Review Article

Seonghyeon Cho[#], Van-An Duong[#], Jeong-Hun Mok, Minjoong Joo, Jong-Moon Park*, and Hookeun Lee*

Enrichment and analysis of glycated proteins

<https://doi.org/10.1515/revac-2022-0036>

received September 03, 2021; accepted December 23, 2021

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 dehydration, 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 autooxidation, 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]. *N*-ε-Carboxymethyl-lysine (CML) and *N*-ε-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

[#] These authors contributed equally to this work.

* **Corresponding author: Jong-Moon Park**, College of Pharmacy, Gachon University, Incheon, South Korea, e-mail: bio4647@naver.com

* **Corresponding author: Hookeun Lee**, College of Pharmacy, Gachon University, Incheon, South Korea, e-mail: hklee@gachon.ac.kr

Seonghyeon Cho, Van-An Duong, Jeong-Hun Mok, Minjoong Joo: College of Pharmacy, Gachon University, Incheon, South Korea

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 A β 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 auto-fluorescence 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 glycosylated 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 glycosylated 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 glycosylated proteins in complex biological samples. This method was used to identify the differential glycation between T1DM patients and normal individuals [43]. An important glycosylated 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 Lectin affinity	Improved peak capacity and optimized selectivity Specifically binds to sugars	Large volumes of data generated Weak binding and requires several kinds of lectin
Depletion 2D-Gel electrophoresis	Enables analysis of low abundant proteins Straightforward and enables separation of complex samples	Unwanted protein loss 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 glycosylated proteins or peptides. BAC can enrich glycosylated 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 glycosylated proteins, or the enrichment of the glycosylated peptides following enzymatic digestion of glycosylated proteins. The enrichment of glycosylated proteins tends to be inefficient, because non-specific binding results in the yield of a lot of unglycosylated proteins, mainly serum albumin [55]. BAC enrichment and isotopic labeling have been applied to

increase the detection efficiency of glycosylated 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 glycosylated proteins, and tends to capture other PTMs. To solve this problem, other protein modifications that are attached to boronic acid can be removed, and glycosylated proteins can then be specifically enriched by boronic acid. Before the glycosylated 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: (a) At low albumin levels, the relative intensity of glycosylated insulin is higher, and (b) at high albumin levels, the relative intensity of glycosylated insulin decreased, as albumin competes for glycation because of its high abundance. I – insulin, Alb – albumin, G – glucose, GI – glycosylated insulin, G-Alb – glycosylated 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 glycosylated 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 glycosylated 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 glycosylated 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 glycosylated peptides of barley proteins and identify 376 glycosylated peptides found in human plasma [72]. Based on this method, T1DM plasma was analyzed and 305 glycosylated 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

Figure 3: 2D-online LC-MS/MS using a boronic acid column for the analysis of glycosylated peptides. Glycosylated peptides are enriched on the first column using BAC. The peak of the glycosylated peptides is detected by UV detector, and eluted glycosylated peptides are resolved by reversed-phase chromatography on a C18 column using a heart-cutting LC mode. Reprinted with permission from Zhang et al. [72]. Copyright 2017, American Chemical Society.

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-pal-mopltar 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

Table 2: Enrichment strategies for glycation proteins and the number of identified glycation proteins and peptides

Sample	Condition	Methods of detection	Method of enrichment	Glycated peptides	Glycated proteins	Ref.
Human blood plasma	<i>In vitro</i> glycation	Automated 4-column capillary LC system and LTQ-XL ion trap MS	Immunodepletion, boronate affinity chromatography, and SCX fractionation	7,749	3,742	[48]
Human Serum	T2DM	Easy nano-ESI-LC 1000 system and Q-exactive orbitrap hybrid MS	Boronate beads	678	411	[49]
Human plasma	<i>In vitro</i> glycation	UltiMate 3000 RSLC nano system, Q-exactive HF orbitrap MS with an EASY spray interface	2D-Online LC	964	161	[72]
Human plasma	T1DM	UltiMate 3000 RSLC nano system, Q-exactive HF orbitrap MS with an EASY spray interface	2D-Online LC	305	46	[28]
RNase	<i>In vitro</i> glycation	Agilent 6340 ion trap LC/MS system equipped with ETD and Agilent HPLC-chip cube MS interface	Boronate affinity chromatography	88	27	[120]
Blood cerebrospinal fluid	<i>In vitro</i> glycation	RP-HPLC and LTQ-orbitrap (HCD-MS2/CID-MS3 neutral loss scanning)	Boronate affinity chromatography	—	50	[121]
Human serum albumin	<i>In vitro</i> glycation	nanoACQUITY UPLC system and hybrid linear ion trap-orbitrap MS	Boronate affinity chromatography	—	35	[122]
Bovine serum albumin	<i>In vitro</i> glycation	UltiMate 3000 RSLC nano system and LTQ orbitrap MS	2D-Online LC and boronate affinity chromatography	—	—	[123]

binding site on HbA1c and forms a soluble complex. Polyhaptenes react with excess anti-HbA1c antibodies to form insoluble complexes. The Ab–polyhapten complex can then be measured turbidimetrically [84]. Glycosylated 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-hydroimidazolone 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]. Glycosylated 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 glycosylated 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

Figure 4: The process by which AGE proteins are detected using MALDI-TOF/MS. A suitable matrix is required to analyze the glycosylated proteins by MALDI-TOF/MS. A laser pulse hits the glycosylated proteins and matrix molecules, which absorbs the laser and transfers energy to the proteins/peptides, which then fly under vacuum to the detector. Reprinted with permission from Kislinger et al. [93]. Copyright 2005, Royal Society of Chemistry.

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 glycosylated 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. Glycosylated insulin, a glycosylated 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 glycosylated 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., ^1H 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 lysozyme's 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, ^{13}C 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 fructoselysine in human lens protein and human skin collagen were measured using SIM-GC-MS [105,106]. The *n*-propyl pentafluoropropionyl 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 *N*-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 glycosylated 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	Lysine-141 of haptoglobin	ESI-MS on a QTRAP 4000 coupled on-line RP-HPLC using timed MRM	Boronate affinity chromatography	[125]
Human plasma	T2DM	Amadori products	UltiMate 3000 RSLC nano system and orbitrap Elite mass spectrometer	Boronate affinity chromatography	[56]
RNase and human serum albumin	<i>In vitro</i> glycation	Amadori products	Micro LC device, Autoflex 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	<i>In vitro</i> glycation	Glycated insulin	Ultraflex MALDI-TOF/MS	Magnetic beads containing immobilized 3-aminophenylboronic acid	[96]
Human serum albumin	<i>In vitro</i> glycation	Glycated albumin	Kyowa Medex AP-960 GA version automated ELBIA system	Enzyme linked boronate-immunoassay	[85]
Mice plasma	Streptozotocin	Glycated albumin	nanoACQUITY UPLC, SYNAPT HDMS system	Depletion and 2D-Gel electrophoresis	[65]
Mouse collagen	Diabetes	Glycated collagen	Fluorescence, LTQ XL linear quadrupole ion-trap MS, and accela 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 nanozyme/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 glycosylated proteins can also be analyzed by LC-MS/MS, revealing the relationship between glycation and diseases [114]. For example, 7,749 unique glycosylated peptides corresponding to 3,742 unique glycosylated 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 glycosylated 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, glycosylated 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 glycosylated 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 glycosylated proteins and the enrichment of non-glycosylated 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.

Funding information: This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. NRF-2017M3D9A1073784) and the Ministry of Science and ICT (No. NRF-2020-R111A1A01074257).

Author contributions: Seonghyeon Cho: writing – original draft and visualization; Van-An Duong: writing – review and editing; Jeong-Hun Mok: writing – original draft; Minjoong Joo: writing – original draft; Jong-Moon Park: supervision and conceptualization; Hookeun Lee: writing – review and editing, project administration and funding acquisition.

Conflict of interest: Authors state no conflict of interest.

Data availability statement: Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

References

- [1] Maillard L. Action of amino acids on sugars. Formation of melanoidins in a methodical way. *Compte-Rendu de l'Academie Des Sci.* 1912;154:66–8.
- [2] Kim C-S, Park S, Kim J. The role of glycation in the pathogenesis of aging and its prevention through herbal products and physical exercise. *J Exerc Nutr Biochem.* 2017;21:55–61. doi: 10.20463/jenb.2017.0027.

- [3] Vlassara H. Advanced glycation end-products and atherosclerosis. *Ann Med*. 1996;28:419–26. doi: 10.3109/07853899608999102.
- [4] Exner M, Hermann M, Hofbauer R, Kapiotis S, Quehenberger P, Speiser W, et al. Genistein prevents the glucose autooxidation mediated atherogenic modification of low density lipoprotein. *Free Radic Res*. 2001;34:101–12. doi: 10.1080/10715760100300101.
- [5] Rabbani N, Ashour A, Thornalley PJ. Mass spectrometric determination of early and advanced glycation in biology. *Glycoconj J*. 2016;33:553–68. doi: 10.1007/s10719-016-9709-8.
- [6] Masania J, Faustmann G, Anwar A, Hafner-Giessauf H, Rajpoot N, Grabher J, et al. Urinary metabolomic markers of protein glycation, oxidation, and nitration in early-stage decline in metabolic, vascular, and renal health. *Oxid Med Cell Longev*. 2019;2019:1–15. doi: 10.1155/2019/4851323.
- [7] Alikhani Z, Alikhani M, Boyd CM, Nagao K, Trackman PC, Graves DT. Advanced glycation end products enhance expression of pro-apoptotic genes and stimulate fibroblast apoptosis through cytoplasmic and mitochondrial pathways. *J Biol Chem*. 2005;280(13):12087–95. doi: 10.1074/jbc.M406313200.
- [8] Nmij H, Mj K, Mc F, Pr N, Cg S, Aj M. Postprandial glucose spikes, an important contributor to cardiovascular disease in diabetes? *Front Cardiovasc Med*. 2020;7:570553–53. doi: 10.3389/fcvm.2020.570553.
- [9] Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification—A role in pathogenesis and antiproliferative chemotherapy. *Gen Pharmacology: Vasc Syst*. 1996;27:565–73. doi: 10.1016/0306-3623(95)02054-3.
- [10] Soboleva A, Vikhnina M, Grishina T, Frolov A. Probing protein glycation by chromatography and mass spectrometry: analysis of glycation adducts. *IJMS*. 2017;18:2557. doi: 10.3390/ijms18122557.
- [11] Monnier VM, Sun W, Sell DR, Fan X, Nemet I, Genuth S. Glucosepane: a poorly understood advanced glycation end product of growing importance for diabetes and its complications. *Clin Chem Lab Med*. 2014;52:21–32. doi: 10.1515/cclm-2013-0174.
- [12] Lederer MO, Bühler HP. Cross-linking of proteins by Maillard processes—characterization and detection of a lysine-arginine cross-link derived from D-glucose. *Bioorg Med Chem*. 1999;7:1081–8. doi: 10.1016/S0968-0896(99)00040-1.
- [13] Ichihashi M, Yagi M, Nomoto K, Yonei Y. Glycation stress and photo-aging in skin. *Anti-Aging Med*. 2011;8:23–9. doi: 10.3793/jaam.8.23.
- [14] Boulanger E, Wautier M-P, Wautier J-L, Boval B, Panis Y, Wernert N, et al. AGEs bind to mesothelial cells via RAGE and stimulate VCAM-1 expression. *Kidney Int*. 2002;61:148–56. doi: 10.1046/j.1523-1755.2002.00115.x.
- [15] Roca F, Grossin N, Chassagne P, Puisieux F, Boulanger E. Glycation: the angiogenic paradox in aging and age-related disorders and diseases. *Ageing Res Rev*. 2014;15:146–60. doi: 10.1016/j.arr.2014.03.009.
- [16] Sanguineti R, Storace D, Monacelli F, Federici A, Odetti P. Pentosidine effects on human osteoblasts *in vitro*. *Ann N Y Acad Sci*. 2008;1126:166–72. doi: 10.1196/annals.1433.044.
- [17] Pigeon H, Zucchi H, Rousset F, Monnier VM, Asselineau D. Skin aging by glycation: lessons from the reconstructed skin model. *Clin Chem Lab Med*. 2014;52:169–74. doi: 10.1515/cclm-2013-0091.
- [18] Dunn EJ, Philippou H, Ariëns RAS, Grant PJ. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia*. 2006;49:1071–80. doi: 10.1007/s00125-006-0197-4.
- [19] Lund T, Svindland A, Pepaj M, Jensen A-B, Berg JP, Kilhovd B, et al. Fibrin(ogen) may be an important target for methylglyoxal-derived AGE modification in elastic arteries of humans. *Diab Vasc Dis Res*. 2011;8:284–94. doi: 10.1177/1479164111416831.
- [20] Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. *Diabetologia*. 2001;44:129–46. doi: 10.1007/s001250051591.
- [21] Schalkwijk CG, Miyata T. Early- and advanced non-enzymatic glycation in diabetic vascular complications: the search for therapeutics. *Amino Acids*. 2012;42:1193–204. doi: 10.1007/s00726-010-0779-9.
- [22] Fishman SL, Sonmez H, Basman C, Singh V, Poretsky L. The role of advanced glycation end-products in the development of coronary artery disease in patients with and without diabetes mellitus: a review. *Mol Med*. 2018;24:59. doi: 10.1186/s10020-018-0060-3.
- [23] Stirban A, Gawłowski T, Roden M. Vascular effects of advanced glycation endproducts: clinical effects and molecular mechanisms. *Mol Metab*. 2014;3:94–108. doi: 10.1016/j.molmet.2013.11.006.
- [24] Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, et al. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proc Natl Acad Sci U S A*. 1994;91:4766–70. doi: 10.1073/pnas.91.11.4766.
- [25] Sciences NA. Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *PNAS*. 1995;92:1794–4. doi: 10.1073/pnas.92.5.1794e.
- [26] Winlove CP, Parker KH, Avery NC, Bailey AJ. Interactions of elastin and aorta with sugars *in vitro* and their effects on biochemical and physical properties. *Diabetologia*. 1996;39:1131–9. doi: 10.1007/BF02658498.
- [27] Monnier VM, Genuth S, Sell DR. The pecking order of skin advanced glycation endproducts (AGEs) as long-term markers of glycemic damage and risk factors for micro- and subclinical macrovascular disease progression in type 1 diabetes. *Glycoconj J*. 2016;33:569–79. doi: 10.1007/s10719-016-9702-2.
- [28] Zhang L, Zhang Q. Glycated plasma proteins as more sensitive markers for glycemic control in type 1 diabetes. *Proteom – Clin Appl*. 2020;14:1900104. doi: 10.1002/prca.201900104.
- [29] Linssen PB, Henry RM, Schalkwijk CG, Dekker JM, Nijpels G, Brunner-La Rocca H-P, et al. Serum advanced glycation endproducts are associated with left ventricular dysfunction in normal glucose metabolism but not in type 2 diabetes: The hoorn study. *Diabetes Vasc Dis Res*. 2016;13:278–85. doi: 10.1177/1479164116640680.
- [30] van der Lugt T, Opperhuizen A, Bast A, Vrolijk MF. Dietary advanced glycation endproducts and the gastrointestinal tract. *Nutrients*. 2020;12:2814. doi: 10.3390/nu12092814.

- [31] Zhang G, Huang G, Xiao L, Mitchell AE. Determination of advanced glycation endproducts by LC-MS/MS in raw and roasted almonds (*Prunus dulcis*). *J Agric Food Chem*. 2011;59:12037–46. doi: 10.1021/jf202515k.
- [32] Thomas CJ, Cleland TP, Sroga GE, Vashishth D. Accumulation of carboxymethyl-lysine (CML) in human cortical bone. *Bone*. 2018;110:128–33. doi: 10.1016/j.bone.2018.01.028.
- [33] Shirakami T, Yamanaka M, Fujihara J, Matsuoka Y, Gohto Y, Obana A, et al. Advanced glycation end product accumulation in subjects with open-angle glaucoma with and without exfoliation. *Antioxidants*. 2020;9:755. doi: 10.3390/antiox9080755.
- [34] Venkatraman J, Aggarwal K, Balaram P. Helical peptide models for protein glycation: proximity effects in catalysis of the Amadori rearrangement. *Chem & Biol*. 2001;8:611–25. doi: 10.1016/S1074-5521(01)00036-9.
- [35] Priego Capote F, Sanchez J-C. Strategies for proteomic analysis of non-enzymatically glycosylated proteins. *Mass Spectrom Rev*. 2009;28:135–46. doi: 10.1002/mas.20187.
- [36] Bilova T, Lukashova E, Brauch D, Greifenhagen U, Paudel G, Tarakhovskaya E, et al. A snapshot of the plant glycosylated proteome: structural, functional, and mechanistic aspects. *J Biol Chem*. 2016;291:7621–36. doi: 10.1074/jbc.M115.678581.
- [37] Soboleva A, Schmidt R, Vikhnina M, Grishina T, Frolov A. Maillard proteomics: opening new pages. *IJMS*. 2017;18:2677. doi: 10.3390/ijms18122677.
- [38] Miller AK, Hambly DM, Kerwin BA, Treuheit MJ, Gadgil HS. Characterization of site-specific glycation during process development of a human therapeutic monoclonal antibody. *J Pharm Sci*. 2011;100:2543–50. doi: 10.1002/jps.22504.
- [39] Poland J, Rabilloud T, Sinha P. Silver Staining of 2-D Gels. In: Walker JM, editor. *The Proteomics Protocols Handbook*. Totowa, NJ: Humana Press; 2005. p. 177–84. doi: 10.1385/1-59259-890-0:177.
- [40] Lapolla A, Brioschi M, Banfi C, Tremoli E, Bonfante L, Cristoni S, et al. On the search for glycosylated lipoprotein ApoA-I in the plasma of diabetic and nephropathic patients. *J Mass Spectrom*. 2008;43:74–81. doi: 10.1002/jms.1274.
- [41] Snow LM, Fugere NA, Thompson LV. Advanced glycation end-product accumulation and associated protein modification in type II skeletal muscle with aging. *J Gerontol A Biol Sci Med Sci*. 2007;62:1204–10. doi: 10.1093/gerona/62.11.1204.
- [42] Chougale AD, Bhat SP, Bhujbal SV, Zambare MR, Puntambekar S, Somani RS, et al. Proteomic analysis of glycosylated proteins from streptozotocin-induced diabetic rat kidney. *Mol Biotechnol*. 2012;50:28–38. doi: 10.1007/s12033-011-9409-3.
- [43] Pereira Morais MP, Marshall D, Flower SE, Caunt CJ, James TD, Williams RJ, et al. Analysis of protein glycation using fluorescent phenylboronate gel electrophoresis. *Sci Rep*. 2013;3:1437. doi: 10.1038/srep01437.
- [44] Scumaci D, Olivo E, Fiumara CV, La Chimia M, De Angelis MT, Mauro S, et al. DJ-1 proteoforms in breast cancer cells: the escape of metabolic epigenetic misregulation. *Cells*. 2020;9:1968. doi: 10.3390/cells9091968.
- [45] Bunai K, Yamane K. Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein proteomics and perspectives. *J Chromatogr B*. 2005;815:227–36. doi: 10.1016/j.jchromb.2004.08.030.
- [46] Gould BJ, Hall PM, Cook JG. A sensitive method for the measurement of glycosylated plasma proteins using affinity chromatography. *Ann Clin Biochem*. 1984;21:16–21. doi: 10.1177/000456328402100103.
- [47] Brooks WLA, Deng CC, Sumerlin BS. Structure–reactivity relationships in boronic acid–diol complexation. *ACS Omega*. 2018;3:17863–70. doi: 10.1021/acsomega.8b02999.
- [48] Zhang Q, Monroe ME, Schepmoes AA, Clauss TRW, Gritsenko MA, Meng D, et al. Comprehensive identification of glycosylated peptides and their glycation motifs in plasma and erythrocytes of control and diabetic subjects. *J Proteome Res*. 2011;10:3076–88. doi: 10.1021/pr200040j.
- [49] Jeon J, Yang J, Park J-M, Han N-Y, Lee Y-B, Lee H. Development of an automated high-throughput sample preparation protocol for LC-MS/MS analysis of glycosylated peptides. *J Chromatogr B*. 2018;1092:88–94. doi: 10.1016/j.jchromb.2018.05.036.
- [50] Takátsy A, Böddi K, Nagy L, Nagy G, Szabó S, Markó L, et al. Enrichment of Amadori products derived from the nonenzymatic glycation of proteins using microscale boronate affinity chromatography. *Anal Biochem*. 2009;393:8–22. doi: 10.1016/j.ab.2009.06.007.
- [51] Choi H, Son SE, Hur W, Tran V-K, Lee HB, Park Y, et al. Electrochemical immunoassay for determination of glycosylated albumin using nanozymes. *Sci Rep*. 2020;10:9513. doi: 10.1038/s41598-020-66446-3.
- [52] Xu Y, Zhang L, Lu H. Use of boronic acid nanoparticles in glycoprotein enrichment. *Methods Mol Biol*. 2013;951:45–55. doi: 10.1007/978-1-62703-146-2_4.
- [53] Son SE, Gupta PK, Hur W, Choi H, Lee HB, Park Y, et al. Determination of glycosylated albumin using a Prussian blue nanozyme-based boronate affinity sandwich assay. *Analytica Chim Acta*. 2020;1134:41–9. doi: 10.1016/j.aca.2020.08.015.
- [54] Liu Y, Ren L, Liu Z. A unique boronic acid functionalized monolithic capillary for specific capture, separation and immobilization of cis-diol biomolecules. *Chem Commun*. 2011;47:5067–9. doi: 10.1039/C0CC005675H.
- [55] Frolov A, Hoffmann R. Analysis of amadori peptides enriched by boronic acid affinity chromatography. *Ann N Y Acad Sci*. 2008;1126:253–6. doi: 10.1196/annals.1433.060.
- [56] Soboleva A, Modzel M, Didio A, Plóciennik H, Kijewska M, Grischina T, et al. Quantification of prospective type 2 diabetes mellitus biomarkers by stable isotope dilution with bi-labeled standard glycosylated peptides. *Anal Methods*. 2017;9:409–18. doi: 10.1039/C6AY02483A.
- [57] Li H, He H, Liu Z. Recent progress and application of boronate affinity materials in bioanalysis. *TrAC Trends Anal Chem*. 2021;140:116271. doi: 10.1016/j.trac.2021.116271.
- [58] Quan C, Alcalá E, Petkovska I, Matthews D, Canova-Davis E, Taticek R, et al. A study in glycation of a therapeutic recombinant humanized monoclonal antibody: where it is, how it got there, and how it affects charge-based behavior. *Anal Biochem*. 2008;373:179–91. doi: 10.1016/j.ab.2007.09.027.
- [59] Xiao H, Chen W, Smeekens JM, Wu R. An enrichment method based on synergistic and reversible covalent interactions for large-scale analysis of glycoproteins. *Nat Commun*. 2018;9:1692. doi: 10.1038/s41467-018-04081-3.
- [60] Soper K. Alternative hedonism, cultural theory and the role of aesthetic revisioning. *Cultural Stud*. 2008;22:567–87. doi: 10.1080/09502380802245829.

- [61] Yamamoto K. Assessment of weak sugar-binding ability using lectin tetramer and membrane-based glycans. In: Hirabayashi J, editor. *Lectins: methods and protocols*. New York, NY: Springer; 2014. p. 413–8. doi: 10.1007/978-1-4939-1292-6_36.
- [62] Klont F, Pouwels SD, Hermans J, van de Merbel NC, Horvatovich P, ten Hacken NHT, et al. A fully validated liquid chromatography-mass spectrometry method for the quantification of the soluble receptor of advanced glycation end-products (sRAGE) in serum using immunopurification in a 96-well plate format. *Talanta*. 2018;182:414–21. doi: 10.1016/j.talanta.2018.02.015.
- [63] Hyung S-W, Piehowski PD, Moore RJ, Orton DJ, Schepmoes AA, Clauss TR, et al. Microscale depletion of high abundance proteins in human biofluids using IgY14 immunoaffinity resin: analysis of human plasma and cerebrospinal fluid. *Anal Bioanal Chem*. 2014;406:7117–25. doi: 10.1007/s00216-014-8058-3.
- [64] Filip S, Vougas K, Zoidakis J, Latosinska A, Mullen W, Spasovski G, et al. Comparison of depletion strategies for the enrichment of low-abundance proteins in urine. *PLoS One*. 2015;10:e0133773. doi: 10.1371/journal.pone.0133773.
- [65] Bhonsle HS, Korwar AM, Kote SS, Golegaonkar SB, Chougale AD, Shaik ML, et al. Low plasma albumin levels are associated with increased plasma protein glycation and HbA1c in diabetes. *J Proteome Res*. 2012;11:1391–6. doi: 10.1021/pr201030m.
- [66] Jankovska E, Svitek M, Holada K, Petrak J. Affinity depletion versus relative protein enrichment: a side-by-side comparison of two major strategies for increasing human cerebrospinal fluid proteome coverage. *Clin Proteom*. 2019;16:9. doi: 10.1186/s12014-019-9229-1.
- [67] Duong V-A, Park J-M, Lee H. Review of three-dimensional liquid chromatography platforms for bottom-up proteomics. *Int J Mol Sci*. 2020;21:E1524. doi: 10.3390/ijms21041524.
- [68] Jandera P, Fischer J, Lahovská H, Novotná K, Česla P, Kolářová L. Two-dimensional liquid chromatography normal-phase and reversed-phase separation of (co)oligomers. *J Chromatogr A*. 2006;1119:3–10. doi: 10.1016/j.chroma.2005.10.081.
- [69] van de Schans MGM, Blokland MH, Zoontjes PW, Mulder PPI, Nielen MWF. Multiple heart-cutting two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry of pyrrolizidine alkaloids. *J Chromatogr A*. 2017;1503:38–48. doi: 10.1016/j.chroma.2017.04.059.
- [70] del Castillo E, Montes-Bayón M, Añón E, Sanz-Medel A. Quantitative targeted biomarker assay for glycated haemoglobin by multidimensional LC using mass spectrometric detection. *J Proteom*. 2011;74:35–43. doi: 10.1016/j.jprot.2010.07.011.
- [71] Petry-Podgórska I, Židková J, Flodrová D, Bobálová J. 2D-HPLC and MALDI-TOF/TOF analysis of barley proteins glycosylated during brewing. *J Chromatogr B*. 2010;878:3143–8. doi: 10.1016/j.jchromb.2010.09.023.
- [72] Zhang L, Liu C-W, Zhang Q. Online 2D-LC-MS/MS platform for analysis of glycosylated proteome. *Anal Chem*. 2018;90:1081–6. doi: 10.1021/acs.analchem.7b03342.
- [73] Leonhardt J, Teutenberg T, Tuerk J, Schlüsener MP, Ternes TA, Schmidt TC. A comparison of one-dimensional and microscale two-dimensional liquid chromatographic approaches coupled to high resolution mass spectrometry for the analysis of complex samples. *Anal Methods*. 2015;7:7697–706. doi: 10.1039/C5AY01143D.
- [74] Meerwaldt R, Links T, Graaff R, Thorpe SR, Baynes JW, Hartog J, et al. Simple noninvasive measurement of skin autofluorescence. *Ann N Y Acad Sci*. 2005;1043:290–8. doi: 10.1196/annals.1333.036.
- [75] Gkogkolou P, Böhm M. Advanced glycation end products. *Dermatoendocrinol*. 2012;4:259–70. doi: 10.4161/derm.22028.
- [76] Monnier VM, Vishwanath V, Frank KE, Elmets CA, Dauchot P, Kohn RR. Relation between complications of type I diabetes mellitus and collagen-linked fluorescence. *N Engl J Med*. 1986;314:403–8. doi: 10.1056/NEJM198602133140702.
- [77] Dekker MAM, den, Zwiers M, Heuvel ER, van den, Vos LC, de, Smit AJ, Zeebregts CJ, et al. Skin autofluorescence, a non-invasive marker for AGE accumulation, is associated with the degree of atherosclerosis. *PLoS One*. 2013;8:e83084. doi: 10.1371/journal.pone.0083084.
- [78] Shah KM, Clark BR, McGill JB, Lang CE, Maynard J, Mueller MJ. Relationship between skin intrinsic fluorescence—an indicator of advanced glycation end products—and upper extremity impairments in individuals with diabetes mellitus. *Phys Ther*. 2015;95:1111–9. doi: 10.2522/ptj.20140340.
- [79] Sell DR, Monnier VM. End-stage renal disease and diabetes catalyze the formation of a pentose-derived crosslink from aging human collagen. *J Clin Invest*. 1990;85:380–4. doi: 10.1172/JCI114449.
- [80] Kouidrat Y, Amad A, Desailoud R, Diouf M, Fertout E, Scoury D, et al. Increased advanced glycation end-products (AGEs) assessed by skin autofluorescence in schizophrenia. *J Psychiatr Res*. 2013;47:1044–8. doi: 10.1016/j.jpsychires.2013.03.016.
- [81] Da Moura Smedo C, Webb M, Waller H, Khunti K, Davies M. Skin autofluorescence, a non-invasive marker of advanced glycation end products: clinical relevance and limitations. *Postgrad Med J*. 2017;93:289–94. doi: 10.1136/postgrad-medj-2016-134579.
- [82] Koetsier M, Lutgers HL, de Jonge C, Links TP, Smit AJ, Graaff R. Reference values of skin autofluorescence. *Diabetes Technol & Therapeutics*. 2010;12:399–403. doi: 10.1089/dia.2009.0113.
- [83] Atzeni IM, Boersema J, Pas HH, Diercks GFH, Scheijen LJLM, Schalkwijk CG, et al. Is skin autofluorescence (SAF) representative of dermal advanced glycation end products (AGEs) in dark skin? A pilot study. *Heliyon*. 2020;6:e05364. doi: 10.1016/j.heliyon.2020.e05364.
- [84] Genc S, Omer B, Aycan-Ustyoğlu E, Ince N, Bal F, Gurdol F. Evaluation of turbidimetric inhibition immunoassay (TINIA) and HPLC methods for glycosylated haemoglobin determination. *J Clin Laboratory Anal*. 2012;26:481–5. doi: 10.1002/jcla.21550.
- [85] Ikeda K, Sakamoto Y, Kawasaki Y, Miyake T, Tanaka K, Urata T, et al. Determination of glycosylated albumin by enzyme-linked boronate immunoassay (ELBIA). *Clin Chem*. 1998;44:256–63. doi: 10.1093/clinchem/44.2.256.
- [86] Ye J, Chen Y, Liu Z. A boronate affinity sandwich assay: an appealing alternative to immunoassays for the determination of glycoproteins. *Angew Chem Int Ed*. 2014;53:10386–9. doi: 10.1002/anie.201405525.

- [87] Brunvand L, Heier M, Brunborg C, Hanssen KF, Fugelseth D, Stensaeth KH, et al. Advanced glycation end products in children with type 1 diabetes and early reduced diastolic heart function. *BMC Cardiovascular Disord.* 2017;17:133. doi: 10.1186/s12872-017-0551-0.
- [88] Hashim Z, Zarina S. Advanced glycation end products in diabetic and non-diabetic human subjects suffering from cataract. *AGE.* 2011;33:377–84. doi: 10.1007/s11357-010-9177-1.
- [89] Ghosh P, Sahoo R, Vaidya A, Cantel S, Kavishwar A, Goldfine A, et al. A specific and sensitive assay for blood levels of glycated CD59: A novel biomarker for diabetes. *Am J Hematol.* 2013;88:670–6. doi: 10.1002/ajh.23478.
- [90] Finco AB, Machado-de-Ávila RA, Maciel R, De Moura J, Billiald P, Stinghen AEM, et al. Generation and characterization of monoclonal antibody against advanced glycation end products in chronic kidney disease. *Biochem Biophysics Rep.* 2016;6:142–8. doi: 10.1016/j.bbrep.2016.03.011.
- [91] Ki H, Oh J, Han G-R, Kim M-G. Glycation ratio determination through simultaneous detection of human serum albumin and glycated albumin on an advanced lateral flow immunoassay sensor. *Lab Chip.* 2020;20:844–51. doi: 10.1039/C9LC00967A.
- [92] Zhang M, Xu W, Deng Y. A new strategy for early diagnosis of type 2 diabetes by standard-free, label-free LC-MS/MS quantification of glycated peptides. *Diabetes.* 2013;62:3936–42. doi: 10.2337/db13-0347.
- [93] Kislinger T, Humeny A, Peich CC, Becker C-M, Pischetsrieder M. Analysis of protein glycation products by MALDI-TOF/MS. *Ann N Y Acad Sci.* 2005;1043:249–59. doi: 10.1196/annals.1333.030.
- [94] Huber B, Pischetsrieder M. Characterization of ascorbylated proteins by immunochemical methods. *J Agric Food Chem.* 1998;46:3985–90. doi: 10.1021/jf9803132.
- [95] Kislinger T, Humeny A, Pischetsrieder M. Analysis of protein glycation products by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Curr Med Chem.* 2004;11:2185–93. doi: 10.2174/0929867043364649.
- [96] Farah MA, Bose S, Lee J-H, Jung H-C, Kim Y. Analysis of glycated insulin by MALDI-TOF mass spectrometry. *Biochimica et Biophysica Acta (BBA) - Gen Subj.* 2005;1725:269–82. doi: 10.1016/j.bbagen.2005.05.028.
- [97] Deng Y, Butré CI, Wierenga PA. Understanding glycation kinetics of individual peptides in protein hydrolysates. *Int Dairy J.* 2019;91:98–109. doi: 10.1016/j.idairyj.2018.10.003.
- [98] Humeny A, Kislinger T, Becker C-M, Pischetsrieder M. Qualitative determination of specific protein glycation products by matrix-assisted laser desorption/ionization mass spectrometry peptide mapping. *J Agric Food Chem.* 2002;50:2153–60. doi: 10.1021/jf011349o.
- [99] Crook AA, Powers R. Quantitative NMR-based biomedical metabolomics: current status and applications. *Molecules.* 2020;25:E5128. doi: 10.3390/molecules25215128.
- [100] Li R, Rajan R, Wong WCV, Reid DG, Duer MJ, Somovilla VJ, et al. *In situ* characterization of advanced glycation end products (AGEs) in collagen and model extracellular matrix by solid state NMR. *Chem Commun.* 2017;53:13316–9. doi: 10.1039/C7CC06624D.
- [101] Lucarelli G, Rutigliano M, Galleggiane V, Giglio A, Palazzo S, Ferro M, et al. Metabolomic profiling for the identification of novel diagnostic markers in prostate cancer. *Expert Rev Mol Diagn.* 2015;15:1211–24. doi: 10.1586/14737159.2015.1069711.
- [102] Maekawa Y, Sugiura M, Takeuchi A, Tomoo K, Ishida T, Kamigauchi M. Study of lysozyme glycation reaction by mass spectrometry and NMR spectroscopy. *Helvetica Chim Acta.* 2010;93:991–8. doi: 10.1002/hlca.200900330.
- [103] Nanni EA, Barnes AB, Griffin RG, Temkin RJ. THz dynamic nuclear polarization NMR. *IEEE Trans Terahertz Sci Technol.* 2011;1:145–63. doi: 10.1109/THZ.2011.2159546.
- [104] Lapolla A, Flamini R, Vedova AD, Senesi A, Reitano R, Fedele D, et al. Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. *Clin Chem Laboratory Med.* 2003;41:1166–73. doi: 10.1515/CCLM.2003.180.
- [105] Dunn JA, Patrick JS, Thorpe SR, Baynes JW. Oxidation of glycated proteins: age-dependent accumulation of N epsilon-(carboxymethyl)lysine in lens proteins. *Biochemistry.* 1989;28:9464–8. doi: 10.1021/bi00450a033.
- [106] Dunn JA, McCance DR, Thorpe SR, Lyons TJ, Baynes JW. Age-dependent accumulation of N epsilon-(carboxymethyl)lysine and N epsilon-(carboxymethyl)hydroxylysine in human skin collagen. *Biochemistry.* 1991;30:1205. doi: 10.1021/bi00219a007.
- [107] Anderson MM, Heinecke JW. Production of N-(carboxymethyl)lysine is impaired in mice deficient in NADPH oxidase: a role for phagocyte-derived oxidants in the formation of advanced glycation end products during inflammation. *Diabetes.* 2003;52:2137–43. doi: 10.2337/diabetes.52.8.2137.
- [108] Wellner A, Huettl C, Henle T. Formation of maillard reaction products during heat treatment of carrots. *J Agric Food Chem.* 2011;59:7992–8. doi: 10.1021/jf2013293.
- [109] Bilova T, Paudel G, Shilyaev N, Schmidt R, Brauch D, Tarakhovskaya E, et al. Global proteomic analysis of advanced glycation end products in the Arabidopsis proteome provides evidence for age-related glycation hot spots. *J Biol Chem.* 2017;292:15758–76. doi: 10.1074/jbc.M117.794537.
- [110] Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, et al. Quantitative screening of advanced glycation end products in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J.* 2003;375:581–92. doi: 10.1042/bj20030763.
- [111] Doll S, Burlingame AL. Mass spectrometry-based detection and assignment of protein posttranslational modifications. *ACS Chem Biol.* 2015;10:63–71. doi: 10.1021/cb500904b.
- [112] Keilhauer EC, Geyer PE, Mann M. HCD fragmentation of glycated peptides. *J Proteome Res.* 2016;15:2881–90. doi: 10.1021/acs.jproteome.6b00464.
- [113] Zhang YV, Rockwood A. Impact of automation on mass spectrometry. *Clinica Chim Acta.* 2015;450:298–303. doi: 10.1016/j.cca.2015.08.027.
- [114] Liu Z, Fan S, Liu H, Yu J, Qiao R, Zhou M, et al. Enhanced detection of low-abundance human plasma proteins by integrating polyethylene glycol fractionation and immunoaffinity depletion. *PLoS One.* 2016;11:e0166306. doi: 10.1371/journal.pone.0166306.
- [115] Kontostathi G, Makridakis M, Bitsika V, Tsolakos N, Vlahou A, Zoidakis J. Development and validation of multiple reaction monitoring (MRM) assays for clinical applications. In: Brun V,

- Couté Y, editors. *Proteomics for Biomarker Discovery: Methods and Protocols*. New York, NY: Springer; 2019. pp. 205–23. doi: 10.1007/978-1-4939-9164-8_14.
- [116] Hernando MD, Suárez-Barcena JM, Bueno MJM, García-Reyes JF, Fernández-Alba AR. Fast separation liquid chromatography–tandem mass spectrometry for the confirmation and quantitative analysis of avermectin residues in food. *J Chromatogr A*. 2007;1155:62–73. doi: 10.1016/j.chroma.2007.02.120.
- [117] Percy AJ, Chambers AG, Yang J, Hardie DB, Borchers CH. Advances in multiplexed MRM-based protein biomarker quantitation toward clinical utility. *Biochimica et Biophysica Acta (BBA) - Proteins Proteom*. 2014;1844:917–26. doi: 10.1016/j.bbapap.2013.06.008.
- [118] Scheijen LJ, Schalkwijk CG. Quantification of glyoxal, methylglyoxal and 3-deoxyglucosone in blood and plasma by ultra performance liquid chromatography tandem mass spectrometry: evaluation of blood specimen. *Clin Chem Lab Med*. 2014;52:85–91. doi: 10.1515/cclm-2012-0878.
- [119] Tareke E, Forslund A, Lindh CH, Fahlgren C, Östman E. Isotope dilution ESI-LC-MS/MS for quantification of free and total N ϵ -(1-Carboxymethyl)-L-Lysine and free N ϵ -(1-Carboxyethyl)-L-Lysine: comparison of total N ϵ -(1-Carboxymethyl)-L-Lysine levels measured with new method to ELISA assay in gruel samples. *Food Chem*. 2013;141:4253–9. doi: 10.1016/j.foodchem.2013.07.003.
- [120] Zhang Q, Tang N, Brock JWC, Mottaz HM, Ames JM, Baynes JW, et al. Enrichment and analysis of nonenzymatically glycosylated peptides: boronate affinity chromatography coupled with electron-transfer dissociation mass spectrometry. *J Proteome Res*. 2007;6:2323–30. doi: 10.1021/pr070112q.
- [121] Priego-Capote F, Ramírez-Boo M, Finamore F, Gluck F, Sanchez J-C. Quantitative analysis of glycosylated proteins. *J Proteome Res*. 2014;13:336–47. doi: 10.1021/pr4000398.
- [122] Priego-Capote F, Scherl A, Möller M, Waridel P, Lisacek F, Sanchez J-C. Glycation isotopic labeling with ^{13}C -reducing sugars for quantitative analysis of glycosylated proteins in human plasma. *Mol & Cell Proteom*. 2010;9:579–92. doi: 10.1074/mcp.M900439-MCP200.
- [123] Pepaj M, Thorsby PM. Analysis of glycosylated albumin by on-line two-dimensional liquid chromatography mass spectrometry. *J Liq Chromatogr & Relat Technol*. 2015;38:20–8. doi: 10.1080/10826076.2013.864980.
- [124] Basu PS, Chatterji S, Batabyal SK. Lectin-based estimation of glycosylated hemoglobin in diabetes mellitus: lectin-based assay of diabetes mellitus. *J Clin Lab Anal*. 2012;26:45–8. doi: 10.1002/jcla.20503.
- [125] Spiller S, Li Y, Blüher M, Welch L, Hoffmann R. Glycosylated lysine-141 in haptoglobin improves the diagnostic accuracy for type 2 diabetes mellitus in combination with glycosylated hemoglobin HbA1c and fasting plasma glucose. *Clin Proteom*. 2017;14:10. doi: 10.1186/s12014-017-9145-1.
- [126] Hudson DM, Archer M, King KB, Eyre DR. Glycation of type I collagen selectively targets the same helical domain lysine sites as lysyl oxidase-mediated cross-linking. *J Biol Chem*. 2018;293:15620–7. doi: 10.1074/jbc.RA118.004829.