distribution, the results obtained from modification of B-chain of insulin were also very similar to those noted on angiotensin II (Figure 6). However, unlike angiotensin II, the experiments on B-chain of insulin illustrated the importance of three-dimensional structure (i.e., folding state) of the polypeptide in significantly influencing the progress of the modification reaction. This was

understood from the observation, where insulin's B-chain underwent modification in a span of about 30 - 60 minutes upon incubating the reaction mix at 60°C with equimolar concentrations of the reactants (see Materials and methods). But, when this reaction was allowed to take place at RT, the modification of B-chain became noticeable after the addition of two fold molar excess of the reagent (PG or CHD) and that too after about 5 hours or subsequent to overnight (~16 hours) incubation only. Under both the circumstances, the polypeptide formed only uncondensed 1:1 adduct with PG ($\Delta M = +134$ Da) or CHD ($\Delta M = +112$ Da). It is important to note that the working concentration for the modification of B-chain of insulin was in the picomole range (see Materials and methods). However, in the case of reactions with 'intact insulin', modification was conspicuous only upon adding ~200 fold molar excess of the reagents and when incubated for ~16 hours at RT.

In contrast to the conditions followed herein for arginine modification in peptides, in a very recently published study, Wanigasekara and Chowdhury (2016) have used 30-fold molar excess of PG or CHD on model and tryptic peptides. Moreover, they have used (0.125 M) potassium bicarbonate medium for carrying out modification with PG, while for modification by CHD, they have utilized (200 mM) sodium hydroxide, as described by Toi *et al.* (1965 and 1967).

PG and CHD do not modify α -NH₂ group of amino acids

Takahashi (1968) reported that PG modifies α -NH₂ group of amino acids as well, especially with dipeptides, PG reacts quite rapidly, resulting in formation of a-keto acyl derivatives, in addition to the modification of sidechain guanidine. In order to verify this, we, therefore, decided to perform acetylation reactions (see Materials and methods) on PG-mod-L-Arg (m/z 309; $[M+H]^+$) and CHD-mod-L-Arg (m/z 287; $[M+H]^+$), since primary amino groups readily undergo acetylation. It was evident from the LC-ESI-MS data that both PG mod-as well as CHD mod- L-Arg underwent acetylation irrespective of any reaction medium, ascertaining the presence of free

α -NH₂ group on each of the modified L-Arg (Figures S2-S4, S6 & S7, Supplementary Material). We also conducted experiments on N-acetyl arginine, which revealed modification of sidechain guanidine by PG and CHD (data not shown). Furthermore, L-Ala and L-Lys were subjected to reactions with PG and CHD in each of the seven media, whose mass spectral data did not show any evidence of modification (data not shown). Hence, our data herein establishes that PG and CHD specifically modifies 'sidechain guanidine' of L-Arg, but not its α -NH₂ group.

In this connection, a recent study reported modifications on N-acetyl arginine by four different 1,2-dicarbonyl compounds using direct infusion mode of ESI-MS; however the modifying reagents, experimental conditions and the outcomes of that study are entirely different from this investigation (Saraiva *et al.*, 2016). Moreover, for interpreting signals in the mass spectrum that correspond to loss of H₂O molecule, Saraiva *et al.* (2016) proposed epoxy moiety containing modified N-acetyl arginine structures; on the other hand, we have chosen Schiff-base type of molecular structures to infer for water condensed arginine-modified products.

Arginine modification of bovine pancreatic RNase A

To understand the extent of arginine modification on the protein, we chose bovine pancreatic ribonuclease A (RNase A) as a model, which has four arginines. Equimolar addition of PG/CHD to the intact protein leads to modification of only one arginine on the protein, whose population was found to be significantly lower as compared to that of native RNase A (data not shown; see Results section). However, addition of ~100 fold molar excess of PG/CHD with reference to protein's concentration yielded good population of modified products; wherein it was found that maximum 3 molecules of PG or CHD add on to the intact protein (see Figure 7). Depending on the folding state of the protein in a given condition, arginines at different sites would have different degrees of surface accessibility. Herein, progress of all reactions on intact RNase A was adjudged after overnight (~16 hours) incubation at RT. Under this circumstance, these

observations suggest that addition of molar excess concentration of PG/CHD causes modification of those arginines that have higher surface accessibility, whereas equimolar concentration of PG/CHD could modify only one of the four arginines having higher degree of surface accessibility on RNase A. Thus, it may be inferred that the three-dimensional structure of the protein is also an important factor in influencing the extent of arginine modification.

Altogether, from the studies carried out herein, it is evident that the four key factors that dictate the extent of arginine modification are: (i) relative concentration of the enzyme/protein with respect to the arginine modifying reagent; (ii) degree of surface-accessibility of arginine(s) on the enzyme/protein, which in turn, is dependent on the 3-dimensional structure or the folding state of the protein, under a given set of conditions, (iii) reaction time and (iv) reaction medium. Indeed, these four aspects are fundamental and are well known (or obvious) to impact the fate of reactions involving proteins. But, thus far, the significance of these factors have not been systematically demonstrated in the previous studies carried out on enzymes or proteins, since several of the past investigations have solely focused on to understand the role of arginine for enzymatic activity. And hence, in such studies the modifying reagent would continued to be added, until a significant reduction in the catalytic activity would be observed; and in many cases, molar excess of the modifier would be added to observe the decrease of the catalytic activity (Takahashi, 1968; Kitson and Knowles, 1971; Pathy and Smith, 1975a; Fleer *et al.*, 1981; Ferti *et al.*, 1981; Suckau *et al.*, 1992; Zappacosta *et al.*, 1997; Wood *et al.*, 1998; Watanabe and Funatsu, 1986; Gould and Norton, 1995). Equimolar concentration of the reactants may not bring about modification of the arginines that are not exposed on the surface of the protein. However, addition of molar excess of the reagent with reference to protein's concentration, can lead to denaturation of the protein, which could bring the buried arginine residues to the surface of the protein. Those surface exposed arginines would then begin to undergo chemical modification. Therefore, it is rather straightforward to anticipate the formation of either 1:1 or 1:2 adduct of

Enzyme: PG or Enzyme:CHD or presence of mixture of both 1:1 and 1:2 types of arginine modified adducts of the enzymes/proteins, upon addition of molar excess of the reagent. Also, it is indeed possible that the condensed products of 1:1 and 1:2 adducts, coexist with the respective uncondensed 1:1 and 1:2 adducts, in the same milieu.

Conclusions

A highlight of this study is that arginine undergoes modification in the solvents, H₂O and ACN:H₂O (1:1). Initially, it was expected that reactions would not take place in these mediums. However, when the pH of the arginine solution in H₂O was found to be >10, we decided to explore the modification reactions in H₂O as well. Further, we conducted reactions in NH₄HCO₃, which has not been reported thus far for performing arginine modification studies of enzymes/proteins and found the products of L-Arg with PG as well as CHD in this buffer. Another unique outcome of this investigation is the detection of 'uncondensed' 1:1 and 1:2 adducts (L-Arg and PG or CHD) in certain mediums, which have not been observed previously on enzymes/proteins. In fact, herein for the first time we present the evidence of detecting 'uncondensed 1:2 adduct' of L-Arg and PG, viz., *m/z* 443, which was originally propounded by Takahashi (1968); however, it is a short-lived species (~30 mins.). The 'uncondensed' 1:1 adduct of L-Arg and PG (i.e., *m/z* 309) too was found to be not a very stable product, but in borate buffer it had very good stability for a long time. Further, L-Arg and CHD formed both condensed and uncondensed 1:1 and 1:2 adducts in borate suggesting that borate stabilizes the 1:1 adduct of L-Arg and PG far better than the 1:1 adduct of L-Arg and CHD. Furthermore, in the case of modification of peptides, predominantly uncondensed 1:1 adduct was observed in this study. In contrast, previous mass spectrometry based reports on arginine modified proteins and peptides have only shown the formation of condensed 1:1 or 1:2 adducts (Krell *et al.*, 1995; Krell *et al.*, 1998; Wood *et al.*, 1998; Wanigasekara and Chowdhury, 2016). Additionally, we find PG and CHD not reacting with α -amino group of amino acids, as observed from the reactions

carried out on L-Ala, L-Lys and PG/CHD-mod-L-Arg, in the mediums chosen in this study. Moreover, acetylation reactions carried out on PG-mod- or CHD-mod- L-Arg confirmed that these two reagents are specific towards modifying only guanidine sidechain of L-Arg, in the reaction mediums studied herein.

Past studies have indeed attempted to understand the role of buffer type/medium in impacting the extent of arginine modification (Takahashi, 1968; Patthy and Smith, 1975a; Vlahos *et al.*, 1985; Wanigasekara and Chowdhury, 2016). Also, in a few studies, structural modifications occurring to guanidine sidechain of arginine in enzymes/proteins due to PG or CHD have been investigated from the mass spectral data of modified intact proteins and proteolytic peptides (Suckau *et al.*, 1992; Zappacosta *et al.*, 1997; Wang *et al.*, 2009; Krell *et al.*, 1995; Krell *et al.*, 1998; Wood *et al.*, 1998). On the other hand, the outcomes of this study are unique because of choosing to experiment on the amino acid L-Arg. The absence of conformational constraint in the case of L-Arg has permitted to visualize multiple products more clearly than the earlier reports. Additionally, the virtue of LC-ESI-MS was instrumental in the identification of multiple products in a single reaction mixture. A key advantage of LC-ESI-MS is that it allows detection of multiple products formed, if any, during the course of the reaction as well as it permits monitoring the stability or lifetime of those products.

Overall, this study demonstrates that LC-ESI-MS should be applicable to investigate biochemical, organic or perhaps even inorganic chemical reactions, which might proceed through multiple steps. In such cases, the LC-ESI-MS data shall be useful to detect and to know about the stability of the intermediate(s) that may be formed during the course of the reaction, thereby eventually enabling 'elucidation of the reaction pathway kinetics'. Additionally, this investigation emphasizes the importance of reaction medium (solvent or buffer) in influencing the progress of chemical or biochemical reactions. Furthermore, the results presented herein may be useful for mass spectrometry based *de novo* peptide or protein sequencing, especially when the unknown peptide or protein has multiple arginines.

Acknowledgements

This work is funded by the Start-Up Research Grant (Young Scientists) No. SB/YS/LS-370/2013, received by VS, from Science and Engineering Research Board (SERB), New Delhi, India. We thank Dr. A.S. Kamalanathan for help during data acquisitions in the high-resolution mass spectrometer (Q/TOF).

Abbreviations

PG, Phenylglyoxal; CHD, 1,2 Cyclohexanedione; RT, Room Temperture; ACN, Acetonitrile; LC, Liquid Chromatography; ESI, Electrospray Ionization; MS, Mass Spectrometry; L-Ala, L-Alanine; L-Lys, L-Lysine; DTT, Dithiothreitol; IAM, Iodoacetamide; TIC, Total Ion Chromatogram; MALDI, Matrix Assisted Laser Desorption/Ionization; Ac_2O , Acetic anhydride; Da, Dalton; UPLC, Ultra Performance Liquid Chromatography; PDA, Photo Diode Array; AJS, Agilent Jet Stream; CID, Collision Induced Dissociation.

Conflict of Interest

The authors do not have any conflict of interest with the contents of this manuscript.

References

- Adak, S., Mazumder, A., and Banerjee, R.K. (1996). Probing the active site residues in aromatic donor oxidation in horseradish peroxidase: involvement of an arginine and a tyrosine residue in aromatic donor binding. *Biochem. J.* 314, 985 -991.
- Corbalan-Garcia, S., Teruel, J.A., and Gomez-Fernandez, J.C. (1996). Involvement of an arginyl residue in the nucleotide- binding site of Ca^{2+} -ATPase from sarcoplasmic reticulum as seen by reaction with phenylglyoxal. *Biochem. J.* 318, 179-186.
- Dong, Q., Liu, F., Myers, A.M., and Fromm, H.J. (1991). Evidence for an Arginine Residue at the Substrate Binding Site of Escherichia coli Adenylosuccinate Synthetase as studied by chemical Modification and Site- directed Mutagenesis. *J. Biol. Chem.* 266, 12228 - 12233.
- Eriksson, O., Fontaine, E., and Bernardi, P. (1998). Chemical Modification of Arginines by 2,3-Butanedione and phenylglyoxal causes closure of the mitochondrial permeability Transition pore. *J. Biol. Chem.* 273, 12669 - 12674.
- Ferti, C., Curti, B., Simonetta, M.P., Ronchi, S., Galliano, M. and Minchiotti, L. (1981). Reactivity of D-Amino Acid Oxidase with 1,2- Cyclohexanedione: Evidence for one Arginine in the Substrate-Binding Site. *Eur. J. Biochem.* 119, 553-557.
- Fleer, E. A., M. Puijk, W. C. Slotboom, A.J., and De Haas, G. H. (1981). Modification of Arginine Residues in Porcine Pancreatic Phospholipase A₂. *Eur. J. Biochem.* 116, 277-284.
- Fligge, T. A., Kast, J., Bruns, K. and Przybylski, M. (1999). Direct Monitoring of Protein-Chemical Reactions Utilising Nanoelectrospray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 10, 112-118.
- Gabel, S. A. and London, R. R. (2008). Ternary borate-nucleoside complex stabilization by Ribonuclease A demonstrates phosphate mimicry. *J. Biol. Inorg. Chem.* 13, 207-217.
- Gadda, G., Negri, A., and Pilone, M.S. (1994). Reaction of Phenylglyoxal with Arginine Groups in D-Amino-acid Oxidase from *Rhodotorula gracili*. *J. Biol. Chem.* 269, 17809-17814.
- Gardner, G., Allen, C. D. and Paterson, D. R. (1983). Modification of Photosystem II by phenylgloxal. *FEBS Lett.* 164, 191-194.
- Gould, A.R. and Norton, R. S. (1995). Chemical modification of cationic groups in the polypeptide cardiac stimulant Anthopleurin-A. *Toxicon.* 33, 187-199.
- Jackson, D.G., and Hersh, L.B. (1986). Reaction of Neutral Endopeptidase 24.11 (Enkephalinase) with Arginine reagents. *J. Biol. Chem.* 261, 8649-8654.
- Kazarinoff, M.N., and Snell, E.E. (1976). D-serine dehydratase from *Escherichia coli*. *J. Biol. Chem.* 251, 6179-6182.
- Kitson, T.M., and Knowles, J.R. (1971). The effect of arginine modification on the pH dependence of pepsin activity. *FEBS Lett.* 16, 337-338.
- Krell, T., Pitt, A. R. and Coggins, J. R. (1995). The use of electrospray mass pectrometry to identify an essential arginine residue in type II dehydroquinases. *FEBS Lett.* 360, 93-96.
- Krell, T., Chackrewarthy, S., Pitt, A.R., Elwell, A. and Coggins, J.R. (1998). Chemical modification monitored by electrospray mass spectrometry: a rapid and simple method for identifying and studying functional residues in enzymes. *J. Peptide Res.* 51, 201-209.
- London, R. E. and Gabel, S. A. (2002). Formation of a Trypsin-Borate-4-Aminobutanol Ternary Complex. *Biochemistry* 41, 5963-5967.
- Mahley, R.W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H., and Gross E.(1977). Gross. Inhibition of Lipoprotein Binding to Cell Surface Receptors of Fibroblasts following Selective Modification of Arginyl Residues in Arginine-rich and B Apoproteins. *J. Biol. Chem.* 252, 7279-7287.
- Masuda, T., Ide, N. and Kitabatake, N. (2005). Structure-Sweetness Relationship in Egg white Lysozyme: Role of Lysine and Arginine Residues on the Elicitation of Lysozyme Sweetness. *Chem. Senses.* 30, 667-681.
- Mendoza, V.L. and Vachet, R.W. (2009). Probing protein structure by Amino Acid-Specific Covalent Labeling and Mass Spectrometry. *Mass Spectrom. Rev.* 28, 785 -815.
- Ni, M. and Beevers, L. (1990). Essential Arginine Residues in the Nitrate Uptake System from Corn Seedling Roots. *Plant Physiol.* 94, 745-751.
- Patthy, L., and Smith, E.L. (1975a). Reversible Modification of Arginine Residues. *J. Biol. Chem.* 250, 557-564.

- Pathy, L., and Smith, E.L. (1975b). Identification of Functional Arginine Residues in Ribonuclease A and lysozyme. *J. Biol. Chem.* 250, 565-569.
- Pullan, L.M., and Noltmann, E.A. (1985). Specific Arginine Modification at the Phosphatase Site of Muscle Carbonic Anhydrase. *Biochemistry*. 24, 635-640.
- Riordan, J.F. (1979). Arginyl Residues And Anion Binding Sites in Proteins. *Mol. Cell Biochem.* 26, 71-92.
- Sancho, J., Medina, M., and Gomez-Moreno, C. (1990). Arginyl groups involved in the binding of *Anabaena* ferredoxin- NADP⁺ reductase to NADP⁺ and to ferredoxin. *Eur. J. Biochem.* 187, 39-48.
- Saraiva, M. A., Borges, C. M. and Helena Florencio, M. (2016). Mass spectrometric studies of the reaction of the blocked arginine with diketonic α -dicarbonyls. *Amino Acids*. 2016, 48, 873-885.
- Suckau, D., Mak, M. and Przybylski, M. (1992). Protein Surface topology-probing by selective chemical modification and mass spectrometric peptide mapping. *Proc. Natl. Acad. Sci. USA*. 89, 5630-5634.
- Takahashi, K., (1968). The Reaction of phenylglyoxal with arginine residues in proteins. *J. Biol. Chem.* 243, 6171 -6179.
- Toi, K., Bynum, E., Norris, E. and Itano, H.A. (1965). Chemical Modification of Arginine with 1,2-Cyclohexanedione. *J. Biol. Chem.* 240, PC3455 - PC3457.
- Toi, K., Bynum, E., Norris, E. and Itano, H.A. (1967). Studies on the Chemical modification of Arginine. *J. Biol. Chem.* 1967, 242, 1036-1043.
- Transue, T. R., Krahn, J.M., Gabel, S. A., DeRose, E. F. and London, R. E. (2004). X-ray and NMR Characterization of covalent complexes of trypsin, Borate, and Alcohols. *Biochemistry*. 43, 2829-2839.
- Traub, P. and Vorgias, C.E. (1984). Differential Effect of Arginine Modification with 1,2-Cyclohexanedione on the capacity of Vimentin and Desmin to assemble into intermediate filaments and to bind to Nucleic Acids. *J. Cell. Sci.* 65, 1-20.
- Vanoni, M.A., Pilone Simonetta, M., Curti, B., Negri, A., and Ronchi, S. (1987). Phenylglyoxal modification of arginines in mammalian D-amino-acid oxidase. *Eur. J. Biochem.* 167, 261-267.
- Vensel, L.A., and Kantrowitz, E.R. (1980). An Essential Arginine Residue in porcine Phospholipase A2. *J. Biol. Chem.* 255, 7306-7310.
- Viale, A.M. and Vallejos, R. H. (1985). Identification of an Essential Arginine Residue in the β Subunit of the Chloroplast ATPase. *J. Biol. Chem.* 1985, 260, 4958-4962.
- Vlahos, C. J., Ghalambor, M. A. and Dekker, E. E. (1985). Evidence for an Essential Arginine Residue in the Active Site of *Escherichia coli* 2-Keto-4-hydroxyglutarate Aldolase. *J. Biol. Chem.* 260, 5480-5485.
- Wang, S., Amornwittawat, N., Juwita, V., Kao, Y., Duman, J.G., Pascal, T.A., Goddard, W.A. and Wen, X. (2009). Arginine, a Key residue for the Enhancing Ability of an Antifreeze Protein of the Beetle *Dendroides canadensis*. *Biochemistry*. 48, 9696-9703.
- Wanigasekara, M. S. K. and Chowdhury, S. M. (2016). Evaluation of chemical labeling methods for identifying functional arginine residues of proteins by mass spectrometry. *Anal. Chim. Acta*. 935, 197-206.
- Watanabe, K. and Funatsu, G. (1986). Involvement of arginine residues in inhibition of protein synthesis by ricin A-chain. *FEBS Lett.* 204, 219-222.
- Weng, L., Heinrikson, R.L., and Westley, J. (1978). Active Site Cysteinyl and Arginyl residues of Rhodanese. *J. Biol. Chem.* 253, 8109-8119.
- Wieth, J.O., Bjerrum, B.J., and Borders JR., C.L. (1982). Irreversible Inactivation of Red Cell Chloride Exchange with Phenylglyoxal, an Arginine- specific Reagent. *J. Gen. Physiol.* 79, 283-312.
- Wood, T.D., Guan, Z., Borders Jr., C.L., Chen, L. H., Kenyon, G.L. and McLafferty, F.W. (1998). Creatine kinase: Essential arginine residues at the nucleotide binding site identified by chemical modification and high-resolution tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA*. 95, 3362-3365.
- Yamamoto, H., and Kawakita, M. (1999). Chemical modification of an arginine residue in the ATP-binding site of Ca²⁺ -transporting ATPase of sarcoplasmic reticulum by phenylglyoxal. *Mol. Cell Biochem.* 190, 169-177.
- Zappacosta, F., Ingallinella, P., Scaloni, A., Pessi, A., Bianchi, E., Sollazzo, M., Tramontano, A., Marrino, G. and Pucci, P. (1997). Surface topology of Minibody by selective chemical modifications and mass spectrometry. *Protein Sci.* 6, 1901-1909.
- Zhang, Q., Tang, N., Brock, J.W.C., Mottaz, H. M., Ames, J. M., Baynes, J.W., Smith, R. D. and Metz, T. O. (2007). Enrichment and Analysis of Non-enzymatically Glycated Peptides: Boronate Affinity Chromatography coupled with Electron Transfer Dissociation Mass Spectrometry. *J. Proteom. Res.* 6, 2323-2330.