



# Observation of the side chain O-methylation of glutamic acid or aspartic acid containing model peptides by electrospray ionization-mass spectrometry



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## ABSTRACT

O-methylation of the side chains of glutamic acid (E) and aspartic acid (D) residues is generally observed modification when an acidified methanol/water (MeOH/dH<sub>2</sub>O) mixture is used as a solvent system during sample preparation for proteomic research. This chemical modification may result misidentification with endogenous protein methylation; therefore, a special care should be taken during sample handling prior to mass spectrometric analysis. In the current study, we systematically examined the extent of E/D methylation and C-terminus carboxyl group of synthetic model peptides in terms of different incubation temperatures, storage times, and added acid types as well as its percentages. To monitor these effects, C-terminus amidated and free acid forms of synthetic model peptides comprised of E or D residue(s) have been analyzed by electrospray ionization-mass spectrometry (ESI-MS). Additionally, LC-MS/MS experiments were performed to confirm the formation of methylated peptide product. The results showed that the rate of methylation was increased as the temperature increases along with prolong incubation times. Moreover, the extent of methylation was remarkably high when formic acid (FA) used as a protonation agent instead of acetic acid (AA). In addition, it was found that the degree of methylation was significantly decreased by lowering acid percentages in ESI solution. More than one acidic residue containing model peptides have been also used to explore the extent of multiple methylation reaction. Lastly, the ethanol (EtOH) and isopropanol (iPrOH) have been substituted separately with MeOH in sample preparation step to investigate the extent of esterification reaction under the same experimental conditions. However, in the positive perspective of view, this method can be used as a simple, rapid and cheap method for methylation of acidic residues under normal laboratory conditions.

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## 1. Introduction

In the past two decades, electrospray ionization-mass spectrometry (ESI-MS) has been recognized as a promising method for the analysis of biological macromolecules [1,2]. In particular, identification and characterization of posttranslational modifications (PTMs) of proteins (phosphorylation, methylation, glycosylation, acetyla-

tion, etc.) can be achieved via tandem mass spectrometry (MS/MS) experiments [3,4]. Among these modifications, protein methylation is one of the most prominent PTM found in eukaryotic cells. It plays vital roles in regulation of histones, DNA repair, RNA processing, and signal transduction [5–9]. Previous reports showed that methylation is generally observed on lysine (K) and arginine (R) residues [8,10]. However, recent works have revealed that glutamic acid (E), aspartic acid (D), glutamine (Q), asparagine (N), cysteine (C), and histidine (H) residues may also undergo methylation [8,11,12]. A very recent report by Wang et al. [13] demonstrated the preference of protein methylation follows K » R > D > N ~ Q ~ H > E > C trend in *Saccharomyces cerevisiae*.

The artificial methylation on E residue as well as at the C-terminus of peptide has been observed for the first time by Haebel et al. [14] during staining procedure of polyacrylamide gels. The staining solution buffer consists of trichloroacetic acid (TCA) and methanol (MeOH) and it was shown that γ-carboxyl group of E con-

**Abbreviations:** AA, acetic acid; CBB, comassie brilliant blue; CID, collision-induced dissociation; D, aspartic acid; E, glutamic acid; ESI-MS, electrospray ionization-mass spectrometry; EtOH, ethanol; FA, formic acid; iPrOH, isopropanol; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI, matrix-assisted laser desorption ionization; MeOH, methanol; MS/MS, tandem mass spectrometry; pE, pyroglutamic acid; PTM, posttranslational modification.

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verted to its corresponding methyl ester form via acid-catalyzed esterification reaction. Additionally, the authors highlighted that the rate of D methylation was too slow compared to the E methylation. They also provide an evidence for ethylation of E when ethanol (EtOH) was used instead of MeOH as a solvent [14]. On the other hand, Sprung et al. [12] have reported that the side chains of E and D can be methylated when MeOH was used as a solvent in protein extraction protocols and gel-staining/destaining buffers of proteomic studies. A short communication by Sumpton and Bienvenut [15] has demonstrated that the use of colloidal Commae brilliant blue (CBB) dissolved in trace amount of phosphoric acid containing MeOH can induce methylation of side chains of acidic residues (E and D) during gel staining procedures. Additionally, it has been shown that MALDI matrix/sample preparation procedures may also cause acidic residues to be methylated [16].

From organic chemistry, it has been well known that the condensation reactions of carboxylic acids with an excess alcohol produce an ester. The reaction mechanism proceeds in the presence of a strong acid, therefore it is also known as an acid-catalyzed esterification (Fischer esterification). Previous studies showed that various carboxylic acids were converted to their corresponding methyl ester derivative under methanol/trimethylchlorosilane solvent system [17–19]. Similarly, E and D residues can be easily converted to their methyl ester form when MeOH has been used as a solvent in acidic media [14,16,20].

In ESI-MS experiments, a mixture with an equal proportion of polar/volatile organic solvent (e.g. MeOH, acetonitrile, or isopropanol) and ultrapure water has been used especially via direct infusions. The organic solvents generally added to the liquid mixture to decrease the surface tension which facilitates formation of gas-phase ions through ESI process. Among these solvents, MeOH is one of the most extensively used organic solvent for solubilising of proteins and peptides prior to mass spectrometric analysis. It is preferred due to its high polarity, volatility, and compatibility. Moreover, in the positive mode MS analysis, the analyte solution is generally acidified with formic acid (FA) or acetic acid (AA) with a range of 0.1–1% to promote ionization.

In our previous studies on the fragmentation reactions of E/D containing model peptides [21,22], we detected  $[M+H+14]^+$  ions in the full scan mass spectra (MS). Further MS/MS experiments have revealed that the side chains of E/D residue were methylated. The formation of unexpected methylated derivative of peptide can be explained by long term storage of ESI solution in an acidified methanolic solution. Therefore, in the current study, we comprehensively investigate the extent of side chain methylation of E/D residues and/or C-terminus carboxyl group of peptide chain in terms of different incubation temperatures, storage times, added acid types and its percentages. Additionally, two adjacent E or D residue containing model peptides have been also used in order to probe the extent of multiple methylation reaction. Lastly, the effects of organic solvent on the esterification reaction have been also studied.

## 2. Experimental

### 2.1. Chemicals and sample preparations

The model peptides (either C-terminus free acid or amide forms) were purchased from GL Biochem Ltd. (Shanghai, China) as a lyophilized powder (purity >90%) and used without any further purification. HPLC-grade methanol (MeOH), and ethanol (EtOH) were provided by Sigma Aldrich. The analytical grade formic acid (FA, purity of 98–100%) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany); acetic acid (100%, puriss) was from Riedel-de Haën; isopropyl alcohol (*i*PrOH, Biochemica)

was from Applichem (Darmstadt, Germany). Throughout the study, ultra-pure water (18.2 M $\Omega$ ) was used (Arium 611 UV, Sartorius AG, Goettingen, Germany). The solid peptide samples were dissolved in a 50:50 (v/v) mixture of organic solvent (methanol, ethanol, or isopropanol) and ultra-pure water just before to use.

### 2.2. Mass spectrometry

Mass spectrometry experiments were conducted on a LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The system was operated in the positive mode and the spray voltage was set to +5.0 kV and the heated capillary temperature at 300 °C. All ion optics was optimized in order to get maximum ion intensity with an auto-tune function of the ion-trap instrument. A 100 pmol  $\mu\text{L}^{-1}$  peptide solutions were introduced into the ion source with an incorporated syringe pump at a flow rate of 5  $\mu\text{L}/\text{min}$ . The default peptide working solutions were prepared in MeOH/ $\text{dH}_2\text{O}/\text{HCOOH}$  (50:50:1, v/v/v) containing mixture. Nitrogen was used as a sheath, an auxiliary, and a sweep gas with a flow rate of 10, 1, 1 (in arbitrary unit), respectively, where helium was used as a collision gas for CID. Full scan MS spectra were acquired in the  $m/z$  range 150–1000 and at least 500 scans were averaged in profile mode. For the tandem mass spectrometry (MS/MS) experiments, the mass isolation window ( $m/z$ ) was set at between 1.0 and 1.4 with an activation time of 30 ms and an activation ( $q$ ) of 0.250. The normalized collision energy was set to 20–26% for the fragmentation of selected precursor ion via (MS/MS). Data acquisition was performed with Xcalibur (ver. 2.0) software data system and all data were further processed using Igor Pro Software package (WaveMetrics, Lake Oswego, OR).

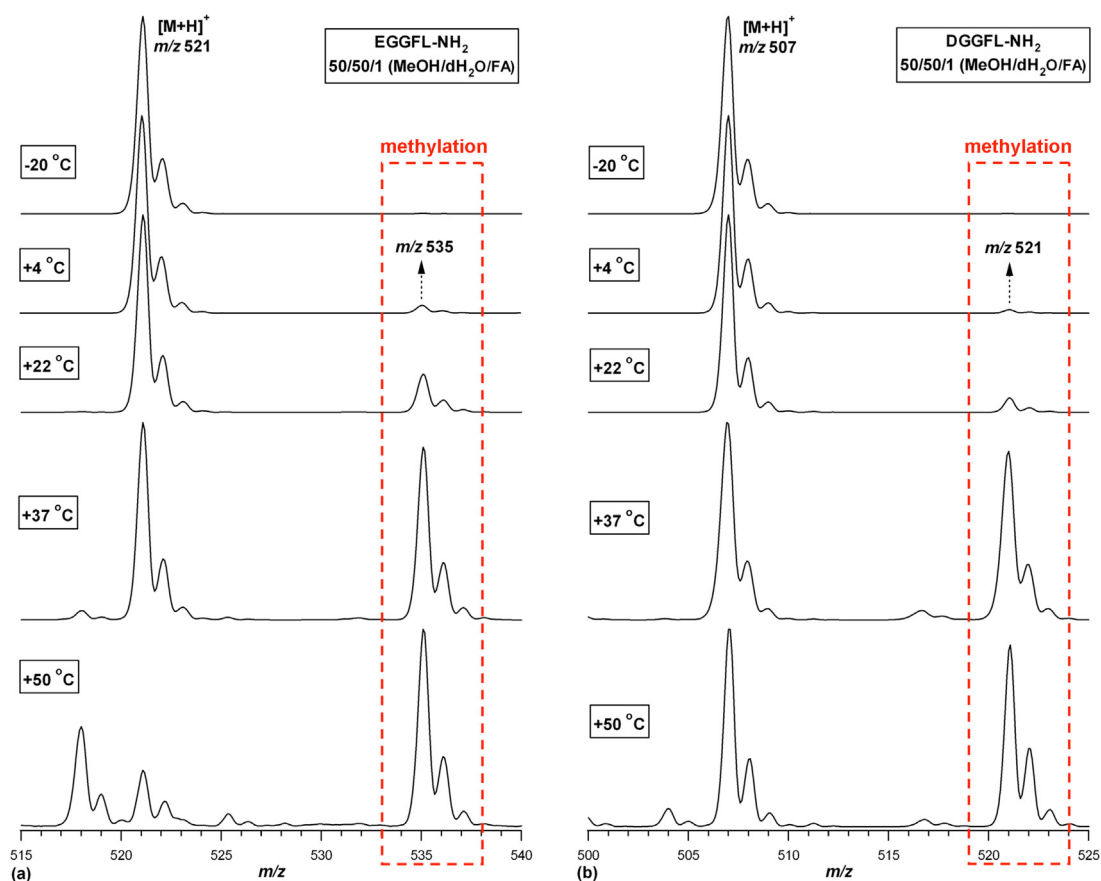
### 2.3. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) experiments were performed on a Dionex Ultimate™ 3000 Nano/Cap system (Dionex, Camberley, UK) using a Kromasil C<sub>18</sub> column (10 cm length  $\times$  2.1 mm i.d., 3.5- $\mu\text{m}$  particle size, 100 Å° pore diameter). The column temperature was maintained at 25 °C. Mobile phases A and B were composed of 0.1% trifluoroacetic acid (TFA) in water and acetonitrile, respectively. Peptides were eluted with a thirty minute gradient program: 15% B for 0 min; followed by a gradient of 15–40% B in 25 min and 80% B for 25.01–30 min. The UV detection wavelength was set to 220 nm. The flow rate was 200  $\mu\text{L}/\text{min}$  and the injection volume was 5  $\mu\text{L}$  which contains approximately 5  $\mu\text{g}$  of peptide sample. Eluted peptides were then splitted with 1/20 ratio from the exit of the detector and were directed into the mass spectrometer equipped with an ESI source. The chromatograms were monitored via on-line recording of the full mass scans within the 150–600 mass range.

## 3. Results and discussion

### 3.1. Effects of different incubation temperatures and storage times on the methylation reaction

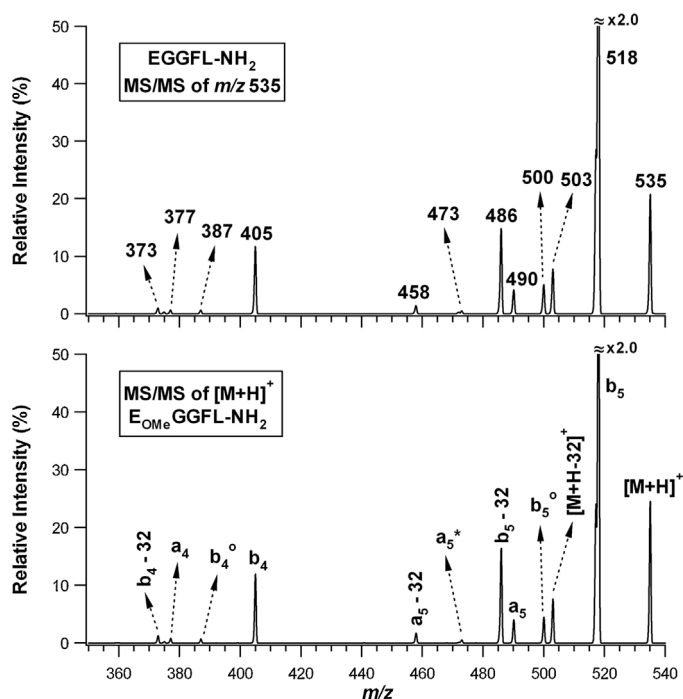
In the first part of this study, EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> synthetic model pentapeptides were primarily used to investigate the effects of incubation temperatures and storage times for the extent of methylation reaction. As noted, these peptides have an amide group rather than free carboxylic acid at their C-terminus; therefore, only possible methylation site is on the side chain of E or D residue. Other amino acid residues within the sequence (G, F, and L) do not bear carboxylic acid group on their side chains; therefore they are inert to methylation. For each peptide sample, five working ESI solutions with a concentration of 100 pmol  $\mu\text{L}^{-1}$  were



**Fig. 1.** Comparison of full scan MS of a) EGGFL-NH<sub>2</sub> and b) DGGFL-NH<sub>2</sub> peptide solutions, recorded after thirty days incubation at  $-20^{\circ}\text{C}$ ,  $+4^{\circ}\text{C}$ ,  $+22^{\circ}\text{C}$ ,  $+37^{\circ}\text{C}$  or  $+50^{\circ}\text{C}$ . The ESI solutions were prepared in 50:50:1 (v/v/v) MeOH/dH<sub>2</sub>O/FA.

freshly prepared in MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) mixture. The prepared peptide samples were immediately infused into the mass spectrometer and full scan MS were acquired in the mass range of  $m/z$  150–600. Then, each of these solutions were allowed to incubate at different temperatures; such as,  $-20^{\circ}\text{C}$ ,  $+4^{\circ}\text{C}$ ,  $+22^{\circ}\text{C}$ ,  $+37^{\circ}\text{C}$ , or  $+50^{\circ}\text{C}$ . For each incubated solutions, full scan MS were recorded over time periods of 1, 3, 7, 14 and 30 days. The obtained full scans MS for EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> peptides over different temperatures and time periods are given in Fig. S1–S4. For simplicity, only comparison of full scan MS recorded at five different temperatures after thirty-day incubation is shown in Fig. 1.

As illustrated in Fig. 1a, the protonated molecular ion,  $[\text{M}+\text{H}]^+$ , of EGGFL-NH<sub>2</sub> is  $m/z$  521. Interestingly, one can note that  $m/z$  535 ion was appeared in the full scan MS of solutions which were incubated at  $+4^{\circ}\text{C}$ ,  $+22^{\circ}\text{C}$ ,  $+37^{\circ}\text{C}$ , and  $+50^{\circ}\text{C}$  for thirty-days (labeled with red-dotted rectangular box). This peak is separated by a +14 Da from  $[\text{M}+\text{H}]^+$  ion and this mass shift is attributed to the methylation. In line with the previous studies [12,20], it was assumed that the side chain of E has been methylated during storage of its ESI solution in MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v). As a result, a methylated peptide product, E<sub>OMe</sub>GGFL-NH<sub>2</sub>, was formed in solution phase. To confirm this, the  $m/z$  535 ion was isolated and allowed to dissociate under low-energy CID which then compared with the MS/MS spectrum of  $[\text{M}+\text{H}]^+$  ion of commercial side-chain methylated pentapeptide, E<sub>OMe</sub>GGFL-NH<sub>2</sub>, (shown in Fig. 2). The fragmentation behavior of  $m/z$  535 ion and  $[\text{M}+\text{H}]^+$  ion of commercial E<sub>OMe</sub>GGFL-NH<sub>2</sub> pentapeptide are virtually identical by having the same fragment ions along with the same relative intensities. The one-to-one similarity of these two mass spectra provided a strong evidence for side chain methylation of E during storage of its ESI



**Fig. 2.** Comparison of MS/MS spectrum of  $m/z$  535 ion observed in the full scan MS of protonated EGGFL-NH<sub>2</sub> and MS/MS spectrum of  $[\text{M}+\text{H}]^+$  ion of protonated E<sub>OMe</sub>GGFL-NH<sub>2</sub>.

solution in MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v). A facile loss of 32 Da (mass of methanol) from [M+H]<sup>+</sup>, *b*, and/or *a* ions unambiguously supports the formation of methylated peptide product. In Fig. 2, a water loss from *b*<sub>5</sub> ion and an ammonia loss from *a*<sub>5</sub> ion are denoted as *b*<sub>5</sub><sup>°</sup> and *a*<sub>5</sub><sup>\*</sup>, respectively.

Similarly, the *m/z* 521 ion was detected while recording the full scan MS of DGGFL-NH<sub>2</sub> at +4 °C, +22 °C, +37 °C, and +50 °C after thirty-day incubation (see Fig. 1b, red-dotted rectangular box). The [M+H]<sup>+</sup> ion of peptide is *m/z* 507 and once again there is +14 Da mass shift which may correspond to the side chain methylation of D residue. Detailed MS/MS experiments showed that MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) solution composition giving rise to formation of D<sub>OMe</sub>GGFL-NH<sub>2</sub> peptide product in solution.

As shown in Fig. 1, neither EGGFL-NH<sub>2</sub> nor DGGFL-NH<sub>2</sub> undergoes methylation when their solutions were stored at –20 °C for thirty days. Additionally, no methylated peptide products were detected even after six-month incubation at –20 °C (spectra not shown). It is obvious that an ESI solution remain stable under this condition. On the contrary, it is apparent that the relative intensities of methylated peptide products have become more significant as the temperature increases. It is worth noting here that the rate of E methylation was rapid in comparison to D methylation for all tested temperatures, except +37 °C, at least when C-terminus amidated model pentapeptides were used. Previous report by Haebel et al. [14] demonstrated that the rate of methylation was selective for E instead of D residue. Our results differ from Haebel et al. [14] report in terms of D methylation.

Overall, these results suggested that methylation of E/D residue within the peptide sequence is temperature dependent process and the rate of reaction is increased as the temperature increases. Moreover, as Fig. S1–4 illustrates, the relative intensities of methylated peptide products greatly dominate the mass spectra with increasing incubation time for all tested temperatures.

On the other hand, the conversion of N-terminus E to pyroglutamic acid (pE) has been reported previously [23–25] through water loss from its  $\gamma$ -carboxyl group; especially when its acidic solution is incubated at elevated temperatures. Therefore, in the current work, in addition to the formation of methylated product, N-terminus pE containing form of peptide (pEGGFL-NH<sub>2</sub>) was also observed at *m/z* 503 while recording full scan MS of +37 °C incubated EGGFL-NH<sub>2</sub> solution. Hence, to gain more insight into the formation of E<sub>OMe</sub>GGFL-NH<sub>2</sub> and pEGGFL-NH<sub>2</sub> in solution phase, both freshly prepared and seven-day +37 °C incubated of EGGFL-NH<sub>2</sub> peptide solutions were injected separately into the high-performance liquid chromatography (HPLC). The comparison of HPLC chromatograms is shown in Fig. 3. Briefly, the retention time for freshly prepared peptide was 7.5 min (see upper panel). However, for seven-day incubated peptide sample, in addition to the unmodified form (with a retention time of 7.7 min), two distinct peaks were also observed at the retention times of 8.5 and 9.5 min (see lower panel). Each eluted peak was introduced directly into the MS and was identified as pEGGFL-NH<sub>2</sub> and E<sub>OMe</sub>GGFL-NH<sub>2</sub>, respectively.

Moreover, EGGFL-OH and DGGFL-OH pentapeptides, where the C-terminus of peptides has free acid functionality, were also analyzed. In this case, two possible methylation sites exist: the side chains of E/D residue and the C-terminus. The peptide solutions were freshly prepared in MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) and their full scan MS were recorded in the mass range of *m/z* 150–600 separately. Then, each peptide solution was incubated for 1, 3, 7, 14, and 30 days at +37 °C. For the best representation of the methylation reaction, +37 °C was selected as a standard temperature. After each incubation time period, the full scan MS were again recorded for each peptide solution. For simplicity, Fig. 4 displays the comparison of full scan MS of EGGFL-OH and DGGFL-OH peptide solutions incubated at +37 °C for thirty days (The obtained full scan MS of

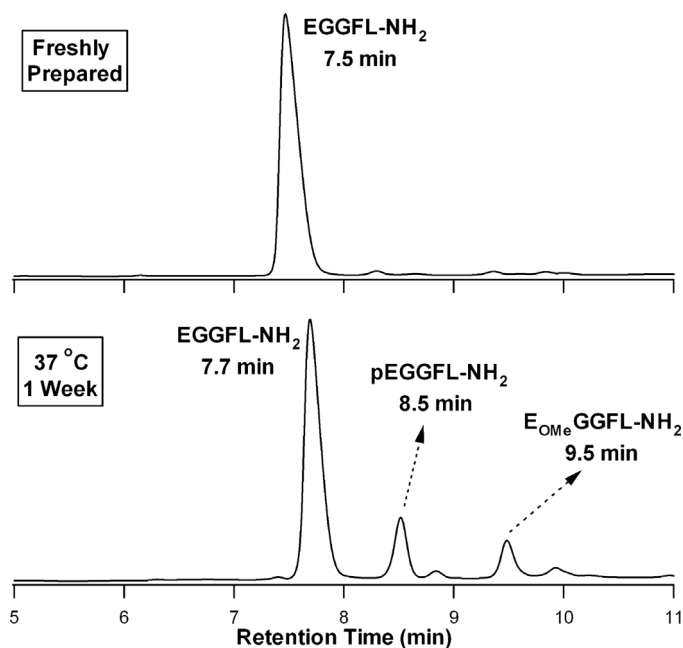


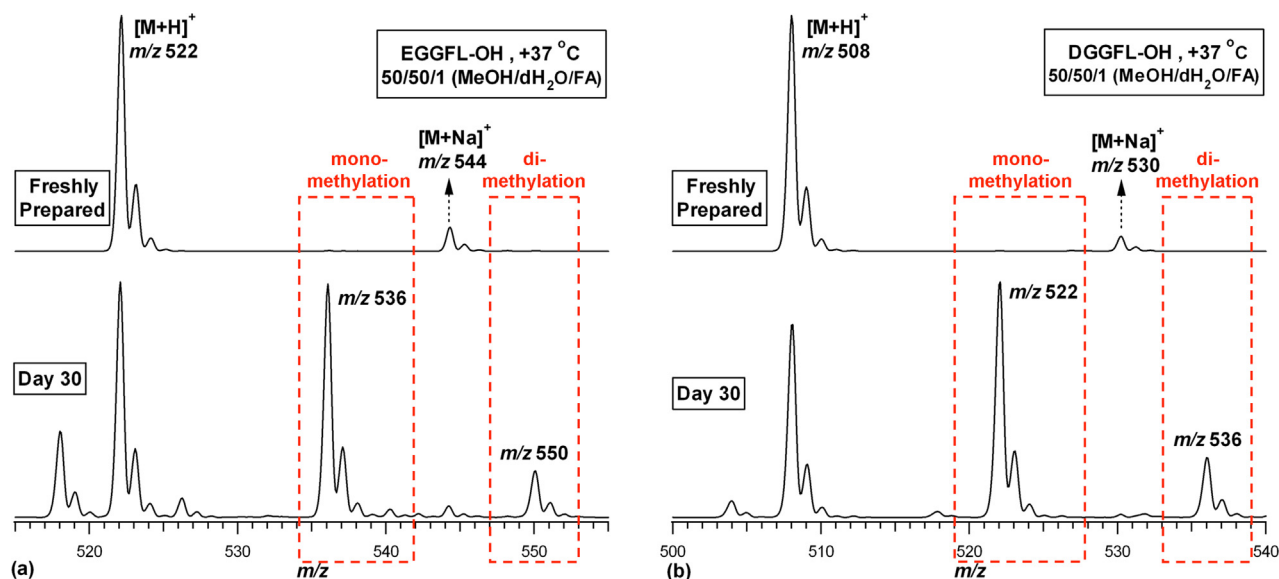
Fig. 3. Comparison of HPLC chromatograms of EGGFL-NH<sub>2</sub> peptide solution for a) freshly prepared and b) incubated for one week at +37 °C.

EGGFL-OH and DGGFL-OH over 1, 3, 7, 14, and 30 days are given in Fig. S5).

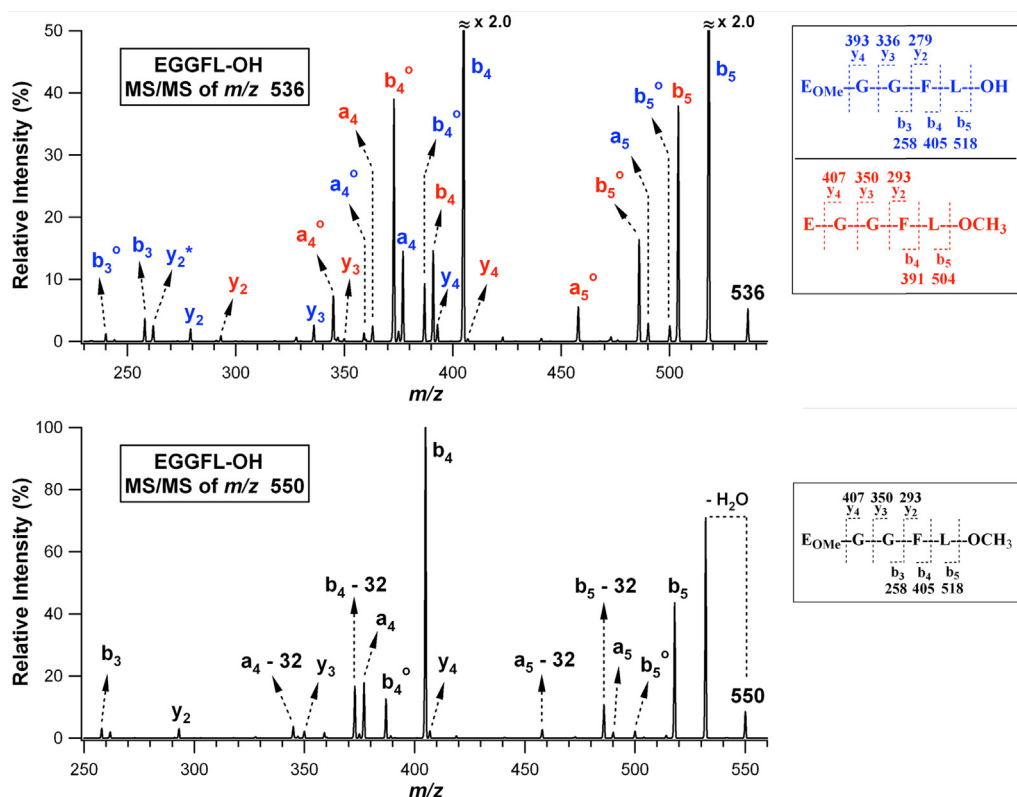
Briefly, the [M+H]<sup>+</sup> ion of EGGFL-OH is *m/z* 522 and it is confirmed by minor sodium-adduct, [M+Na]<sup>+</sup>, at *m/z* 544 (Fig. 4a). It can be noticed that the *m/z* 536 appeared in the full scan MS of EGGFL-OH when its aqueous solution was incubated at +37 °C for thirty-days, while it was originally absent for freshly prepared one. This +14 Da mass shift corresponds to the mono-methylation form of peptide, either on the side chain of E (E<sub>OMe</sub>GGFL-OH) or at the C-terminus of peptide (EGGFL-OMe). To support this observation, the ion at *m/z* 536 was further selected and allowed to fragment via CID. As shown in the upper panel of Fig. 5, the MS/MS spectrum of *m/z* 536 ion consists of several of *b*, *a*, and *y*-type ions which confirms that two mono-methylated peptide products have been formed at the same time during storage its solution in an acidified MeOH/dH<sub>2</sub>O (50:50, v/v) mixture. Moreover, the ion at *m/z* 550 was also observed in the full scan MS which corresponds to the formation of di-methylated peptide product, E<sub>OMe</sub>GGFL-OMe, in solution. The di-methylated form was further confirmed through assigning its sequence specific ions through its MS/MS spectrum (see lower panel of Fig. 5). In particular, the superscripts “\*” and “°” denotes neutral loss of one ammonia (–17 Da) and one water (–18 Da) molecule from fragment ions. It can be noticed here that the relative intensity of mono-methylated derivative’s product ion is higher than di-methylated one.

Likewise, the same behavior was also observed for DGGFL-OH peptide (see Fig. 4b). Briefly, both two mono-methylated peptide products (D<sub>OMe</sub>GGFL-OH and DGGFL-OMe) were observed at *m/z* 522. Additionally, di-methylated peptide product (D<sub>OMe</sub>GGFL-OMe) was also detected at *m/z* 536. In here, it can be noticed that methylation rate of E or D is not significant when C-terminus free acid forms of pentapeptides were used.

Furthermore, to test the methylation with different peptide sequence, AAAXAAA-NH<sub>2</sub> and AAAXAAA-OH (X is E or D) synthetic model heptapeptides have been utilized. A 100 pmol  $\mu\text{L}^{-1}$  peptide solutions were freshly prepared in MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) and their full scan MS were recorded. Again, the peptide solutions were incubated at +37 °C for thirty days and then their full scan MS were acquired. The comparison of the recorded spectra for freshly



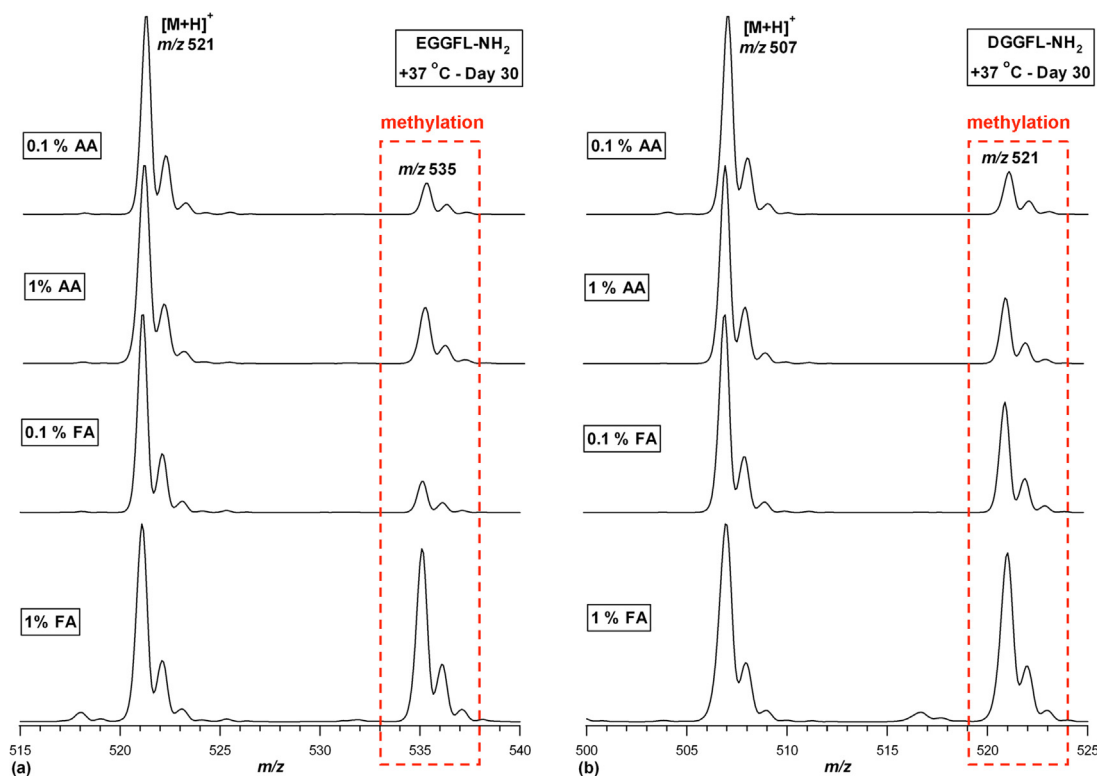
**Fig. 4.** Comparison of full scan MS for freshly prepared and incubated at +37 °C for thirty days of a) EGGFL-OH and b) DGGFL-OH peptide solutions. The ESI solutions were prepared in 50:50:1 (v/v/v) MeOH/dH<sub>2</sub>O/FA.



**Fig. 5.** MS/MS spectra of  $m/z$  536 (mono-methylated) and  $m/z$  550 (di-methylated) ions, derived from EGGFL-OH. The peptide solution was prepared in 50:50:1 (v/v/v) MeOH/dH<sub>2</sub>O/FA.

prepared and thirty-day incubated peptide samples were shown in Fig. S6 and S7. The  $m/z$  587 and 573 ions were appeared in the full scan MS of AAAEAAA-NH<sub>2</sub> and AAADAAA-NH<sub>2</sub>, respectively, which reflects the methylation of acidic residues. It is obvious that the extent of D methylation is low compared to the E methylation and this behavior can be explained by neighboring amino acid effect.

On the other hand, the  $m/z$  588 (mono-methylated) and  $m/z$  602 (di-methylated) ions were observed for AAAEAAA-OH; while  $m/z$  574 (mono-methylated) and  $m/z$  588 (di-methylated) ions were detected for AAADAAA-OH. It is clear to see that the relative intensities of mono-methylated peptide product ions are more abundant than the di-methylated peptide products both for E and D residue containing model heptapeptides.



**Fig. 6.** Comparison of full scan MS for thirty-day incubated at +37 °C of a) EGGFL-NH<sub>2</sub> and b) DGGFL-NH<sub>2</sub> peptide solutions. The ESI solutions were prepared in a mixture of MeOH/dH<sub>2</sub>O (50:50, v/v) containing 0.1% AA, 1% AA, 0.1% FA or 1% FA, respectively.

### 3.2. Effects of added acid type and its percentages on the methylation reaction

In positive-ion mode ESI-MS analysis, a trace amount of acid is usually added to the aqueous solution of analyte to facilitate protonation. Therefore, in the second part of the study, we studied the influence of added acid types as well as their percentages on the methylation of E/D containing peptide. To investigate these effects, formic acid (FA) and acetic acid (AA) with a range of 0.1–1.0% have been used. At +25 °C, the tabulated acidities (pKa) of FA and AA are 3.75 and 4.76, respectively, in water [26]. Similar to the previous section, we have used EGGFL-NH<sub>2</sub>, DGGFL-NH<sub>2</sub>, EGGFL-OH and DGGFL-OH synthetic model pentapeptides and +37 °C was selected as a standard temperature in order to best representation of the methylation reaction.

For the purpose of comparison, we prepared peptide solutions in a mixture of MeOH/dH<sub>2</sub>O (50:50, v/v) containing either 1.0% of FA or AA. The pH of each solution was measured and their pKa values were calculated as 4.71 and 5.76, respectively at normal laboratory conditions. The full scan MS were acquired for EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> over time periods of 1, 3, 7, 14, and 30 days. Fig. 6 shows the comparison of thirty-day incubated EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> peptide solutions which were prepared either in MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) or MeOH/dH<sub>2</sub>O/AA (50:50:1, v/v/v) (Other full scan mass spectra are illustrated in Figs. S8 and S9). It is clearly demonstrated that the rate of E/D methylation was low when 1.0% AA was used instead of 1.0% FA (see Fig. 6) due to the low acidity of former compared to latter one.

To probe the effect of added acid percentage on the rate of methylation reaction, we prepared ESI peptide solutions in MeOH/dH<sub>2</sub>O (50:50, v/v) mixture containing 0.1% FA or AA and their full scan MS were recorded immediately after preparation. After 1, 3, 7, 14, and 30 day incubation periods, full scan MS were again acquired (The obtained mass spectra are illustrated in Fig. S10

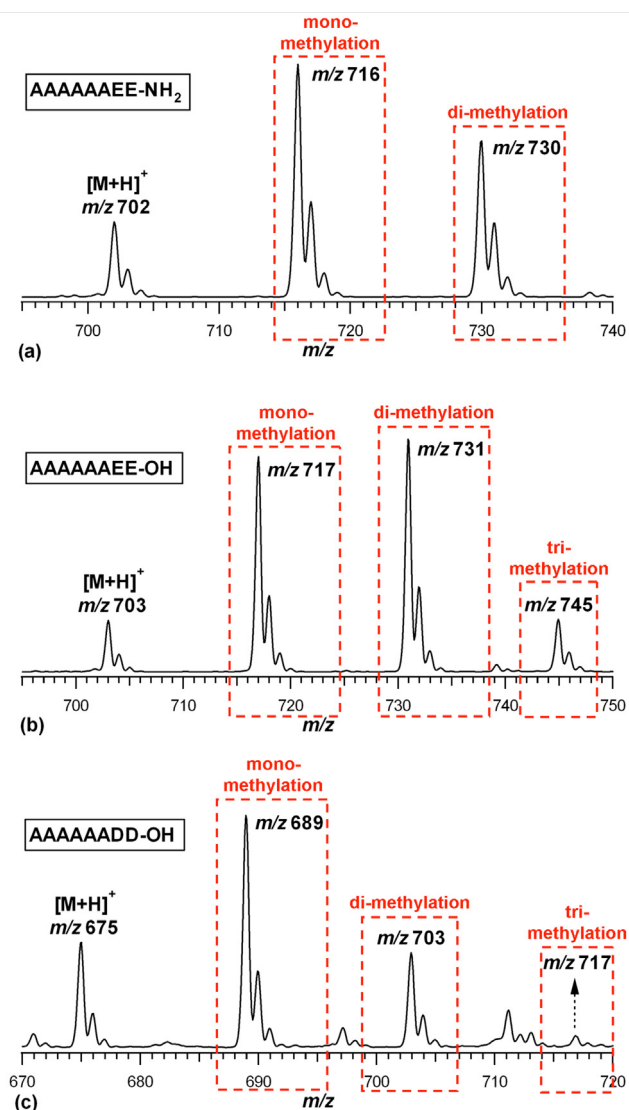
and S11). As Fig. 6a displays, the extent of methylation was nearly same when 0.1% FA or AA was used for EGGFL-NH<sub>2</sub>. On the contrary, the rate of D methylation was low when 0.1% AA was used instead of 0.1% FA (Fig. 6b).

Additionally, we have used EGGFL-OH and DGGFL-OH model pentapeptides where there were two potential methylation sites in each peptide. The obtained results demonstrated that the rate of mono- and di-methylation was significantly reduced when 0.1% acid was used instead of 1.0%. Additionally, the extent of methylation was low for AA containing mixtures compared to the FA (Fig. S12–15). It can be concluded that methylation of E/D residue was significantly decreased if the peptide ESI solutions were prepared in 0.1% AA containing MeOH/dH<sub>2</sub>O (50:50, v/v) mixture.

### 3.3. Methylation of multiple acidic residues

To investigate the extent of methylation of more than one acidic residue containing peptides, AAAAAAEE-NH<sub>2</sub>, AAAAAAEE-OH, and AAAAAADD-OH synthetic model hexapeptides have been used. The C-terminus amidated peptide has two potential methylation sites (7<sup>th</sup> and 8<sup>th</sup> acidic residues); whereas C-terminus free acid forms comprise of three potential methylation sites (two adjacent acidic residues positioned at 7<sup>th</sup> and 8<sup>th</sup> and C-terminus of peptide). The ESI peptide solutions were prepared freshly in a mixture of MeOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) and their full scan mass spectra were recorded in the range of 150–750 Da. The peptides were then allowed to incubate at +37 °C for thirty-days and their full scan MS again acquired.

For AAAAAAEE-NH<sub>2</sub>, in addition to the [M+H]<sup>+</sup> ion at m/z 702, two isomeric mono-methylated peptide products (AAAAAAE<sub>OMe</sub>E-NH<sub>2</sub> or AAAAAAEE<sub>OMe</sub>-NH<sub>2</sub>) were observed at m/z 716, after thirty-day incubation at +37 °C (Fig. 7a). This ion was further selected and allowed to dissociate under low-energy CID conditions. The obtained MS/MS spectra consist of several sequence



**Fig. 7.** The full scan MS of a) AAAAAEE-NH<sub>2</sub>, b) AAAAAEE-OH, and c) AAAAAADD-OH incubated for thirty-day at +37 °C. The ESI solutions were prepared in 50:50:1 (v/v/v) MeOH/dH<sub>2</sub>O/FA.

specific ions, i.e.  $b_7$  ions at  $m/z$  570 and 556 can be accepted as characteristic fragment ions for AAAAAE<sub>OMe</sub>E-NH<sub>2</sub> and AAAAAE<sub>OMe</sub>E-NH<sub>2</sub>, respectively. Similarly, di-methylated peptide product (AAAAAE<sub>OMe</sub>E<sub>OMe</sub>-NH<sub>2</sub>) ion was also detected at  $m/z$  730 and its sequence was confirmed by subsequent MS/MS experiment. It is apparent that the rate of di-methylation is low compared to the rate of mono-methylation.

Additionally, the [M+H]<sup>+</sup> ion of AAAAAEE-OH is  $m/z$  703 (see Fig. 7b). After thirty-day incubation at +37 °C, mono-, di-, and tri-methylated peptide products were detected at  $m/z$  717, 731, and 745, respectively. Briefly, three mono-methylated isomeric peptide, namely AAAAAE<sub>OMe</sub>E-OH, AAAAAE<sub>OMe</sub>-OH, and AAAAAE-OMe, were formed in solution. The formations of these three isomeric peptide derivatives have been verified via a careful interpretation of their corresponding low-energy CID-MS/MS spectra. On the other hand, three di-methylated peptide products (AAAAAE<sub>OMe</sub>E<sub>OMe</sub>-OH, AAAAAE<sub>OMe</sub>E-OMe, and AAAAAE<sub>OMe</sub>-OMe) were also formed in solution ( $m/z$  731) and their sequences were verified via MS/MS experiments. Lastly, tri-methylated peptide product (AAAAAE<sub>OMe</sub>E<sub>OMe</sub>-OMe) ion was detected at  $m/z$  745 and the sequence was confirmed by monitoring its characteristic  $b_8$

and  $y_2$  ions at  $m/z$  713 and 319, respectively in its MS/MS spectrum (spectrum not shown). It is obvious that the relative intensity of tri-methylated peptide product ion was low compared to the mono- and di-methylated ions.

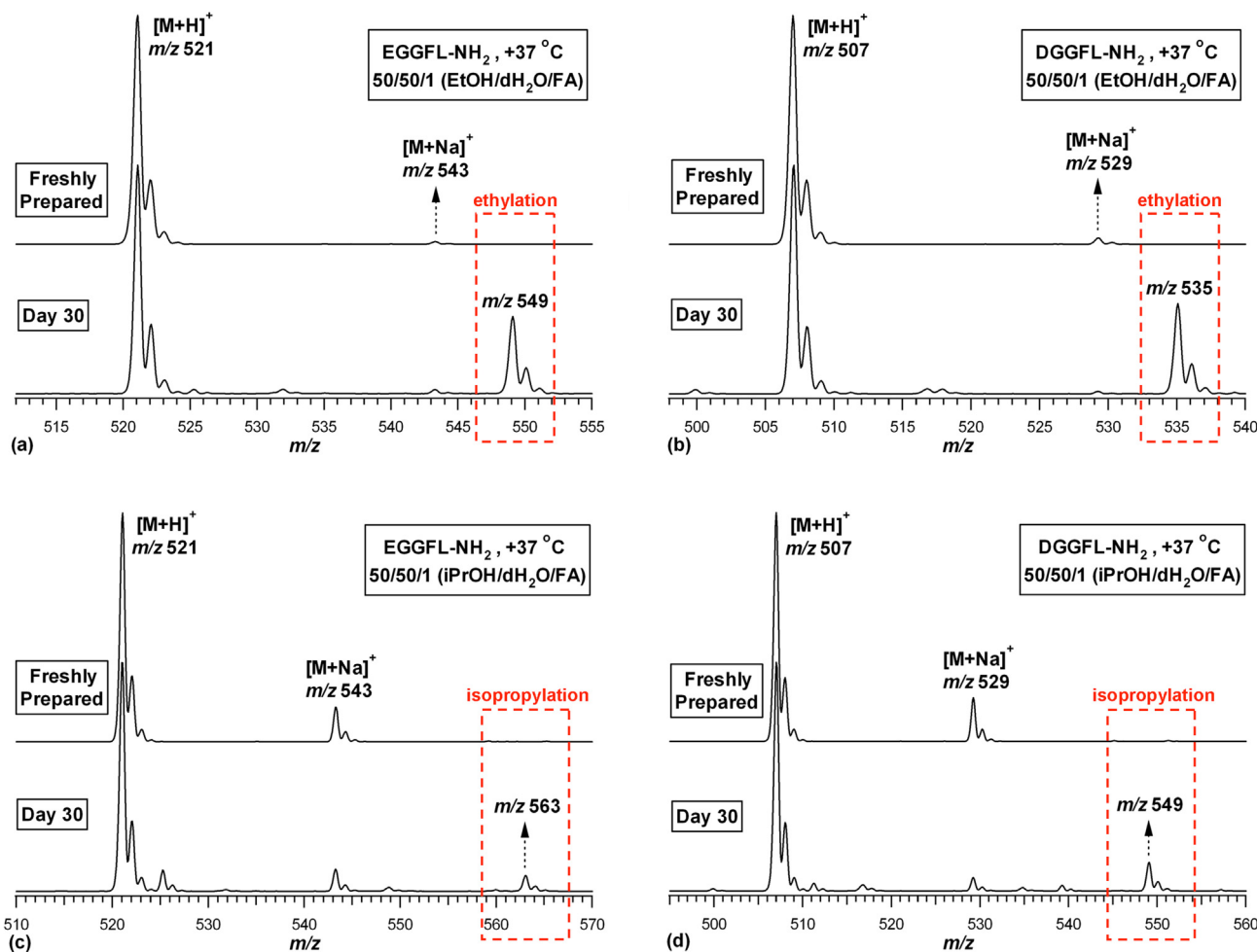
Likewise, AAAAAADD-OH model hexapeptide was also methylated when its ESI solution was incubated at +37 °C for one month. As shown in Fig. 7c, the [M+H]<sup>+</sup> ion is  $m/z$  675 and mono-, di-, and tri-methylated peptide products were detected at  $m/z$  689, 703, and 717, respectively. The formation of these peptide products was further verified through MS/MS experiments along with assigning their sequence characteristic ions. The resulting full scan MS for AAAAAADD-OH showed that the rate of mono-methylation is high compare to the di- and tri-methylation. Di-methylation and tri-methylation on E residue is more obvious compared to the D residue where the rate of D mono-methylation is higher compared to the E mono-methylation.

#### 3.4. Effects of different organic solvent on the esterification reaction

To investigate the effect of different organic solvents on the esterification reaction, the methanol was substituted with ethanol (EtOH) or isopropanol (iPrOH) in sample preparation steps. These solvents were used to compare the effects of primary (MeOH or EtOH) and secondary alcohol (iPrOH) on the esterification reaction of E/D residues as well as C-terminus of peptide. For that purposes, EGGFL-NH<sub>2</sub>, DGGFL-NH<sub>2</sub>, EGGFL-OH, and DGGFL-OH synthetic model pentapeptides have been used. For each peptide sample, a final concentration of 100 pmol  $\mu\text{L}^{-1}$  was freshly prepared in a mixture of EtOH/dH<sub>2</sub>O/FA or iPrOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) separately and their full scan MS were acquired immediately. For the best representation of the esterification reaction, the incubation temperature and added acid were selected as +37 °C and 1% FA, respectively. Then, each solution was allowed to incubate at +37 °C for 1, 3, 7, 14, and 30 days. After specified time periods, the full scan MS were again recorded.

For ethylation reaction, the comparison of full scan MS obtained from freshly prepared and thirty-day incubated EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> peptide solutions were shown in Fig. 8a and b (The obtained full scan MS of EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> for 1, 3, 7, 14, and 30 days are given in Fig. S16). For EGGFL-NH<sub>2</sub>, the [M+H]<sup>+</sup> ion has a mass of  $m/z$  521 with minor sodium adduct, [M+Na]<sup>+</sup>, at  $m/z$  543. It can be noticed that the  $m/z$  549 ion was observed for incubated peptide sample which has a mass difference of +28 Da with respect to the [M+H]<sup>+</sup> ion (see Fig. 8a). This mass shift can be attributed to the ethylation of E residue. To support this assumption,  $m/z$  549 ion was further selected and fragmented via low-energy CID. The resultant MS/MS spectrum showed that  $b_3$ ,  $b_4$ , and  $b_5$  ions were shifted by +28 Da compared to the same ions for unmodified peptide (spectrum not shown). Therefore, this solid evidence proved that the side chain of E residue was ethylated. Similarly,  $m/z$  535 was appeared in the full scan MS of DGGFL-NH<sub>2</sub> for thirty-day incubated solution where it was originally absent for freshly prepared one (see Fig. 8b). Detailed MS/MS experiment showed that D residue was also ethylated when the peptide solution was prepared in EtOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v). The rate of D ethylation is higher compared to the E ethylation. The obtained results on ethylation were in good agreement with the previous studies [14,16,27].

Additionally, EGGFL-OH and DGGFL-OH model pentapeptides were also analyzed for ethylation reaction. For these peptides, we expected to observe both mono- and di-ethylated peptide products. As Fig. S17 illustrates, mono-ethylated and di-ethylated peptide products were appeared with a mass increase of +28 Da and +56 Da, respectively, in the full scan MS of EGGFL-OH and DGGFL-OH. Briefly, mono-ethylated (E<sub>OMe</sub>GGFL-OH or EGGFL-OEt)



**Fig. 8.** Comparison of full scan MS for freshly prepared and incubated at +37 °C for thirty days of a) EGGFL-NH<sub>2</sub>, b) DGGFL-NH<sub>2</sub> peptide solutions prepared in 50:50:1 (v/v/v) EtOH/dH<sub>2</sub>O/FA, and c) EGGFL-NH<sub>2</sub>, d) DGGFL-NH<sub>2</sub> peptide solutions prepared in 50:50:1 (v/v/v) iPrOH/dH<sub>2</sub>O/FA.

and di-ethylated (E<sub>OEt</sub>GGFL-OEt) peptide products were observed at  $m/z$  550 and 578, respectively. Similarly, for DGGFL-OH, mono-methylated (D<sub>OEt</sub>GGFL-OH or DGGFL-OEt) and di-ethylated (D<sub>OEt</sub>GGFL-OEt) peptide products were detected at  $m/z$  536 and 564, respectively. The formation of each of these peptide products were identified via MS/MS experiments followed by monitoring their sequence characteristic ions. It is interesting to note that the degree of mono-ethylation on D residue is greater than mono-ethylation on E residue. However, the extent of di-ethylation was nearly same after thirty-day incubation. Moreover, as expected, the relative intensities of di-ethylated peptide products were low compared to the mono-ethylated peptide products for both peptides.

Furthermore, to explore the effect of secondary alcohol on the esterification reaction, four ESI peptide solutions were freshly prepared in a mixture of iPrOH/dH<sub>2</sub>O (50:50, v/v) containing 1% of FA to a final concentration of 100 pmol  $\mu\text{L}^{-1}$  and their full scan MS were immediately acquired in the mass range of  $m/z$  150–620. After 1, 3, 7, 14, and 30 days incubation period, the full scan MS were again recorded and compared, as Fig. 8c illustrates (The obtained full scan MS of EGGFL-NH<sub>2</sub> and DGGFL-NH<sub>2</sub> for 1, 3, 7, 14, and 30 days are given in Fig. S18). For EGGFL-NH<sub>2</sub>, the  $[M+H]^+$  ion is  $m/z$  521 along with its sodium adduct at  $m/z$  543. It can be noticed that  $m/z$  563 was appeared in the full scan MS which corresponds to a mass shift of +42 Da. Similarly, the  $[M+H]^+$  ion of DGGFL-NH<sub>2</sub> is  $m/z$  507 and  $m/z$  549 was detected with a mass increase of +42 Da (see Fig. 8d). In line with methylation and ethylation reactions, we

assumed that the side chains of E/D residues were isopropylated when their peptide solutions were prepared in iPrOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) after storage at +37 °C over a month. Further MS/MS experiments have confirmed that  $b_3$ ,  $b_4$ , and  $b_5$  ion series were shifted by +42 Da with respect to original peptide sequence. As Fig. S18 illustrates, the isopropylation was began after seven-day incubation both for E and D containing model pentapeptides. However, the rate of isopropylation was markedly low compared to the ethylation and methylation. This aspect can be explained by steric hindrance behavior of iPrOH compare to the MeOH and EtOH. Hence, the rate of esterification of the side chains of E/D residues as well as C-terminus of peptide became slower when iPrOH was used. Additionally, our results differ from Haebel et al. [14] report in terms of isopropylation. The authors previously used iPrOH as an organic solvent to test esterification of E/D residues. However, they did not observe any isopropylated peptide product.

Moreover, EGGFL-OH and DGGFL-OH model pentapeptides were also utilized for examining mono- and di-isopropylation. The peptide samples were prepared freshly in iPrOH/dH<sub>2</sub>O/FA (50:50:1, v/v/v) mixture and full scan MS were recorded immediately. Then, the samples were incubated at +37 °C over 1, 3, 7, 14, and 30 days and the full scan MS were again recorded after each time period. The comparison of full scan MS generated from incubated peptide samples over different time intervals were shown in Fig. S19. The  $m/z$  564 and 550 were detected for EGGFL-OH and DGGFL-OH solutions, respectively, after 7-day incubation. These ions corre-



spond to the mono-isopropylation; either on the side chains of E/D residues or on the C-terminus of peptide. Further MS/MS experiments have revealed that both two mono-isopropylated peptide products were formed during storage. However, it is interesting to note that di-isopropylated peptide products ( $m/z$  606 for EGGFL-OH and  $m/z$  593 for DGGFL-OH) were absent for thirty-day incubated peptide samples. Our further experiments showed that formation of di-isopropylated peptide products requires at least three-month incubation at +37 °C.

#### 4. Conclusion

This work presents a detailed investigation of *O*-methylation on the side chains of E/D residue and C-terminus of peptide as a function of different incubation temperatures (−20 °C, +4 °C, +22 °C, +37 °C, or +50 °C), storage times (1, 3, 7, 14, and 30 days), added acid type and its percentages (0.1–1% FA or AA) by using synthetic model peptide series. The methylation was confirmed by +14 Da mass shift in peptide *b* and/or *y* ion series as well as an exclusive loss of methanol (−32 Da) from *b* and/or *a* ions upon CID.

The obtained results have revealed that methylation of the side chains of E/D residues and C-terminus is temperature dependent process and the heating an acidified ESI solution greatly enhances the rate of reaction. On the other hand, no methylated peptide products were observed if peptide ESI solutions were stored −20 °C for a month. The results have also demonstrated that long incubation times remarkably favor the formation of methylated products.

On the other hand, the extent of methylation was found to decrease when 1.0% AA used instead of 1.0% FA for all tested peptides and this aspect can be explained low acid strength of former with respect to latter one. Additionally, the usage of 0.1% FA or AA significantly diminishes the formation of methylated products. These results collectively suggested that peptide solution storage conditions can effect methylation reaction. To avoid this unwanted modification, the peptide ESI solutions must be acidified with 0.1% AA just prior to use through MS analysis and should be stored at −20 °C for further use.

The extent of multiple methylations was also studied by using two E/D containing model peptides. The resulting mass spectra showed that mono- and di-methylated peptide products were observed for AAAAAEE-NH<sub>2</sub> peptide. In addition, we detected mono-, di-, and tri-methylated peptide products of AAAAAEE-OH and AAAAAADD-OH. However, the rate of tri-methylation is low compared to the mono- and di-methylation. Finally, MeOH was replaced with EtOH or *i*PrOH to examine the effect of organic solvent type on the esterification reactions. The obtained results showed that the ethylation (+28 Da mass shift) is more obvious than the isopropylation (+42 Da mass shift). This behavior can be explained by bulky nature *i*PrOH compare to the EtOH which directly influences the kinetics of esterification reaction.

In conclusion, preparing ESI solutions of E/D residue containing synthetic peptides in an acidified MeOH/dH<sub>2</sub>O mixture can induce generation of methylated peptide products. This artificial chemical modification during sample preparation causes misidentification of endogenous methylation of peptides/proteins; hence a special care must be taken while evaluating MS data through search algorithms. Taken together, on the other hand, the aforementioned procedure provides good yields of methylated peptide products for further MS/MS studies, especially while studying synthetic model peptides.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2016.12.043>.

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