Original Article

Rapid pretreatment for multi-sample analysis of advanced glycation end products and their role in nephropathy

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Advanced glycation end products (AGEs), produced by the Maillard reaction between carbohydrates and proteins, may be involved in diabetes and its complications. Accurate quantification of AGEs *in vivo* can demonstrate the relation between AGEs and pathological conditions, but it is not widely used in clinical practice because of the multiple pretreatment steps before analyses. We developed a fully automated solid-phase extraction system (FSPES) to simplify rate-limiting pretreatment using a cation exchange column. We applied this device to evaluate AGEs in nephropathy. Among the standard samples, we used arginine, lysine, N^{ε}-(carboxymethyl)lysine (CML), N^{ω}-(carboxymethyl)arginine (CMA), N^{ε}-(carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) for FSPES. We analyzed the coefficient of variation (CV) by mass spectrometry. FSPES performed column operations rapidly at a pressure three times higher compared with the conventional method. FSPES stably performed pretreatment. CV results for CML, CMA, CEL, and MG-H1 measurements in bovine and human serum were the same as those in the conventional pretreatment. Among the AGE structures we measured, CML and CEL increased with the decline in kidney function. The CML and CEL levels of patients with nephropathy were significantly higher than those in normal subjects. Thus, FSPES is useful for clarifying the relation between AGEs and various pathological conditions.

***Key Words*: advanced glycation end products, oxidation, nephropathy, solid-phase extraction**

The incidence of lifestyle-related diseases is increasing annually owing to the diversification of lifestyles. The associated increase in medical expenses for their treatment puts pressure on the government's financial resources. The number of patients with diabetes mellitus, one of the most common lifestyle-related diseases, is increasing. Along with genetic and environmental factors, most patients with type 2 diabetes have lifestyle-related factors. When diabetes develops, it is not limited to prolonged high blood glucose levels but eventually leads to the development of diabetic complications, such as nephropathy. The onset of diabetic complications must be prevented because they are difficult to cure. However, the early detection of complications is also difficult because they are not accompanied by pain.

A wide variety of sugars, proteins, and lipids are present in living organisms and food. The Maillard reaction occurs when the carbonyl group of reducing sugars reacts with the amino groups of amino acids and proteins to form advanced glycation end products (AGEs). Research has demonstrated that N^{ε}-(carboxymethyl)lysine (CML) is generated from the Amadori product by oxidation with hydroxyl radicals[1] and peroxynitrite,[2] which is known as an oxidative marker *in vivo*. N^{ε}-(carboxyethyl) lysine (CEL) and N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) are generated from methylglyoxal *via* the glycolysis pathway.[3] Furthermore, N^{ω}-(carboxymethyl)arginine (CMA) is generated especially in the glycation of collagen with glyoxal,[4] demonstrating that metabolic disorders can be evaluated by detecting each AGE simultaneously.

The accumulation of AGEs in the body increases with age and with the onset of lifestyle-related diseases, such as diabetes, as reported in a study that compare patients and healthy individuals.[5,6] Especially poor glycemic control induces kidney failure. In Japan, the number of diabetes-induced dialysis patients is increasing.[7] The level of AGE in kidney of diabetic nephropathy rats increases in accordance with the level of urinary proteins.[8] Hemoglobin A1c (HbA1c) is an Amadori product that has been measured as a typical marker of glycemic control in diabetes; accurately predicting diabetic complications remains difficult.[9] AGEs are expected to predict the onset of complications and improve the quality of life of patients with lifestyle-related diseases, followed by a reduction in medical expenses. However, unlike HbA1c, the structure and characteristics of AGEs produced by each metabolic pathway are unknown, and the measurement of biological AGEs remains challenging and often not accurately evaluated.[10–12] In addition, many pretreatments, such as reduction, acid hydrolysis, application of pretreatment columns, concentration, and re-solubilization, are required to evaluate biological AGEs accurately,[13] making it difficult to analyze many clinical samples. In particular, the cation exchange column cannot conduct many samples at once because it requires considerable processing time and is prone to human error owing to the need for multiple procedures.

To evaluate the correlation between disease and AGEs, we focused on the process of the cation exchange column, which is a part of the pretreatment and the most time-consuming step, and verified the automation of the process. Specifically, we compared with the conventional method the appropriate aspirating pressure in the suction step of the automated pretreatment system, location of the column, and presence or absence of disease. Our results showed that the concentrations of arginine (Arg), lysine (Lys), CML, CMA, CEL, and MG-H1 in the serum should be measured. We also evaluated their relation with nephropathy.

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Materials and Methods

We purchased the following chemicals: Calf serum (Biowest, Kansas, MO); CML and CEL (Poly Peptide Laboratories, Strasbourg, France); Arg and Lys (Wako, Osaka, Japan); isotope-labelled internal standards (ISTDs) of [$^2H_2$]-CML, [$^2H_4$]-CEL, and [$^2H_3$]-MG-H1 (Poly Peptide Laboratories, Strasbourg, France), [$^{13}C_6$] lysine and [$^{13}C_6$] arginine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) were purchased. Meanwhile, MG-H1,[14] CMA,[4] and [$^{13}C_6$]-CMA[4] were synthesized as previously described.

Pretreatment of serum for measurement of AGEs. Serum was pretreated as previously described.[13] Briefly, 5 µl serum was mixed with 20 µl H_2O. The samples were reduced with $NaBH_4$ (2.5 µl of 1 M $NaBH_4$ in 0.1 M NaOH) in 25 µl of 200 mM sodium borate buffer (pH 9.1) at 25°C for 4 h. After, 0.01 nmol of [$^2H_2$] CML, [$^2H_4$] CEL, [$^2H_3$] MG-H1, [$^{13}C_6$] CMA, and 5 nmol of [$^{13}C_6$] lysine and [$^{13}C_6$] arginine were added to the pellets, which were then hydrolyzed with 1 ml of 6 M HCl at 100°C for 18 h. After the hydrolysis, the samples were dried. For cation exchange, the dried samples were resuspended in 1 ml of 0.1% trifluoroacetic acid and passed over a Strata X-C column (Phenomenex, Torrance, CA), which had been pre-washed with 1 ml of methanol and equilibrated with 1 ml of 0.1% trifluoroacetic acid. The column was then washed with 3 ml of 2% FA and eluted with 2 ml of 7% ammonia. The eluted samples were dried.

Optimization of vacuum pressure settings for Strata X-C columns. To optimize the vacuum pressure settings for the Strata X-C columns, we used a vacuum manifold system that was experimentally arranged by Shimadzu Corporation (Kyoto, Japan). This system was equipped with a vacuum manifold, column holder, tube holder, electro-pneumatic regulator, pressure gauge, vent valve, and control unit (Fig. 1A and B). We used a diaphragm pump (DTC-22; ULVAC, Kanagawa, Japan) for decompression. In this system, vacuum pressure settings, including the setting pressure (P1), time to reach the setting pressure (t1), duration of time that negative pressure is applied to the columns (t2), and time to reach atmospheric pressure (t3), were automatically controlled. The protocol for the vacuum manifold system was the same as that for the equivalent manual method mentioned above, except for the vacuum manifold system and elution volume. For the vacuum pressure settings, P1 was set at −80, −60, −40, or −20 kPa. The other parameters were set as fixed values: t1, t2, and t3 were 10, 40, and 5 s, respectively. The volume of the 7% NH_4OH aq. for elution was 2 ml. The processing trials using standard AGEs were repeated 4 times.

Solid-phase extraction-based pre-treatment for liquid chromatography with mass spectrometry using FSPES. Based on the optimization of vacuum pressure settings for Strata X-C columns, the automation settings were applied for the fully automated solid-phased extraction system (FSPES) developed by Shimadzu Corporation. Briefly, the vacuum pressure settings were set to −60 kPa for 1 min (t1, t2, and t3 were 10, 45, and 5 s, respectively) for the conditioning, washing, and elution steps (Fig. 1C). These settings were controlled by the same components with ones used in the optimization of vacuum pressure settings for Strata X-C columns; an electro-pneumatic regulator, a pressure gauge, a vent valve, and a control unit. The FSPES is an automated system for solid-phase extraction (SPE)-based pre-treatment for ESI-quadrupole TOF analysis (LC-ESI-QTOF). FSPES is designed to fit column formats (1 ml SPE columns) and

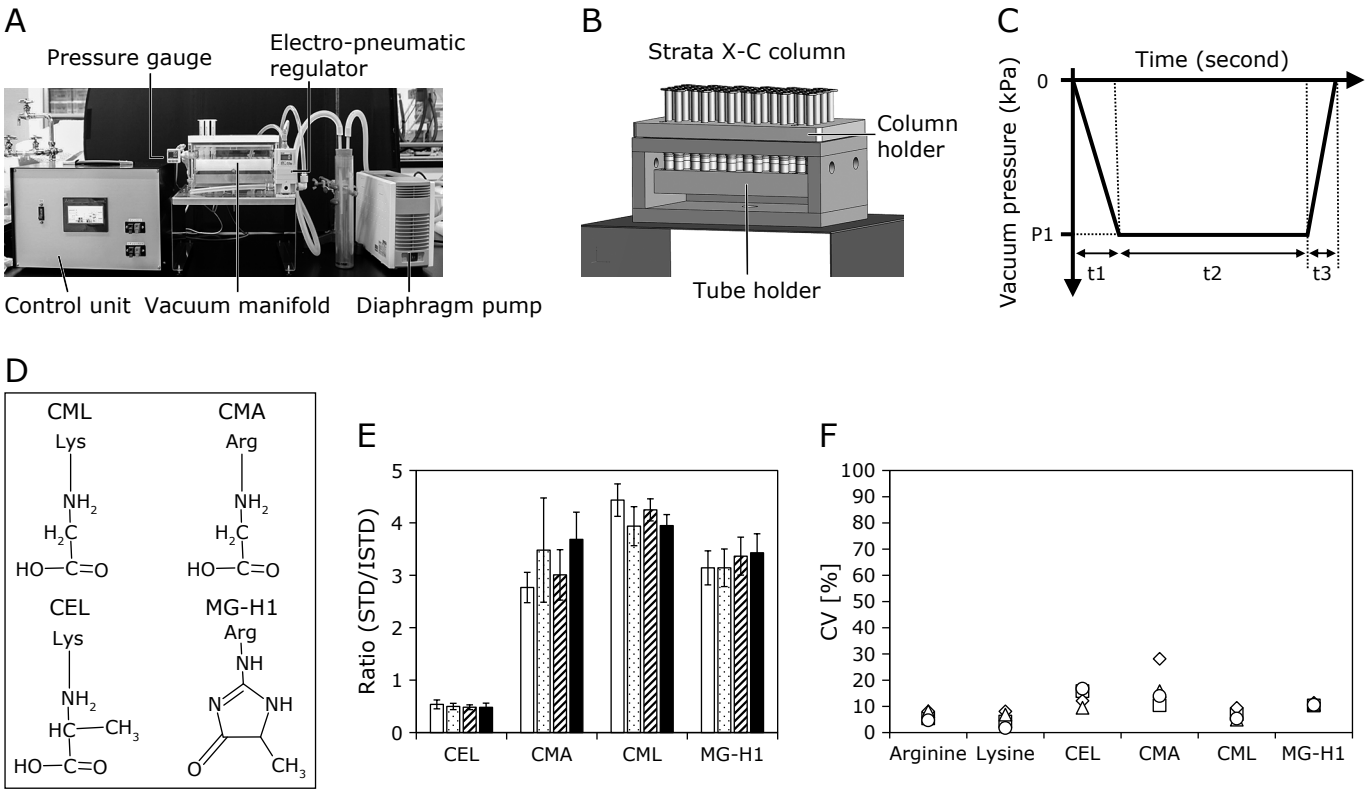

Fig. 1. Experimentally arranged vacuum manifold system. Front side of the vacuum manifold system (A). 3D-CAD image of the vacuum manifold (B). Schematic image of vacuum pressure settings (C). Chemical structures of CML, CEL, CMA, and MG-H1 (D). Comparison of each pressure (–20/–40/–60/–80 kPa) with FSPES of vacuum manifold system. Open bars (n = 4), –20 kPa. Dotted bars (n = 4), –40 kPa. Diagonal striped bars (n = 4), –60 kPa. Closed bars (n = 4), –80 kPa. Mean ± SD (E). CV scores in AGEs and amino acids in each pressure. Square (n = 4), –20 kPa. Diamond (n = 4), –40 kPa. Triangle (n = 4), –60 kPa. Circle (n = 4), –80 kPa (F).

can process 1–48 columns in a serial process. We placed the tubes containing pre-treated samples in a workplace in the FSPES and then loaded the samples into Strata X-C columns using a pipetting unit. Conditioning, washing, and elution were conducted using syringe, filtration, and carrier units. These steps were conducted automatically. The extracts were collected in 2 ml tubes. The protocol with minor modifications was transferred to FSPES. The volume of the pre-treated sample applied to Strata X-C columns was 950 µl, and the volume of the 7% NH_4OH aq. for elution was 1 ml. The processing trials using calf serum were repeated 4 times.

Measurement of AGEs with LC-MS-MS. The dried samples were resuspended in 0.2 ml of 20% MeCN containing 0.1% FA and filtered through 0.45 µm filter (Merck Japan, Tokyo, Japan). The samples were subjected to LC-ESI-QTOF.[15] LC was conducted on a ZIC®-HILIC column (150 × 2.1 mm, 5 µm) (Merck Japan). The mobile phase was collected using solvents A (H_2O containing 0.1% FA) and B (MeCN containing 0.1% FA). The flow rate was 0.2 ml/min and the column was kept at 40°C. The retention times for the four AGEs and two amino acids were approximately 12–15 min. These AGEs, amino acids, and the standard were detected by electrospray ionization and positive ion mass spectrometric multiple reaction monitoring. The parent ions of CML and [$^2H_2$] CML had mass values of 205 (*m*/*z*) and 207 (*m*/*z*), respectively, and those of CEL and [$^2H_4$] CEL, 219 (*m*/*z*) and 223 (*m*/*z*), respectively. Mass values for MG-H1 and [$^2H_3$] MG-H1 were 229 (*m*/*z*) and 232 (*m*/*z*); for CMA and [$^{13}C_6$] CMA, 233 (*m*/*z*) and 239 (*m*/*z*); for lysine and [$^{13}C_6$] lysine, 147 (*m*/*z*) and 153 (*m*/*z*); and for arginine and [$^{13}C_6$] arginine, 175 (*m*/*z*) and 181 (*m*/*z*), respectively.[16]

Human clinical study. All human clinical experimental protocols were approved by the ethics review committee of Tokai University (approval number: 17092). A total of 41 subjects (31 with nephropathy and 10 in the healthy group) were admitted to Fukuoka University Hospital (Fukuoka, Japan) in 2017. Healthy subjects did not have a pathological history of diabetes mellitus. Table 1 gives age, the number of healthy and nephropathy, eGFR score, and nephropathy stage.

Statistical analysis. Data are expressed as mean ± SD (Fig. 1E and 2C). Statistical significance was determined using one-way analysis of variance (Kruskal–Wallis tests) with Bonferroni test (Fig. 3A–C) (*p<0.05, **p<0.01). We examined the correlation between the estimated glomerular filtration rate (eGFR) and CML or CEL content in the serum (Fig. 3D) for statistical significance using Spearman's rank correlation coefficient (p<0.001). Statistical analyses were conducted using EZR (ver. 4.0.0).[17]

Table 1. Clinical characteristics of patients with nephropathy and healthy subjects

		Nephropathy			
	Healthy	G2	G3	G4	G5
	(*n* = 10)	(*n* = 18)	(*n* = 5)	(*n* = 4)	(*n* = 4)
Age (years)	26–48	17–73	57–67	59–71	43–71
Men	9 (90%)	4 (100%)	1 (50%)	2 (50%)	9 (50%)
eGFR (ml/min/1.73 m^2)		64.9–80.2	45.7–53.5	17.8–24.2	1.7–11.6
Diabetic nephropathy		0	1 (14%)	0	9 (50%)

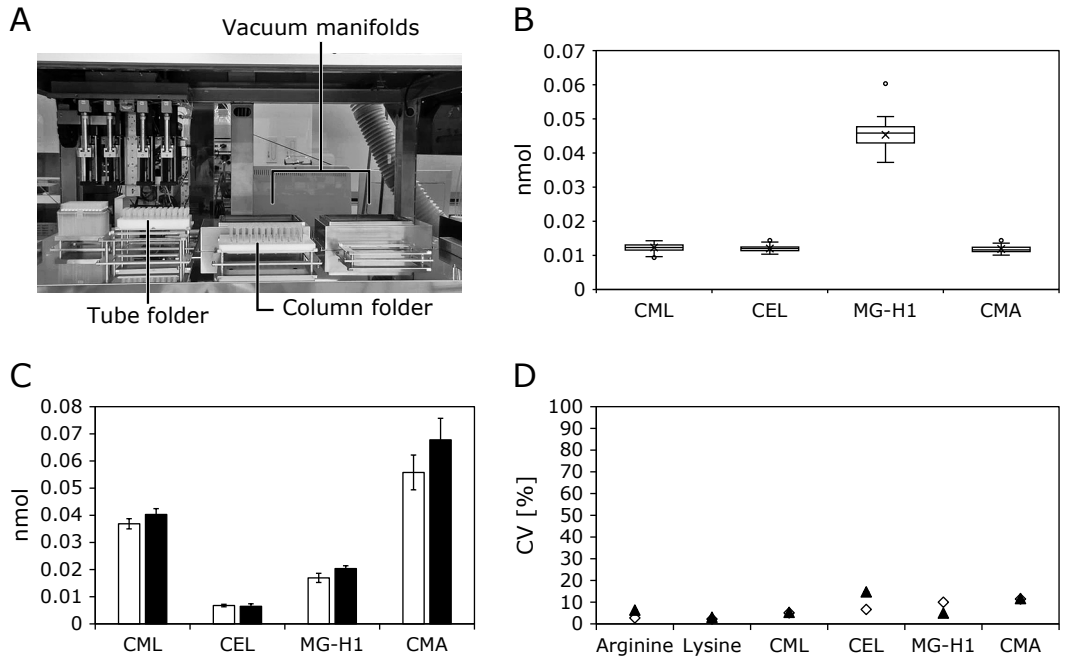

Fig. 2. Overview of fully automated solid phase extraction system (FSPES). Internal structure of FSPES (A). Evaluation of dependency in 48 locations for FSPES. AGEs measured by LC-MS-MS. 0.001 nmol CML, CEL, and CMA were added, and 0.05 nmol MG-H1 was added (B). Accuracy comparison of cation exchange between FSPES and manual method in CS. Open bars (*n* = 4), FSPES. Closed bar (*n* = 4), manual method. Mean ± SD (C) (*n* = 4). CV scores between FSPES and manual method. Open diamond (*n* = 4), FSPES. Closed triangle (*n* = 4), manual method (D).

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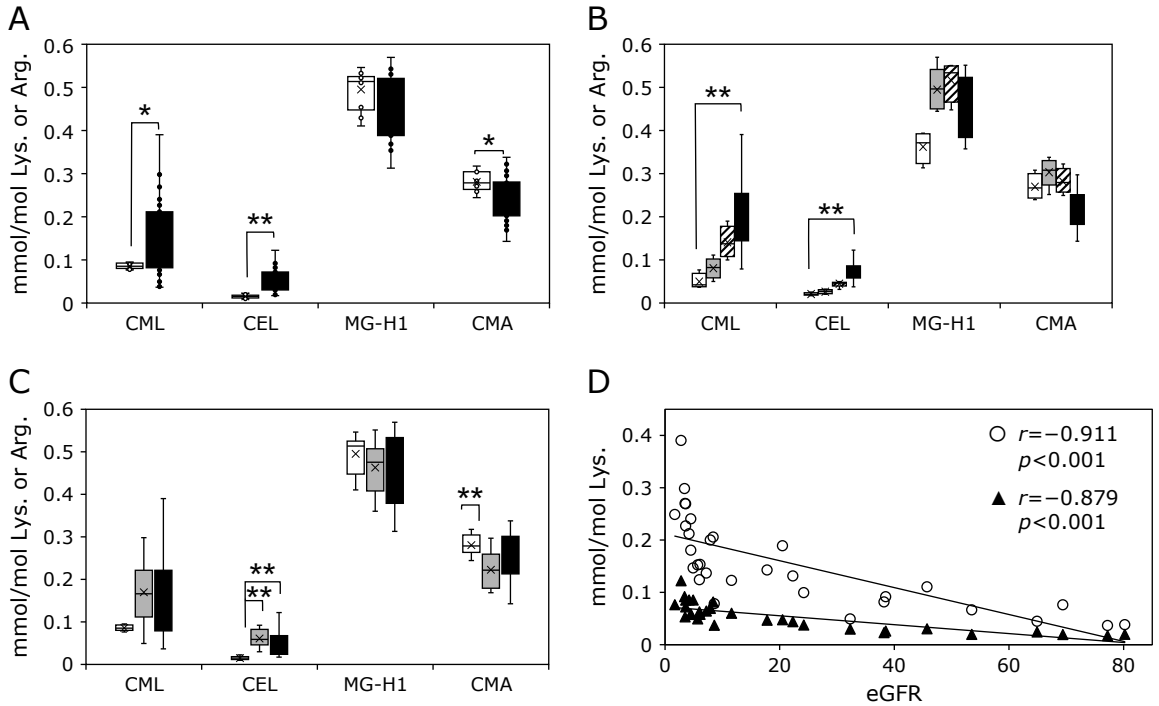

Fig. 3. Evaluation of AGEs content in human serum. Cation exchanges of sample performed using FSPES. AGEs measured by LC-MS-MS. Comparison of AGEs content between healthy subjects (open bars, $n = 10$) and nephropathy patients (closed bars, $n = 31$). *p<0.05, **p<0.01 (A). AGEs content with eGFR stage. Open bars ($n = 4$), G2 eGFR (60–89 ml/min/1.73 m^2). Dotted bars ($n = 5$), G3 eGFR (30–59 ml/min/1.73 m^2). Diagonal striped bars ($n = 4$), G4 eGFR (15–29 ml/min/1.73 m^2). Closed bars ($n = 18$), G5 eGFR (<15 ml/min/1.73 m^2). **p<0.01 (B). Comparison of AGEs content between healthy subjects (open bars, $n = 10$) and diabetic nephropathy patients (dotted bars, $n = 10$) and other nephropathy patients (closed bars, $n = 21$), **p<0.01 (C). Correlation between eGFR and serum CML or serum CEL content. Open circle ($n = 31$), CML content ($r = –0.911$, p<0.001). Closed triangle ($n = 31$), CEL content ($r = –0.879$, $p \leq 0.001$) (D).

Results

Optimization of vacuum pressure. AGEs structures such as CEL, CMA, CML, and MG-H1 as shown in Fig. 1D were measured by LC-MS/MS. If the vacuum pressure of the column operation is high, then the processing speed is high. However, owing to the high vacuum pressure, AGEs may pass through the resin of the column without being adsorbed. Therefore, the appropriate vacuum pressure was verified by using only the suction part of the FSPES. Since it is possible to process from −20 to −80 kPa in FSPES, vacuuming was performed using −20, −40, −60, and −80 kPa. After the calf serum was pretreated, it was applied to a cation exchange column with FSPES, and AGEs were measured using LC-MS-MS. The amount of bound AGE in the column did not change even when the vacuum pressure was changed (Fig. 1E). The coefficient of variation (CV) of each AGE content demonstrated that the variation did not increase even when the vacuum pressure was increased (Fig. 1F).

Measurement of variation by position in FSPES. As shown in Fig. 2A, FSPES consists of a tube folder, vacuum manifolds, and a column folder, designed to process up to 48 samples simultaneously. We verified whether processing could be performed uniformly at 48 locations without the location having effects. The standard AGEs were subjected to a cation exchange column with FSPES and quantified using LC-MS-MS. As shown in Fig. 2B, 0.01 nmol of CML, CEL and CMA and 0.05 nmol of MG-H1 were measured in 48 locations, and the SD of CML, CEL, MG-H1, and CMA were 0.001, 0.001, 0.004 and 0.001, respectively.

Next, the same pretreatment was performed using calf serum. Since a large amount of serum was expected to use for development of the extraction system, commercially available calf serum was chosen to demonstrate the CV values. The cation exchange column was conducted using the conventional method and FSPES, and the AGE contents were compared by LC-MS-MS. In the conventional method, the vacuum pressure of the suction part was −20 kPa, whereas FSPES was run at −60 kPa. No significant difference was observed between the conventional method and FSPES (Fig. 2C), and the CV values were almost the same (Fig. 2D).

Measurement of clinical samples by FSPES. Using FSPES, we attempted to quantify the AGEs in multiple clinical samples. Because an increase in AGEs in patients with kidney failure has been reported, we analyzed sera from patients with nephropathy. The sera of healthy subjects and nephropathy patients were reduced, acid-hydrolyzed, treated with FSPES as described in the Methods section, and measured by LC-MS-MS. As shown in Fig. 3A, the sera of patients with nephropathy were significantly increased in CML and CEL compared with healthy subjects.

We divided the nephropathy patients according to their eGFR stage (G2 to G5), which is an important indicator of renal function. We observed a significant increase in CML and CEL between G2 and G5 stage (Fig. 3B). Patients with nephropathy were divided into diabetic nephropathy and non-diabetic nephropathy groups and compared with healthy subjects. The results showed that CEL was significantly increased by nephropathy, whereas no significant difference was observed with or without diabetes (p<0.05) (Fig. 3C). In contrast, CMA was significantly decreased by diabetic nephropathy whereas no significant difference was observed without diabetes (p<0.05) (Fig. 3C). We also examined the correlation between eGFR and CML or CEL. As shown in Fig. 3D, eGFR was significantly anticorrelated with CML ($r = −0.911$, p<0.001) and CEL ($r = −0.879$, p<0.001).

Discussion

Worldwide, the number of patients with kidney failure is increasing and various kinds of lifestyle-related diseases have a negative impact on kidney function. In particular, diabetes mellitus is a leading cause of end-stage kidney disease. In Japan, the number of patients receiving dialysis treatment was approximately 340,000 in 2019.[18] Research has identified several causes of renal disorders, such as lifestyle-related diseases and chronic glomerular nephropathy. Diabetic nephropathy, a lifestyle-related disease, is a major cause of nephropathy onset and diabetic nephropathy usually develops over 10 years of diabetic history. The onset of complications has individual differences; for example, some patients with diabetes develop nephropathy after less than a few years whereas others do not even after 10 years.

Given the lack of subjective symptoms in the early stages of nephropathy, it is often discovered after renal damage has progressed to an irreversible state. An increase in the number of patients leads to an increase in medical expenses for dialysis. Therefore, the early detection of nephropathy is important because it is possible to stall the progression and onset of nephropathy by taking preventive measures and countermeasures. Prevention can lead to a healthy life and reduce medical expenses.

The FSPES was developed for multiple clinical analyses. Based on the stable CV values even at high vacuum pressure, we showed that stable processing can be performed even if the vacuuming pressure is increased. The fluctuation of the CV value was small at 48 locations of the FSPES, indicating that analysis could be performed at any location. Regarding the effect of the cation exchange column between the conventional method and FSPES, although the method of the aspiration part was different, no significant difference was found in the quantitative results of AGEs in the protein, demonstrating that FSPES can be used in the same way as the conventional method.

Our study confirmed that processing multiple samples, which is difficult when using the conventional method, can be completed more easily with FSPES. Indeed, when we used FSPES to investigate changes in AGEs in biological samples, we found that CML, which is an oxidation marker produced from lysine residues, and CEL, which increases in dyslipidemia, showed a significant difference between the healthy subjects and patients with nephropathy. Based on the increased CML and CEL with the onset of nephropathy, oxidation and abnormality of lipid metabolism may be involved in the pathogenesis of kidney failure.

Our evaluation of eGFR stages showed a significant increase in G5 stage for CML and CEL compared with G2 stage, and these AGEs were highly anticorrelated with eGFR. Compared with healthy subjects, patients with diabetic nephropathy and non-diabetic nephropathy recorded a significant increase in CEL. However, no significant differences were found between patients with diabetic nephropathy and non-diabetic nephropathy. These results demonstrated the potential relation between severe renal damage and CML and CEL. CMA was significantly decreased with diabetic nephropathy. We previously observed that CMA level was decreased in rat lenses with type 1 diabetes (unpublished observation). Thus, CMA may have different properties from other AGEs such as CML and CEL.

Although large-scale clinical trial to investigate the relationship between AGEs and diseases was difficult, the newly developed FSPES can solve the difficult pretreatment process for measurement of AGEs, and helps in the analysis of many clinical samples. Future research may thus clarify the relation between various AGEs and pathological conditions more precisely.

Author Contributions

SK, HS, KI, and KT: acquisition, analysis and interpretation of data. RN and HN: study concept and design and interpretation of data.

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Abbreviations

AGEs	advanced glycation end products
CEL	N^{ε}-(carboxyethyl)lysine
CMA	N^{ω}-carboxymethyl)arginine
CML	N^{ε}-(carboxymethyl)lysine
CV	coefficient of variation
FSPES	solid-phase extraction system
LC-ESI-QTOF	liquid chromatography electrospray ionization-quadrupole time of flight
MG-H1	N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine

Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1 Nagai R, Ikeda K, Higashi T, *et al*. Hydroxyl radical mediates N^{ε}-(carboxymethyl)lysine formation from Amadori product. *Biochem Biophys Res Commun* 1997; **234**: 167–172.
2 Nagai R, Unno Y, Hayashi MC, *et al*. Peroxynitrite induces formation of N^{ε}-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. *Diabetes* 2002; **51**: 2833–2839.
3 Berner AK, Brouwers O, Pringle R, *et al*. Protection against methylglyoxal-derived AGEs by regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology. *Diabetologia* 2012; **55**: 845–854.
4 Kinoshita S, Mera K, Ichikawa H, *et al*. N^{ω}-(carboxymethyl)arginine is one of the dominant advanced glycation end products in glycated collagens and mouse tissues. *Oxid Med Cell Longev* 2019; **2019**: 9073451.
5 Duff M, Demidova O, Blackburn S, Shubrook J. Cutaneous manifestations of diabetes mellitus. *Clin Diabetes* 2015; **33**: 40–48.
6 Morcos M, Sayed AA, Bierhaus A, *et al*. Activation of tubular epithelial cells in diabetic nephropathy. *Diabetes* 2002; **51**: 3532–3244.
7 Iwase M, Komorita Y, Ohkuma T, *et al*. Incidence of stroke and its association with glycemic control and lifestyle in Japanese patients with type 2 diabetes mellitus: The Fukuoka diabetes registry. *Diabetes Res Clin Pract* 2021; **172**: 108518.
8 Nangaku M, Miyata T, Sada T, *et al*. Anti-hypertensive agents inhibit *in vivo* the formation of advanced glycation end products and improve renal damage in a type 2 diabetic nephropathy rat model. *J Am Soc Nephrol* 2003; **14**: 1212–1222.
9 Yamanaka M, Matsumura T, Ohno R, *et al*. Non-invasive measurement of skin autofluorescence to evaluate diabetic complications. *J Clin Biochem Nutr* 2016; **58**: 135–140.
10 Miki Hayashi C, Nagai R, Miyazaki K, *et al*. Conversion of Amadori products of the maillard reaction to N^{ε}-(carboxymethyl)lysine by short-term heating: possible detection of artifacts by immunohistochemistry. *Lab Invest* 2002; **82**: 795–808.

doi: 10.3164/jcbn.21-175

11 Nakano M, Kubota M, Owada S, Nagai R. The pentosidine concentration in human blood specimens is affected by heating. *Amino Acids* 2013; **44**: 1451–1456.
12 Nagai R, Ikeda K, Kawasaki Y, *et al*. Conversion of Amadori product of Maillard reaction to N^{ε}-(carboxymethyl)lysine in alkaline condition. *FEBS Lett* 1998; **425**: 355–360.
13 Shirakawa J, Arakawa S, Tagawa T, *et al*. *Salacia chinensis* L. extract ameliorates abnormal glucose metabolism and improves the bone strength and accumulation of AGEs in type 1 diabetic rats. *Food Funct* 2016; **7**: 2508–2515.
14 Yamaguchi H, Nagai M, Sugawa H, Yasuda H, Nagai R. Development of a conventional immunochemical detection system for determination of N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine in methylglyoxal-modified proteins. *Glycoconj J* 2021; **38**: 293–301.
15 Ohno R, Ichimaru K, Tanak S, *et al*. Glucoselysine is derived from fructose and accumulates in the eye lens of diabetic rats. *J Biol Chem* 2019; **294**: 17326–17338.
16 Sugawa H, Yachi A, Fujimoto Y, Nagai R. Accumulation of N^{ε}-(carboxyethyl) lysine in *Caenorhabditis elegans* is correlated with the formation of ketone body. *J Biochem* 2021; **170**: 587–592.
17 Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013; **48**: 452–458.
18 Nitta K, Masakane I, Hanahusa N, *et al*. 2019 Annual dialysis data report, JSDT renal data registry. *J Jpn Soc Dial Ther* 2020; **53**: 579–632.