Review Article

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Enrichment and analysis of glycated proteins

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Abstract: Glycation is a spontaneous post-translational modification of lysine, arginine, and the N-terminus of proteins. Protein glycation is closely related to the pathogenesis of human diseases, including diabetes, Alzheimer’s disease, renal disease, and cancer. The levels of advanced glycation end products (AGEs) are positively correlated with the progression of many diseases. However, it remains challenging to analyze glycation-related products, such as reactive carbonyl species, Schiff bases, Amadori compounds, and AGEs, because of their high heterogeneity. Many analysis methods, such as fluorescence detection, immunoassays, and liquid chromatography-tandem mass spectrometry, have attempted to correlate glycation products with diseases. Some enrichment methods have been used to increase the probability of detection of glycated proteins due to their low abundance in blood plasma. This review summarizes the enrichment and analysis methods that are currently used to identify glycation as a disease biomarker in exploratory studies.

Keywords: glycation, AGEs, diabetes, LC-MS/MS, enrichment

1 Introduction

Glycation is a spontaneous post-translational modification (PTM) of lysine, arginine, and the N-terminus of proteins. Glycation was first discovered by Maillard in 1912, and the reaction during cooking that gives food a brownish color and enhanced flavor is known as the Maillard reaction [1]. The active carbonyl group of the sugar and the nucleophilic free amino group of the protein react to form an unstable Schiff base. The Schiff base is rearranged to produce ketoamines or Amadori products. After dehydration and rearrangement, Amadori products undergo other reactions, such as cyclization, oxidation, and dehydroxylation, to form more stable advanced glycation end products (AGEs) [2]. AGEs generation usually occurs in long-lived proteins, such as collagen, myelin, and plasminogen activator [3]. Oxidative stress also plays a significant role in the generation and accumulation of AGEs. During glucose autoxidation, the production of many reactive oxygen species (ROS) is facilitated by the action of non-enzymatic covalent binding of glucose molecules to proteins that produce AGEs [4]. Methylglyoxal (MG), 3-deoxyglucosone, and glyoxal (GO) are reactive carbonyl species (RCS) that are mainly derived from oxidative degradation or autooxidation of Amadori products, which typically lead to molecular cross-linking. Glycation products are divided into two main categories: early stage glycation adducts and AGEs. Early stage glycation adducts are typically bound to lysine residues, forming a Schiff base and fructosamine which is an Amadori compound [5]. Nε-Carboxymethyllysine (CML) and Nε-carboxyethyl-lysine (CEL) are created by the oxidative degradation of fructosamine, GO, and other compounds. CML and CEL are major quantitative lysine-derived AGEs [6], which are non-fluorescent and non-crosslinked, and are produced from intermediate GO. CML and CEL are typically found in patients with diabetes or high oxidative stress. Once CML reacts with collagen, apoptosis is induced when the collagen is added to human dermal fibroblasts [7]. CML is linked to increased fasting and postprandial glucose exposure, insulin resistance, and cardiovascular disease [8]. MG is formed by non-enzymatic fragmentation of triosephosphate, which is a highly reactive α-oxoaldehyde [9]. Methylglyoxal-derived hydroimidazolone-1 (MG-H1) is a major quantitative arginine-derived AGE, which is formed from MG [10]. Glucosepane is a significant quantitative cross-link product that is formed during protein glycation, and consists of a mixture of four diastereoisomers with a seven-member
ring made of glucose that intersects two amino acid side chains of lysine and arginine [11]. As glucosepane is unstable during acid hydrolysis and absorbs only short wavelength UV light, liquid chromatography-mass spectrometry (LC-MS) is commonly used for its analysis [12]. Pentosidine is a low-level pentose sugar-derived glycation cross-link product, and an intense fluorophore. Pentosidine levels reflect pentose phosphate pathway activity [13]. The glyoxal-lysine dimer and methylglyoxal-lysine dimer form non-fluorescent protein cross-links. The process of glycation-related product generation is shown in Figure 1.

AGEs combine with receptors for advanced glycation end products (RAGEs) to cause cell dysfunction or glycated protein dysfunction. RAGE is a cell surface molecule that recognizes AGEs. The action of AGE and RAGE causes aging by altering the function of cells and organs, primarily via the inflammatory pathways. RAGE regulates several crucial cell processes, including inflammation, apoptosis, ROS signaling, proliferation, autophagy, and aging [14,15].

Glycation of collagen leads to the development of fibrosis in diabetes, atherosclerosis, and skin aging [16,17]. When glycation occurs in fibrinogen, it leads to impaired fibrinolysis, formation of a less thrombogenic fibrin network, and vascular dysfunction [18,19]. When AGES accumulate, proteins cross-link with AGES, increasing their stiffness and resistance to cross-linked protein removal, thus interrupting the function of organs and tissues [20]. Accumulation of AGES in the extracellular matrix cross-linking with the extracellular matrix reduces the elasticity of the extracellular matrix [21]. The level of AGES is correlated with the progression of several diseases. Hyperglycemia is the most prominent feature of diabetes, and promotes glycation by increasing the amount of glucose in the blood [22]. An increase in circulating AGES during hyperglycemia, which is commonly observed in diabetic patients, is thought to contribute to the development of complications [23]. Glycation occurs in Aβ and tau, which are proteins involved in Alzheimer’s disease (AD), resulting in the formation of neurofibrillary tangles and senile plaques in the brain tissue [24,25]. The accumulation of ROS increases the oxidation of proteins, and promotes the production of RCS, which are the precursors of glycation [26]. A recent study aimed to identify the skin collagen AGES and other variables that are most relevant to the index of past glycemia, as well as the risk of developing type 1 diabetes mellitus (T1DM) and complications, and identified hemoglobin A1c (HbA1c) [27]. To improve the currently available glycemic biomarkers, it is necessary to explore other glycated plasma proteins to

Figure 1: Process glycation related product generation. RCS’ are initially covalently bonded, forming the early stages of glycation products (Schiff bases and Amadori compounds), and finally AGES.
find biomarkers that are relevant to glycemic levels [28]. Other AGEs are being studied because the accumulation of AGEs such as pentosidine, CML, and CEL, in addition to HbA1C, is accelerating in patients with type 2 diabetes mellitus (T2DM) [29].

AGEs can be detected by immunoassay or by measuring their auto-fluorescence. Enzyme-linked immunosorbent assays (ELISAs) are a typical immunoassay technique, and along with fluorescence assays are easy to use and fast [30]. The main AGEs, including CML, CEL, and MG, can be measured using ELISA methods based on antibodies for CML or MG derivatives [31]. In particular, as CML is non-fluorescent, it is typically quantified by competition ELISA [32]. However, immunoassays only enable quantitative analysis of some AGEs. Several protein-binding forms of AGEs have been chemically defined for only a small subset of AGEs, and may only react with a few specific AGEs as antigens. Measuring AGE auto-fluorescence is often used as a non-invasive technique for measuring skin AGE levels, for example AGEs in the fingertip skin [33]. However, some AGEs do not fluoresce, limiting the use of auto-fluorescence as a tool for quantifying accumulating AGEs. The preferred method for measuring AGE levels has rapidly become liquid chromatography-tandem mass spectrometry (LC-MS/MS), as it can detect AGEs regardless of their ability to fluoresce or the availability of specific antibodies. However, glycation may form isomeric products with various modifications in one protein, or alternative modification on the same amino acid residue, resulting in highly heterogeneous AGE structures [34–36]. LC-MS/MS enables the analysis of the limited properties of protein glycation at the intact protein level. To enable the complementary evaluation of the modification pattern, proteins are subjected to enzymatic digestion, and LC-MS/MS analysis is performed at the peptide level [37]. In this review, we aim to highlight methods for analyzing glycation adducts and AGEs in exploratory studies.

2 Glycation enrichment assay

Two-dimensional gel electrophoresis (2D-GE) or autofluorescence can be used to analyze PTMs, including glycation. However, these methods have low sensitivity and are non-specific. Mass spectrometers are a highly sensitive and specific alternative detector. However, human serum is a complex matrix, and glycated proteins are relatively low abundant. Therefore, enrichment is required to detect AGEs and glycation adducts by mass spectrometry. Enrichment is also carried out to separate critical PTMs that affect diseases and biological function from non-critical PTMs [38]. The following subsection introduces various enrichment methods used for the analysis of AGEs and glycation adducts, which are summarized in Table 1.

2.1 2D-GE

2D-GE can detect low-abundance proteins by separating out the proteome over two dimensions. Typically, 2D gels are stained with either Coomassie blue or with colloidal silver. Although silver staining is more sensitive, Coomassie blue is more compatible with mass spectroscopy (MS) detectors, enabling the combination of 2D-GE and MS techniques [39]. Several studies have performed MALDI-TOF/MS following enrichment of glycated proteins in the plasma of diabetic patients [40–42]. Fluorescent boronic acid from Flu-PAGE and Flu-BLOT can be used to visualize and identify glycated proteins in complex biological samples. This method was used to identify the differential glycation between T1DM patients and normal individuals [43]. An important glycated site was identified in the breast cancer cell line MCF-7 using 2D-GE, in-gel digestion, and nano LC-MS/MS analysis [44]. Although 2D-GE can separate and analyze complex biological samples, it is of limited use for resolving low-abundance proteins, particularly in

Table 1: Strengths and limitations of different enrichment methods for glycation adducts

| Glycation adduct enrichment methods | Strength | Limitation |
|------------------------------------|----------|------------|
| Boronate affinity                  | Specifically and strongly binds to cis-diol group | Also captures other PTMs with a cis-diol group |
| 2D-Online LC                       | Improved peak capacity and optimized selectivity | Large volumes of data generated |
| Lectin affinity                    | Specifically binds to sugars | Weak binding and requires several kinds of lectin |
| Depletion                          | Enables analysis of low abundant proteins | Unwanted protein loss |
| 2D-Gel electrophoresis             | Straightforward and enables separation of complex samples | Unable to analyze total protein |
complex matrices where the dynamic range of protein abundances is very large [45].

2.2 Affinity chromatography

Boronate affinity chromatography (BAC) is a popular enrichment method for glycated proteins or peptides. BAC can enrich glycated peptides by attaching the OH of boronic acid to the 1,2- or 1,3-cis-diol group according to the pH [46]. This occurs through the formation of boronate esters with 1,2- or 1,3-diols that are present on the substrate [47]. Boronic acid can be packed on a column or tip, on the filter during filter aided sample preparation, or by binding boronic acid to nanoparticles [48–54]. Automation can be used during boronic acid enrichment to increase reproducibility and reduce the workflow time [49]. Two BAC approaches are the enrichment of glycated proteins, or the enrichment of the glycated peptides following enzymatic digestion of glycated proteins. The enrichment of glycated proteins tends to be inefficient, because non-specific binding results in the yield of a lot of unglycated proteins, mainly serum albumin [55]. BAC enrichment and isotopic labeling have been applied to increase the detection efficiency of glycated human serum albumin in T2DM [56]. BAC is particularly effective at the specific capture, recognition, and labeling of cis-diol-containing proteins because of its pH-controlled, covalent, and reversible binding properties [57]. However, because the BAC method captures all modifications with 1,2 or 1,3-cis diol groups, it cannot specifically capture glycated proteins, and tends to capture other PTMs. To solve this problem, other protein modifications that are attached to boronic acid can be removed, and glycated proteins can then be specifically enriched by boronic acid. Before the glycated form of a basic recombinant humanized monoclonal antibody (rhuMab) was enriched with BAC, size exclusion chromatography, and ion-exchange chromatography, it was treated with N-glycosidase F to remove N-glycans that attach to boronic acid [58]. Another affinity chromatography enrichment method, the lectin-based method, is commonly used to recognize the structure of a specific glycan depending on the type of lectin [59]. Con A binding α-d-mannosyl and α-d-glucosyl residues are mainly used for lectin enrichment [60]. However, lectin has a relatively low binding power, resulting in a low enrichment efficiency [61]. Furthermore, mixing several lectins can result in non-specific binding. ELISA is most often used as a quantitative assay, but can also be used as an enrichment

![Figure 2](image-url)

**Figure 2**: (a) At low albumin levels, the relative intensity of glycated insulin is higher, and (b) at high albumin levels, the relative intensity of glycated insulin decreased, as albumin competes for glycation because of its high abundance. I – insulin, Alb – albumin, G – glucose, GI – glycated insulin, G-Alb – glycated albumin. Reprinted with permission from Bhonsle et al. [65]. Copyright 2012, American Chemical Society.
method prior to LC-MS/MS. Soluble RAGE, a low-abundance protein, was enriched using immunoprecipitation based on ELISA and then analyzed quantitatively by LC-MS/MS [62]. Depletion and fractionation methods are also effective at reducing sample complexity [63]. In the case of plasma, low-abundance proteins can be masked by highly abundant proteins such as IgG and albumin, and as a result are not detected by MS. To improve LC-MS/MS coverage, highly abundant proteins should be removed by depletion [64]. As shown in Figure 2, albumin depletion facilitated the detection of low-abundance proteins, such as glycated insulin [65]. However, depletion kits or columns can also cause the loss of target proteins, and their use should be accompanied with carefully considered controls [66].

### 2.3 2D-Online LC

2D-Online LC is a state-of-the-art LC technology that is used to analyze complex samples using two channels. Various 2D-LC combinations have been reported for the global proteomic analysis, such as strong cation exchange chromatography (SCX)-reversed phase liquid chromatography (RPLC), strong anion exchange chromatography (SAX)-RPLC, hydrophilic interaction chromatography (HILIC)-RPLC, RPLC-RPLC [67], and normal phase LC-RPLC [68]. However, only some of them have been used for the enrichment and analysis of glycated proteins. There are two types of 2D-online LC methods: comprehensive mode, which elutes all the analytes from the first column onto the second column, and heart-cutting mode, which elutes some analytes at specific retention times from the first column onto the second column [69]. HbA1C was successfully separated using 2D-LC combining BAC and SCX, and quantitative analysis was performed by multiple reaction monitoring (MRM) [70]. Another study used 2D-LC with an SCX column and a MALDI-TOF/MS [71]. Zhang et al. used a boronate affinity enrichment column to enrich glycated peptides, with a second reversed-phase nano-LC C18 column in-line with the mass spectrometer. A schematic diagram of 2D-online LC is shown in Figure 3. In another study, a reversed-phase nano-LC C18 column was used to separate the glycated peptides of barley proteins and identify 376 glycated peptides found in human plasma [72]. Based on this method, T1DM plasma was analyzed and 305 glycated peptides and 290 differentially expressed peptides were identified [28]. A disadvantage of 2D-online LC is the long run time, which is exacerbated by long column equilibration times [73]. The comprehensive mode also generates large amounts of data, which can complicate data analysis.

### 3 Glycation assays

AGEs and glycation adducts relating to aging and disease can be quantified as biomarkers. However, it remains unclear which AGEs are associated with aging and disease. Until recently, it was known that the amount of AGEs in the body increases when a disease occurs, but not the identity of the disease-specific AGEs. Therefore, many researchers in the field of proteomics are trying to identify specific AGEs as biomarkers for aging and...
disease. In this section, the AGEs and glycation adducts analysis methods that are currently used in exploratory studies are introduced. Table 2 summarizes the current AGEs assays.

### 3.1 Fluorescence methods

The skin is an excellent sample for the direct detection of glycation and AGEs using minimally or non-invasive technique [74]. The accumulation of AGEs in the skin was first studied using auto-fluorescence or intrinsic fluorescence [75]. A correlation was identified between skin collagen fluorescence as a surrogate marker of the advanced Maillard reaction in vivo and the severity of diabetic complications in T1DM [76]. Skin auto-fluorescence increases in subclinical and clinical arteriosclerosis, independent of known risk factors such as diabetes and kidney disease [77]. Patients with diabetes and diabetes complications have higher intrinsic skin fluorescence levels than normal people [78]. The levels of AGEs in the fingertip skin, measured by auto-fluorescence, are higher in patients with exfoliation syndrome and glaucoma than in non-glaucomatous control [33]. Pentosidine can be separated by chromatography and monitored for fluorescence emissions as a quantitative assay [32,79]. The amount of AGEs obtained from skin fluorescence in patients with schizophrenia is higher than that in normal patients [80]. Skin auto-fluorescence is used to measure the amount of AGEs over the long term, and accurately represents cumulative metabolic stress [81]. However, pigmentation occurs in the non-palmoplantar parts of people with dark skin, which leads to inaccuracies in skin auto-fluorescence readings [82]. Skin auto-fluorescence is associated with fluorescence in the dermis of dark-skinned individuals, but experiments have confirmed that it is not associated with CML, pentosidine, and MG-H1 due to high intra-individual dispersion [83]. There are also many non-fluorescent AGEs. As all AGEs cannot be analyzed by auto-fluorescence, it is impossible to perform an accurate quantitative analysis of all AGEs using fluorescence. Even if AGEs are fluorescent, this technique only enables quantitative analysis of AGEs, but not qualitative analysis.

### 3.2 Immunoassays

HbA1c can be analyzed by turbidimetric inhibition immunoassay. An anti-HbA1c antibody reacts with a single
binding site on HbA1c and forms a soluble complex. Polyhaptens react with excess anti-HbA1c antibodies to form insoluble complexes. The Ab–polyhapten complex can then be measured turbidometrically [84]. Glycated proteins are selectively enriched using immunochemical methods, such as enzyme-linked boronate-immunoassay (ELBIA) [85]. To overcome the shortcomings of the traditional immunoassay method, the boronate affinity sandwich assay can be used to enrich proteins with cis-diol in complex samples [86]. Several AGE immunoassay kits using this technology have been introduced. ELISA has high sensitivity because of the high catalytic efficiency of enzymes and high binding specificity of antibodies. Through ELISA, universal AGEs and specific AGE classes, such as CML, CEL, and MG, have been successfully identified. MG-H1 is measured by immunoassay because it has cross-reactivity and sensitivity to anti-hydromidazolone antibodies [87]. Using ELISA, the levels of AGES and CELs in the lens of the eye were found to be higher in diabetic patients than in control patients [88]. Glycated CD59 protein in the serum or plasma was detected using the sandwich ELISA format, with high reproducibility and sensitivity [89]. The inverse correlation between CML and glomerular filtration rate, and the correlation between CML and chronic kidney disease phases, were observed using competitive ELISA [90]. The AGES in the hippocampal tissue of patients with AD were analyzed using a glycation-specific antibody to immunostain the neurofibrillary tangles and senile plaques containing pyrraline and pentosidine [25]. However, this method lacks specificity, and cannot transmit information about AGE classes that may have clinical diagnostic potential [10]. Immunoassays require high sample concentrations, and complex enzyme reactions, including specific hydrolysis and oxidation of glycated peptides [91]. Immunoassays cannot simultaneously quantify several AGES, and require prior knowledge of biomarkers and their corresponding antibodies [92].

3.3 MALDI-TOF/MS

MALDI-TOF/MS is a promising tool for the analysis of AGES, and is capable of analyzing peptides and proteins up to 300 kDa in size with minimal sample preparation. Because of poorly resolved peaks, intact AGE analysis is not possible by MALDI-TOF/MS. Therefore, MALDI-TOF/MS is usually used for site-specific AGE analysis [93]. The process of analyzing AGE protein with MALDI-TOF/MS is summarized in Figure 4. Several novel AGES have been identified using MALDI-TOF/MS [94,95]. MALDI-TOF/MS is particularly well-suited for AGE analysis because it can be used to analyze intact proteins regardless of the AGE structure. Unlike other mass detection methods, it can detect proteins up to 300 kDa in size, and produces spectra with a reduced number of peaks. However, the resolution of MALDI-TOF/MS depends on the range of molecular masses analyzed, and the isotopic resolution ranges from 500 to 5,000 Da [93]. MALDI-TOF/MS analysis revealed that the glycated protein produced by MG or glucose has a wide peak, and heterogeneous product peak. As a result, the mass difference caused by modifications by early and advanced glycation products, such as Amadori products, early stage Maillard products, and post-Amadori modifications, is used to estimate the average level of protein in the presence of heterogeneous peaks. Glycated insulin, a glycated hormone, was enriched by magnetic beads with immobilized 3-aminophenylboronic acid and analyzed by MALDI-TOF/MS [96]. Using UPLC-TOF/MS, glycation kinetics were studied by quantifying glycated peptides [97]. However, the resolution of MALDI-TOF/MS is not sufficient at mass ranges that are suitable for identifying intact AGE structures. Therefore, peptide mapping was introduced in MALDI-TOF/MS analysis of AGE proteins. Peptide mapping requires the digestion of proteins with enzymes, such as trypsin, to lower the mass range of the analytes so that they enter the isotopic resolution of
MALDI-TOF/MS, and enables site-specific analysis of AGES. A limitation of MALDI-TOF/MS is that it cannot measure the exact quantities of glycation adducts, because fragments of different molecular weights can be decomposed and ionized differently [98].

3.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy uses the magnetic properties of an atom to measure the NMR signal generated by the excitation of nuclei in a magnetic field. The structure can be analyzed using isotope labeling (e.g., $^1$H and $^{13}$C) and measurement of the distance between atoms in the molecule [99]. NMR spectroscopy provides detailed chemical information for biological fluid and tissue that is almost native. The detection of specific AGES depends on the stability of the modification. Although NMR spectroscopy is much less sensitive than other detectors, it has the advantage of being applicable to native AGES following simple treatment, and without consequent possible decomposition [100]. NMR spectroscopy can identify all the AGES produced in a single sample [101]. Using $^{13}$C-glucose, six $^{13}$C labeled signals were obtained for lysozyme using $^{13}$C NMR spectroscopy for in vitro glycation. This allowed the estimation of changes in the structural environment of lysozymes' AGE adducts [102]. A previous study identified the AGES and their molecular dynamics using solid-state NMR at the atomic level [100]. However, solution-state NMR analysis can only analyze small molecules and soluble proteins, and a large number of samples is required to obtain acceptable NMR signals. In particular, $^{1}$H NMR requires a large sample volume because the natural abundance of $^{13}$C is very low [99]. Macromolecules are difficult to analyze by NMR because of their complexity and difficulty in interpretation. To overcome the low sensitivity of NMR spectroscopy, optical pumping and dynamic nuclear polarization are used to improve its sensitivity [103].

3.5 Gas chromatography-mass spectrometry (GC-MS)

GC-MS is capable of determining the composition of a compound within a reasonable time, and usually results in sharp and reproducible peaks, allowing accurate quantitative analysis. GC-MS uses two main methods: full-scan mode and selected ion monitoring (SIM) mode. The full scan mode is typically used for qualitative analysis, while the SIM mode is used for quantitative analysis. Carbohydrate intermediate-related glycation was identified and quantified using GC-MS. RCS, such as MG and GO, were quantified in diabetic patients using SIM-GC-MS [104]. The amount of CML and fructoselysin in human lens protein and human skin collagen were measured using SIM-GC-MS [105,106]. The n-propyl pentfluoropropionyl derivative of CML, which is produced by mouse neutrophils, was analyzed in negative ion mode by SIM-GC-MS using isotope dilution [107]. The CML of carrots was quantified using SIM-GC-MS with Nε-carboxymethylornithine as an internal standard [108]. However, GC-MS can only analyze thermally stable and volatile compounds. While amino acids and sugars can be derivatized, substances such as glycosides are unstable at the temperature used for elution.

3.6 LC-MS/MS

LC-MS/MS is a useful approach for comparing the source and physiological state of AGES. Analysis of glycation-related products by LC-MS/MS enables measurement of the changes in the function of proteins related to glycation, because the formation and decomposition of AGES depend on the protein sequence and structure [109]. As shown in Tables 2 and 3, LC-MS/MS is the most commonly used methodology for quantitative and qualitative analysis of AGES. Enrichment is also primarily achieved by packing resins into columns, which are compatible with LC-MS/MS. LC-MS/MS provides high sensitivity, and several different analytical methods are available [110]. To achieve high-throughput analysis, it is essential to conduct simultaneous qualitative and quantitative analyses. The quantitative method is crucial, and must have a high sensitivity and specificity for protein glycation adduct analytes. LC-MS/MS enables unbiased analysis of PTMs, like glycation [111], and can be used to analyze glycated peptides by adjusting the normalized collision energy applied during higher-energy collisional dissociation fragmentation. LC-MS/MS also enables the determination of the residue where glycation occurs, and various glycated derivatives can be used to study the characteristics of the glycated peptide [112]. Although not all processes during sample preparation have been automated, LC-MS/MS has the advantage of being easier to automate than other assays [113]. The number of glycated proteins in human plasma is approximately 1,100 [48]. Despite its strengths, LC-MS/MS does not provide biological information on modified proteins and their exact areas of impact [37]. Because of the dynamic range of human plasma, the analysis of glycated proteins by LC-MS/MS is still dependent on a suitable enrichment technique. If the glycated protein can be selectively...
Table 3: Enrichment strategy used in the quantitative analysis of glycated proteins

| Sample                  | Condition | Target                          | Methods of detection                                                                 | Method of enrichment                                      | Ref. |
|-------------------------|-----------|---------------------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------|------|
| Human HbA1C             | T2DM      | HbA1C                           | Ion-exchange HPLC                                                                    | Lectin (concanavalin A)                                    | [124]|
| Rats HbA1C              | Streptozotocin | Fructosyllysine 12 AGES | 2690 separation module and Quattro Ultima QQQ MS                                     | Boronate affinity chromatography                           | [110]|
| Human HbA1C             | DM        | HbA1C                           | Shimadzu LC-10 AD, ICP-ORS-MS and ESI-MS                                             | Boronate affinity chromatography and strong cation exchange | [70] |
| Human plasma            | T2DM      | HbA1C                           | ESI-MS on a QTRAP 4000 coupled on-line RP-HPLC using timed MRM                       | Boronate affinity chromatography                           | [125]|
| Human plasma            | T2DM      | Lysine-141 of haptoglobin       | UltiMate 3000 RSLC nano system and orbitrap ELMA mass spectrometer                 | Boronate affinity chromatography                           | [56] |
| RNase and human serum albumin | In vitro glycation | Amadori products | Micro LC device, Autosflex II MALDI                                                  | Boronate affinity tip                                      | [50] |
| rhuMAb                  | Sugar cell culture | rhuMAb | Agilent 1100 HPLC system and Finnigan classic LCQ ion trap mass spectrometer          | Boronate affinity chromatography, size exclusion chromatography, and ion exchange chromatography | [58] |
| Bovine insulin          | In vitro glycation | Glycated insulin | UltiFlex MALDI-TOF/MS                                                                | Magnetic beads containing immobilized 3-aminophenylboronic acid | [96] |
| Human serum albumin     | In vitro glycation | Glycated albumin | Kyowa Medex AP-960 GA version automated ELBIA system                                 | Enzyme linked boronate-immunoassay                         | [85] |
| Mice plasma             | Streptozotocin | Glycated albumin | nanoACQUITY UPLC, SYNAPL HDMS system                                                  | Depletion and 2D-Gel electrophoresis                       | [65] |
| Mouse collagen          | Diabetes   | Glycated collagen | Fluorescence, LTQ XL linear quadrupole ion-trap MS, and acela 1250 LC                 | Fractions by C8 reverse-phase HPLC and 2D-gel electrophoresis| [126]|
| Barley                  | Brewing    | Glycated barley protein         | 2D-HPLC Ultimate system 3000 and Applied Biosystems 4700 Proteomics Analyzer         | 2D-HPLC                                                    | [71] |
| Human serum albumin     | —          | Glycated albumin | Electrochemical analyzer and UV-vis spectroscopy                                     | Antibody urchin-like Pt nanoyzme/boronic acid agarose bead complex | [51] |
| Human serum albumin     | —          | Glycated albumin | Electrochemical analyzer and UV-vis spectroscopy                                     | Nanoparticle with boronic acid                             | [53] |
enriched and analyzed, low-abundance glycated proteins can also be analyzed by LC-MS/MS, revealing the relationship between glycation and diseases [114]. For example, 7,749 unique glycated peptides corresponding to 3,742 unique glycated proteins were identified using depletion, enrichment, and fractionation in succession [48]. MRM mode is typically used for the quantitative analysis of disease biomarkers in biofluids, including blood plasma. In many cases, the target substance can be precisely and effectively detected using a stable isotope-labeled standard [115]. MRM mode enables high-sensitivity and selective analysis on a quadrupole instrument, based on the parent and fragment ions of an analyte [116]. Standard curves made with stable isotope-labeled peptide standards can be used to achieve more accurate quantification of the analyte [117]. RCS, which has high reactivity with proteins, can be stabilized with EDTA and accurately quantified using MRM mode with a relatively short analysis time [118]. RCS, including GO and MG, from plasma in T1DM and normal individuals were analyzed using MRM mode, and a comparative analysis revealed higher levels of RCS in T1DM. Glucosepane, a fructosamine-derived AGE, is correlated with the progression of T2DM, and MG-H1 was correlated with insulin resistance according to MRM analysis [6].

4 Authors’ perspectives and concluding remarks

AGEs are associated with aging and disease. There are many methods for the detection of glycated proteins in exploratory studies. The use of skin auto-fluorescence detection, which is currently widely used for detecting skin glycation, can only represent the total amount of AGEs in the skin. The best way to analyze glycation-related products in human plasma is currently LC-MS/MS because it can simultaneously perform quantitative and qualitative analysis, and has high sensitivity. Furthermore, glycated site profiling on peptide can be performed by LC-MS/MS. One study compared the analysis of CML and CEL by LC-MS/MS, GC/MS, and ELISA, and found that LC-MS/MS was more reproducible than GC/MS. Moreover, LC-MS/MS has better reproducibility and specificity than ELISA [119]. However, the complexity of human plasma makes it challenging to analyze low-abundance glycation-related products. Furthermore, the current enrichment methods cannot specifically enrich glycation adducts, as other PTMs are enriched simultaneously. 2D-Online LC and BAC are often used to enrich glycation products. 2D-Online LC facilitates simultaneous enrichment and analysis of glycated peptides. BAC has high selectivity as a method for capturing cis-diol, and can be packed into a column for LC-MS/MS, which means that BAC is frequently used for quantitative glycation analysis in tips, nanoparticles, beads, immunoassays, and columns. Affinity enrichment methods, such as depletion, must be optimized to reduce the loss of glycated proteins and the enrichment of non-glycated proteins. Due to the growing importance of LC-MS/MS in glycation-related product analysis, LC-MS/MS compatibility should be considered during the development of new enrichment methods. Finding unique disease-specific AGE biomarkers will enable accurate diagnosis and prognostic prediction for a variety of important human diseases. Since the increased AGEs in the body is common in several diseases, the overall change in the amount of AGEs is inappropriate to be used as a biomarker for a specific disease. It is therefore urgently necessary to continue identifying disease-specific AGE biomarkers and glycation sites using LC-MS/MS in exploratory studies.

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