Biochimica et Biophysica Acta 1840 (2014) 818–829

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Detection of oxidized and glycated proteins in clinical samples using mass spectrometry — A user's perspective☆

Paul J. Thornalley [a,b,*], Naila Rabbani [a,b]

[a] Clinical Sciences Research Laboratories, Warwick Medical School, University of Warwick, University Hospital, Coventry CV2 2DX, UK
[b] Warwick Systems Biology Centre, Coventry House, University of Warwick, Coventry CV4 7AL, UK

ARTICLE INFO

Article history:
Received 23 February 2013
Received in revised form 17 March 2013
Accepted 24 March 2013
Available online 2 April 2013

Keywords:
Protein glycation
Methylglyoxal
Protein oxidation
Mass spectrometry
Dicarbonyl stress
Oxidative stress

ABSTRACT

Background: Proteins in human tissues and body fluids continually undergo spontaneous oxidation and glycation reactions forming low levels of oxidation and glycation adduct residues. Proteolysis of oxidised and glycated proteins releases oxidised and glycated amino acids which, if they cannot be repaired, are excreted in urine.
Scope of Review: In this review we give a brief background to the classification, formation and processing of oxidised and glycated proteins in the clinical setting. We then describe the application of stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS) for measurement of oxidative and glycation damage to proteins in clinical studies, sources of error in pre-analytic processing, corroboration with other techniques – including how this may be improved – and a systems approach to protein damage analysis for improved surety of analyte estimations.
Major conclusions: Stable isotopic dilution analysis LC-MS/MS provides a robust reference method for measurement of protein oxidation and glycation adducts. Optimised pre-analytic processing of samples and LC-MS/MS analysis procedures are required to achieve this.
General significance: Quantitative measurement of protein oxidation and glycation adducts provides information on level of exposure to potentially damaging protein modifications, protein inactivation in ageing and disease, metabolic control, protein turnover, renal function and other aspects of body function. Reliable and clinically assessable analysis is required for translation of measurement to clinical diagnostic use. Stable isotopic dilution analysis LC-MS/MS provides a "gold standard" approach and reference methodology to which other higher throughput methods such as immunoassay and indirect methods are preferably corroborated by researchers and those commercialising diagnostic kits and reagents. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

Proteins in human tissues and body fluids continually suffer spontaneous modifications by reactions with oxidants, saccharides and saccharide derivatives. This forms oxidised and glycated amino acid residues within proteins [1–3].

1.1. Classification of glycated and oxidised proteins

Glycation adducts are classified into two groups: early glycation adducts – Schiff's base and Amadori products or fructosamine residues formed at early stages of glycation processes – also called the Maillard reaction; and advanced glycation endproducts (AGEs) — other glycation adducts initially considered to be formed in later or advanced stages of the Maillard reaction but are rather formed in both early and advanced stages [4]. Major glycation adducts in physiological systems, N_{ε}-fructosyl-lysine (FL) residues and methylglyoxal derived hydroimidazolone N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (MG-H1), are formed by non-oxidative processes [5,6]. Some AGEs are formed by oxidative

Abbreviations: AASA, aminoadipic semialdehyde; AGE, advanced glycation endproduct; AOPP, advanced oxidation protein product; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; BUL, bilateral ureteral ligation; BNX, bilateral nephrectomy; CEL, Nε-(1-carboxyethyl)lysine; CML, N_{ε}-carboxymethyl-lysine; CRF, chronic renal failure; 3DG-H, 3DG-H1 (N_{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine) and related structural isomers 3DG-H2 and 3DG-H3 see [1]; ESI, electrospray ionisation; FCR, fractional clearance rate; FL, N_{ε}-fructosyl-lysine; GSA, glutamic semialdehyde; FN3K, fructosamine-3-kinase; FN3KRP, fructosamine-3-kinase related protein; G-H1, N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine; GOLD, glyoxal-derived lysine dimer, 1,3-di(N^{ε}-lysino)imidazolium salt; HD, hemodialysis; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDL, low density lipoprotein; MALDI, matrix assisted laser desorption ionisation; MetSO, methionine sulfoxide; MG, methylglyoxal; MG-H1, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N^{ε}-lysino)-4-methyl-imidazolium salt; MRM, multiple reaction monitoring; MSR, methionine sulfoxide reductase; NFK, N-formylkynurenine; 3-NT, 3-nitrotyrosine; PD, peritoneal dialysis; SAF, skin autofluorescence; TFA, trifluoroacetic acid; UPLC, ultra high performance liquid chromatography

☆ This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

* Corresponding author at: Clinical Sciences Research Laboratories, Warwick Medical School, University of Warwick, University Hospital, Coventry CV2 2DX, UK.
E-mail address: P.J.Thornalley@warwick.ac.uk (P.J. Thornalley).

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.03.025

processes and are called glycoxidation products [4]. Examples are N_{ε}-carboxymethyl-lysine (CML) [7] and pentosidine [8].

A classification in protein oxidation research that has been used is the term "advanced oxidation protein products" (AOPPs). AOPP measurement is an indirect measure of protein oxidation: a measure of the ability of oxidised proteins to oxidise iodide to iodine, with measurement of iodide formation by quantitation of the tri-iodide ion I_3^- by spectrophotometric measurement at 340 nm [9–11]. This is a mechanism of formation related definition where the molecular nature of AOPPs remains unclear. This is thought to be related to N-chloramines derivatives formed by oxidation of protein with hypochlorite generated by myeloperoxidase [12]. Dityrosine and pentosidine have bene claimed to contribute to the AOPP response but independent measurements show that their low levels in plasma cannot account for the absorbance at 340 nm found in the AOPP measurement [13]. The molecular species contributing to the AOPP response are most readily formed by the hydrogen peroxide/myeloperoxidase/ chloride which forms hypochlorite [12]. A recent study has claimed that AOPP activity in plasma is due to oxidised fibrinogen on the basis of AOPP activity correlating to fibrinogen concentration but confirmation of molecular nature of the oxidant is required [14]. Molecular characterisation of AOPP(s) of fibrinogen and possibly other proteins remain to be disclosed.

Oxidation and glycation of proteins form oxidation and glycation adducts, respectively, which are part of the peptide backbone of the protein and are appropriately called oxidation and glycation adduct residues. Measurement of these is often normalised to the amount of related unmodified amino acid in the protein sample. For example, estimates of methionine sulfoxide (MetSO) residues are often reported as mmol/mol methionine and estimates of FL residues as mmol/mol lysine [1]. In some clinical research areas – particularly studies of renal failure – oxidation and glycation adduct residues have been called "protein-bound" adducts. This nomenclature is not strictly correct as part of the oxidation and glycation adduct is a residue of the protein precursor. Nomenclature and collective terms commonly used in protein oxidation and glycation research are given in Table 1. Important oxidised and glycated amino acid residues are given in Fig. 1.

1.2. Endogenous repair of oxidised and glycated proteins

It was initially thought that protein oxidation and glycation gives rise to adducts that remain in organs and body fluids throughout life and accumulate therein. This is only correct for oxidation and glycation adducts that are chemically stable, not repaired *in situ* and formed on lived-lived proteins. Examples are dityrosine and CML residues on articular cartilage in good skeletal health [19]. For most oxidised and glycated proteins, however, this is not the case. Oxidation and glycation adducts are repaired *in situ* or the oxidised and glycated proteins are degraded and replaced by *de novo* protein synthesis. MetSO residues are repaired by reduction to methionine residues by methionine sulfoxide reductases (MSRs) [20] and FL residues are repaired to lysine residues by fructosamine-3-phosphokinase [21]. This is of current clinical importance and impact as the universally used marker of glycemic control, glycated hemoglobin HbA_{1c}, is hemoglobin modified mainly on β-val-1 and α-lys-66 with 1-deoxyfructosyl residues [22]. The extent of glycation of hemoglobin is a balance between rate of glycation by glucose exposure in the 6–8 weeks prior to blood sampling and the rate of de-glycation by fructosamine-3-phosphokinase [23]; and also influenced by red blood cell turnover [24]. Where there is slow dynamic reversibility of adduct formation – such as for MG-H1 formation from methylglyoxal [25] – sustained decrease of the modifying agent – as may likely be achieved by induction of expression of glyoxalase 1 which metabolises methylglyoxal [26] – will provide for *in situ* repair of the modified amino acid residue. Examples of repair of oxidation and glycation adducts are given in Table 2.

1.3. Turnover of oxidised and glycation proteins and measurement of protein oxidation and glycation in the steady-state

Oxidised and glycated proteins that are not repaired are usually degraded and replaced. Measurement of oxidised and glycated proteins, therefore, is an estimate of the steady-state level dependent on the rate of formation, rate of repair or further modification and rate of turnover or clearance of the protein substrate. Proteolytic degradation of oxidised and glycated proteins by cellular proteolysis gives rise to release and excretion of oxidised and glycated amino acids, also called oxidation and glycation free adducts [1]. Oxidised and glycated proteins are often predicted to have distorted or damaged structures [6,29,30] and therefore may be targeted for proteolysis by the proteasome [31–33]. Lysosomal proteolysis is also important for degradation of long-lived cellular proteins, endocytosed extracellular proteins [34] and chaperone-mediated autophagy of cellular proteins [35]. Release of glycation and oxidation free adduct from cells and tissues [1] and decrease of oxidised and glycated proteins with increased cellular 20S proteasome activity [31,32] is consistent with targeting of proteins damaged in this way for proteasomal degradation. Oxidation and glycation free adducts are also formed by direct oxidation and glycation of amino acids and are also absorbed from the gastrointestinal tract from digested damaged proteins in ingested foods [16]. They are the major form by which oxidation and glycation adducts are excreted from the body [1,36,37]. A scheme of multi-compartment formation and gastrointestinal tract absorption of oxidized and glycated proteins and their proteolytic processing in the body and renal excretion of free adducts is given in Fig. 2.

Estimation of protein oxidation and glycation adduct content of proteins in the steady-state suggests that the extent of modification is influenced by both changes in rate of oxidation and glycation and also rates of protein turnover. In the case of plasma protein, the level of

Table 1
Classification terms of protein oxidation and glycation research.

Term	Definition	Comment	Reference
Oxidation/glycation adduct residues	Product of oxidation/glycation of precursor amino acid residues in a protein or peptide	Also termed "protein bound" oxidation/glycation adducts	[1,4,15]
Oxidation/glycation free adducts	Oxidised/glycated amino acids formed by release of oxidation/ glycation adduct residues from proteins and direct oxidation and glycation of amino acids.	Also absorbed from food and are the major form of oxidation/ glycation free adduct excretion in urine.	[1,4,15]
Oxidised/glycated peptides	Peptides (molecular mass <10 kDa)[a] containing oxidation/ glycation adduct residue(s)	Initially mistaken for oxidation/ glycation free adducts	[16,17]
Early glycation adducts	Schiff's base and Amadori products formed in early stages of the Maillard reaction		[4]
Advanced glycation endproducts (AGEs)	Non-early glycation adducts formed in early and late, advanced stages of the Maillard reaction.	Initially defined as "brown fluorescent pigments which crosslink proteins"	[4,18]
Advanced oxidation protein products (AOPPs)	Measure of ability of protein oxidation products to oxidise iodide to iodine – thought to be activity of N-chloramine derivatives	Molecular components of AOPPs are unknown.	[11,12]

[a] Molecular mass cut-off is arbitrary.

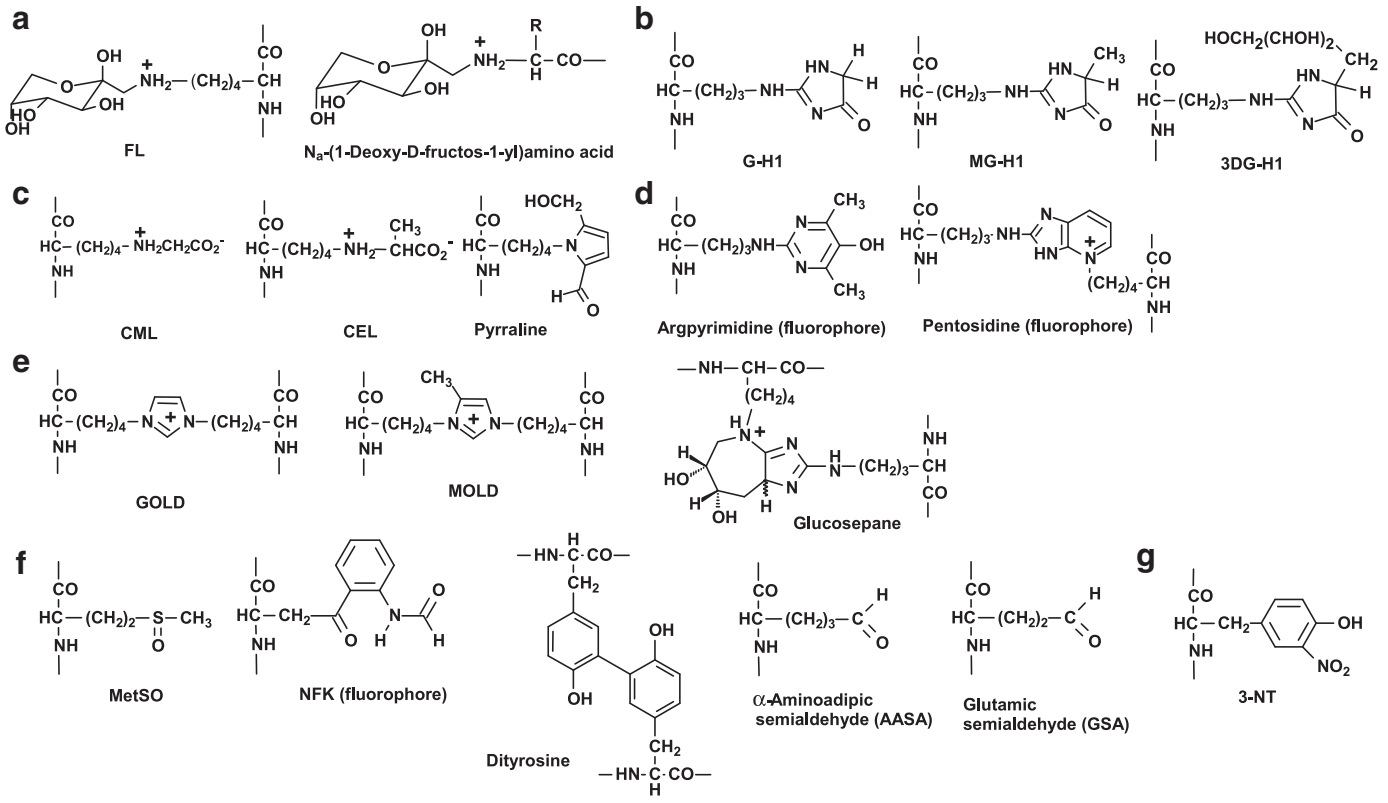


Fig. 1. Protein glycation, oxidation and nitration adduct residues in physiological systems. a. Early glycation adducts — FL and N_{α}-(1-deoxy-D-fructos-1yl)amino acid residues. Advanced glycation endproducts: b. Hydroimidazolones, c. Monolysyl d. Fluorophores, AGEs, e. Non-fluorescent crosslinks, f. Oxidation adducts, and g. Nitration adduct. Protein glycation, oxidation and nitration adduct residues are shown. For the corresponding free adducts at physiological pH, the N-terminal amino group is protonated $-NH_3^+$ and the C-terminal carbonyl is a carboxylate $-CO_2^-$ moiety.

oxidised and glycated proteins is influenced by the rates of oxidation and glycation of protein in the plasma compartment and also by the rate of clearance of plasma proteins or plasma fractional clearance rate (FCR). Examples of effects of change in FCR of plasma protein are increased plasma protein glycation and oxidation adduct residues in: (i) diabetic patients with microalbuminuria (a "leaky" glomerular filter) where tightening of the glomerular filter by treatment with an angiotensin blocker decreased clearance of FL-modified albumin and increased plasma protein FL residue content [38]; and (ii) liver cirrhosis where decreased albumin concentration slowed albumin catabolism, extending half-life and increase glycation and oxidation damage markers [16]. Increased extravasation of plasma protein post-surgery may also decrease plasma protein oxidation and glycation adduct residue contents [37]. Albumin glycated to minimal extent, as found *in vivo* [16,39], has a half-life similar to unglycated albumin and is metabolized in the kidney and elsewhere [40]. Albumin glycated highly by glucose *in vitro* (containing, for example, *ca.* 40 molar equivalents of glycation adducts) is not a good model of glycated albumin *in vivo* and has a very short plasma half-life with clearance from circulation in the liver [41] which is not the fate of glycated albumin *in vivo* [16]. Similarly highly oxidised and glycated low density lipoprotein (LDL) are not appropriate models of the minimal oxidised and glycated LDL found in the circulation *in vivo* [42]. For example, LDL highly glycated by methylglyoxal is severely structurally impaired and rapidly cleared from circulation by scavenger receptors [43] whereas LDL modified minimally by methylglyoxal as *in vivo* has an unveiled proteoglycan binding site and exhibits increased affinity for the arterial wall with no significant change in plasma half-life [44]. Extent of protein glycation and oxidation is, therefore, a critical influence in protein function and most proteins tend to be minimally oxidised or glycated *in vivo*.

The extent of modification of proteins *in vivo* by glycation and oxidation was assessed by measuring protein glycation and oxidation of purified proteins — such as for haemoglobin and apolipoprotein B100 of low density lipoprotein [13,44] and by measuring mass

Table 2
Processes of protein oxidation and glycation adduct repair.

Adduct	Repair enzyme or process	Comment	Reference
MetSO	Methionine sulfoxide reductase (MSR)	Requires thioredoxin co-factor.	[20]
FL and other Amadori products of hexoses and riboses	Enzymatic: Fructosamine-3-kinase (FN3K) and fructosamine-3-kinase related protein (FN3KRP). Non-enzymatic: reversal of Amadori rearrangement with carbon-2 epimeric mixture.	ATP co-factor. Initial product is the pyranose of furanose 3-phosphate which fragments to lysine residue, phosphate and related 3-deoxyhexosone or 3-deoxypentosone.	[21,27]
Hydroimidazolones MG-H1, G-H1, 3DG-H1 and structural isomers	Slow, dynamic reversal of the glycation process.	Half-lives at pH 7.4 and 37 °C: MG-H1, 12 days; G-H1, 69 days; 3DG-H1, 17 days.	[1,25]
3-Nitrotyrosine	Nitratase activity with conversion to tyrosine with or without 3-aminotyrosine.		Reviewed in [28].

*Molecular mass cut-off is arbitrary.

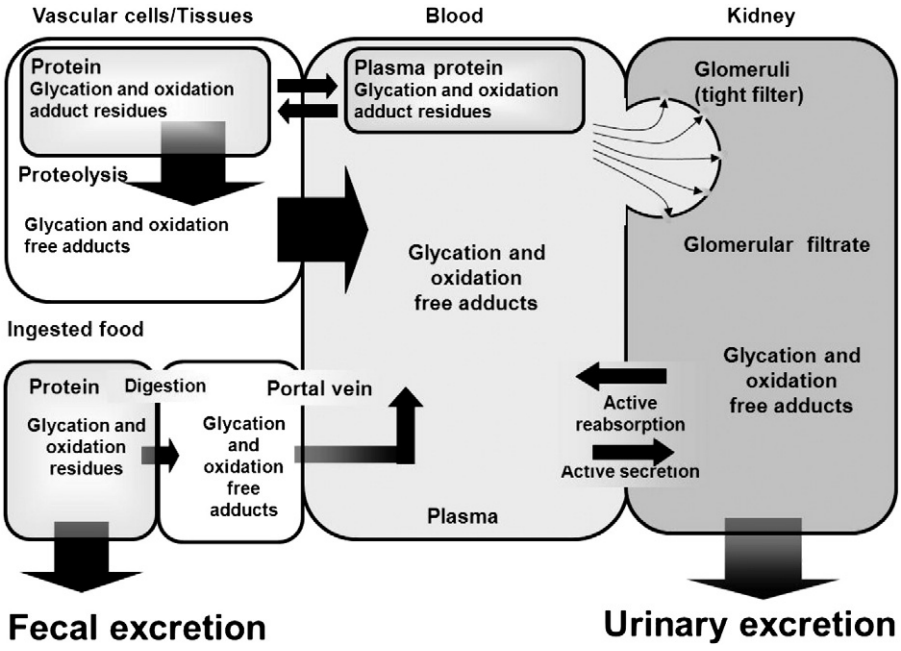


Fig. 2. Multi-compartment model of formation, proteolytic processing and excretion of proteins damaged by glycation and oxidation and nitration.

increment on molecular mass for serum albumin [16,39]. Application of quantitative high resolution mass spectrometry to studies of protein oxidation and glycation will facilitate measurement of extent of protein oxidation and glycation in proteome-wide analysis in the future. Internal standardization by stable isotope labelled proteins will be required for robust quantitation [45].

Where the rate of modification of a protein is constant, the extent of protein modification is expected to be proportional to mean plasma residence of the protein. This was found to be the case for oxidation of apolipoprotein B100 of LDL [46]. Trace modification of proteins by oxidation and glycation adduct residues may, therefore, provide surrogate markers of plasma proteomics dynamics.

1.4. Renal clearance and urinary excretion of protein oxidation and glycation free adducts

Oxidation and glycation free adducts in plasma are filtered through renal glomeruli and may be reabsorbed by organic anion and cation transporters in the renal tubular system. There may also be some active secretion from the renal circulation, The plasma concentrations of oxidation and glycation free adducts are, therefore, sensitive to renal clearance and accumulate markedly in experimental total loss of clearance – as in bilateral nephrectomy (BNX), and also in experimental tubular obstruction – as in bilateral ureteral ligation (BUL) [37]. A lower plasma increase of oxidation and glycation free adducts in plasma in BUL than in BNX provides evidence of the marked extent to which the kidney removes these damaged amino acids from the circulation. Protein oxidation and glycation free adducts accumulate in plasma clinically in chronic renal failure prior to the need for dialysis treatment where the accumulation is due to loss of renal clearance rather than to increase in flux of adduct formation. In stage 5 chronic kidney disease with requirement for renal replacement therapy by hemodialysis (HD) or peritoneal dialysis (PD), the increase in plasma concentration of oxidation and glycation free adducts before a dialysis session is profound – up to 50-fold – and decreases evidently during dialysis due to the clearance provided by ultrafiltration through the dialysis membrane in HD and peritoneal membrane in PD. In contrast, the change in plasma protein adduct residues is relatively modest proportionately. This reflects the major role of the kidney in clearance of oxidation and glycation free adducts and the kidney as a relatively minor metabolic fate of albumin [36]. Estimates of oxidation and glycation free adduct levels in plasma or other body fluids are reported per unit volume in concentration units (nM or nmol/L). Characteristic renal clearances of oxidation and glycation free adducts in healthy human subjects are given in Table 3. The low renal clearance of MetSO reflects metabolism to methionine by renal MSRs and the high clearance of MetSO in PD patients reflects the escape of MetSO from renal repair by ultrafiltration into the peritoneal cavity.

Urinary excretion of oxidation and glycation free adducts is an estimate of total body oxidative and glycation damage — with the qualifiers of effects of oxidation and glycation adduct repair leading to underestimates and contributions of oxidation and glycation adducts absorbed form the gastrointestinal tract from digested damaged proteins in ingested foods leading to overestimates. Estimates of oxidation and glycation free adducts in urine are reported amount or per mg creatinine for time point or "spot" urine collection or amount per 24 h for timed urine collections (typically nmol per mg creatinine or nmol/24 h). Characteristic urinary excretion rates of oxidation and glycation free adducts in healthy human subjects are given in Table 4.

Protein oxidation and glycation in human physiology, therefore, is a multi-compartmentalised dynamic process where levels of oxidised

Table 3
Clinical renal clearance of oxidation and glycation free adducts in chronic renal failure, peritoneal dialysis and healthy subjects.

Subject group	Clearance (ml/min)		
	Control (n = 8)	CRF (n = 7)	PD (n = 8)
FL	64.5 ± 6.0	10.6 ± 4.4***	66.7 (37.8–222.2)
CML	73.5 ± 6.3	28.0 ± 3.5***	42.3 (14.0–75.4)**
CEL	84.6 ± 10.9	25.4 ± 4.7***	9.2 (5.6–45.0)***
G-H1	48.0 ± 6.1	14.9 ± 3.3***	15.0 ± 1.7**
MG-H1	41.2 (22–121)	9.8 (7–38)**	16.8 ± 3.8**
3DG-H	38.1 (25–92)	26.2 ± 6.7*	15.6 (3.9–33.5)**
Pentosidine	18.7 ± 2.6	22.1 ± 3.1	13.1 (4.4–26.9)
MetSO	2.4 ± 0.3	1.9 ± 0.2	41.7 ± 5.5***

Data are mean ± SD or median or [minimum–maximum]. Significance: *, ** and *** indicate $P < 0.05$, $P < 0.01$and $P < 0.001$, respectively, with respect to the normal control subjects. Data from [36].

Table 4
Clinical urinary excretion of oxidation and glycation free adducts in chronic renal failure, peritoneal dialysis and healthy subjects.

Subject group	24 h Urinary excretion (nmol/mg creatinine)	
	Control (n = 12)	Type 1 diabetes (n = 21)
FL	4.3 ± 2.6	22.3 ± 7.9***
CML	1.19 ± 0.53	2.56 ± 1.37***
CEL	2.64 ± 1.10	3.26 ± 1.80
G-H1	1.54 ± 0.59	1.22 ± 0.39
MG-H1	2.3 ± 1.2	34.8 ± 24.9***
3DG-H	4.40 ± 1.82	8.33 ± 1.52***
Argpyrimidine	0.70 ± 0.40	0.80 [0.16–5.18]
MOLD	0.027 ± 0.011	0.040 ± 0.014**
Pentosidine	0.022 ± 0.014	0.118 [0.033–0.654]***
MetSO	0.018 ± 0.009	0.080 ± 0.035***
NFK	3.6 ± 1.7	20.3 ± 10.8***
3-NT	0.034 ± 0.014	0.036 ± 0.018
Dityrosine	0.186 ± 0.086	0.267 ± 0.066**

Data are mean ± SD or median or [minimum–maximum]. Significance: *, ** and *** indicate $P < 0.05$, $P < 0.01$and $P < 0.001$, respectively, with respect to the normal control subjects. Data from [13].

and glycated proteins and related analytes measured are influenced by the type of sample (protein, ultrafiltrate and compartment), modified protein half-life and the chemical half-life of the adduct residue. Experimental factors that influence estimates of protein oxidation and glycation analytes are stability of analytes in pre-analytical processing and storage, and specificity, sensitivity and reproducibility of the analytical method used.

2. Application of LC-MS/MS to detection and quantitation of protein oxidation and glycation

To characterise protein oxidation and glycation processes in physiological systems analytical methods applicable to the detection and quantitation of oxidised and glycated proteins and oxidised and glycated amino acids are required. In selecting an analytical technique to use for detection of analytes it is important that it has high sensitivity and specificity for protein oxidation and glycation adduct analytes. In selecting a technique for total or partial separation of analytes it is important that protein oxidation and glycation status remains unchanged during this pre-analytical processing. Polyacrylamide gel electrophoresis has been widely used to separate and detect proteins and applications have been developed to resolve and detect oxidised proteins and glycoxidation adduct residues of proteins [47,48]. Recent research has shown that protein oxidation occurs during electrophoresis and although this can be decreased by gel preparation with reagent solution gassing with nitrogen or argon, significant oxidation still occurs [49]. A faster method of separation technique with low pH to stabilise oxidation and glycation adducts is preferable.

The best available analytical method to quantify protein oxidation and glycation adducts is stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS). This provides high security of estimates on the basis of analytical method [1]. Nevertheless there are still issues involving appropriate use and pre-analytic processing that may lead to insecure estimation of protein oxidation and glycation adducts. For surety of estimates fully validated procedures are required.

Oxidised and glycated amino acids are detected and quantified by stable isotopic dilution analysis LC-MS/MS. Analytes and isotopic standards are ionised by positive electrospray ionisation (ESI) and detected by multiple reaction monitoring (MRM). There is a prior chromatographic step with ultra high performance liquid chromatography (UPLC) where analytes are resolved from the related unmodified amino acid or compound that may oxidise to them in the ESI source (for example, tyrosine is resolved form dityrosine) to avoid artifactual overestimation from in-source formation. Chromatography is performed on Hypercarb™ graphic columns: two columns (first column, 2 × 50 mm; second column, 250 mm). The columns are initially in series and later the second column is then switched out of the flow to elute strongly hydrophobic analytes. This allows for analysis of analytes of markedly different hydrophobicity. The mobile phase is initially 0.1% trifluoroacetic acid (TFA) and then contains a custom gradient of acetonitrile. The Hypercarb graphitic matrix is strongly hydrophobic such that it retains even the most hydrophilic amino acids without derivatisation. This permits the initial 0.8 ml min of eluate containing non-volatile salts to be diverted to waste. This excludes non-volatile sample components from the mass spectrometer ESI source and preserves mass spectrometric detection sensitivity. The down-side of this is that Hypercarb columns retain hydrophobic compounds very strongly and relatively severe washing solvent is required to regenerate the columns between each run. Experience has shown that 50% tetrahydrofuran with 0.1% TFA is a good decontamination solvent.

In MRM mode, each analyte is detected by selective ion monitoring of its molecular ion in the first mass analyser and major fragment ion in the second mass analyser after fragmentation with argon atoms in the collision cell. Each analyte, therefore, is detected by characteristic chromatographic retention time, molecular ion mass/charge ratio m/z and fragment ion m/z. This detector response is normalised to the added stable isotopic standard response which has the same retention time as the normal analyte and a different molecular ion or molecular ion and fragment ion m/z value. Data are collected sequentially and rapidly for many mass transitions (up to 32 transitions on some instruments which has increased to several hundred on latest models) such that multiple mass chromatograms may be recorded for each analyte and isotopic standard. Examples of MRM detection conditions are given in Table 5. Analyte content is determined from the analyte/internal standard peak response peak area ratio by reference to calibration curves recorded under the same conditions with the same content of internal standard as the samples and known amounts of normal reference standard analyte.

Oxidation and glycation free adduct concentrations are determined in physiological fluids by assay of oxidised and glycated amino acids in ultrafiltrate with added stable isotopic standards. Ultrafiltrates are prepared at 4 °C by microspin ultrafiltration with a 3 kDa cut-off filter for urine and a higher cut-off, 10 kDa cut-off filter for plasma, serum or other fluid. The higher cut-off ultrafilter for the latter samples increases rate of ultrafiltrate preparation with minor loss of small molecular mass protein content. In early studies with immunoassay of glycation adducts in peripheral venous plasma fractionated by gel filtration, the low molecular mass fraction contained AGE free adducts which the investigators incorrectly called “AGE peptides” [50]. We have examined plasma for the presence of AGE peptides by enzymatically digesting 10 kDa ultrafiltrate and analysed for glycated and oxidised amino acid content in excess of the free adducts. We found no evidence for AGE peptides or highly oxidised peptides in venous plasma but there was evidence of glycated and damaged peptides in portal venous plasma, presumably from absorption of peptides from digested food proteins [16]. Immunoassay of “AGE peptides”, therefore, probably detected AGE free adducts.For assay of oxidation and glycated adduct residues in proteins, protein substrates are hydrolyzed and the oxidation and glycation adducts are thereafter quantified in the hydrolysate. Enzymatic hydrolysis is used because acid hydrolysis leads to destruction of glycation analytes and poor analytical recoveries [1]. Exhaustive enzymatic hydrolysis is achieved by sequential addition of pepsin (substituted by collagenase when extracellular matrix proteins are analysed), pronase E and added together prolidase and aminopeptidase [51]. Good recovery of amino acids is achieved with proteins minimally modified by oxidation and glycation found *in vivo* [1,25,51]. This has been corroborated in other laboratories [52]. Enzymatic hydrolysis is inefficient for proteins modified highly by glycation adducts – such as in some experimental preparations of proteins highly modified by

Table 5
Mass spectrometric multiple reaction monitoring detection of protein oxidation and glycation adducts.

Analyte group	Analyte	Parent ion Da)	Fragment ion (Da)	CE (eV)	Neutral fragment loss(es)
Amino acids	Arg	175.2	70.3	15	H_2CO_2, $NH_2C(=NH)NH_2$
	Lys	147.1	84.3	15	H_2CO_2, NH_3
	Met	150.0	104.2	11	H_2CO_2
	Tyr	182.1	136.2	13	H_2CO_2
	Trp	205.0	159.1	15	H_2CO_2
Fructosamines	FL	291.0	84.3	31	H_2CO_2, fructosylamine
Hydroimidazolones[a]	G-H1	215.0	100.2	14	$NH_2CH(CO_2H)CH_2CH{=}CH_2$
	MG-H1	229.2	114.3	14	$NH_2CH(CO_2H)CH_2CH{=}CH_2$
	3DG-H	319.1	114.8	20	$NH_2CH(CO_2H)CH_2CH{=}CH_2$
Monolysyl AGEs	CEL	219.2	130.1	13	$NH_2CH(CH_3)CO_2H$
	CML	204.9	130.1	12	$NH_2CH_2CO_2H$
	Pyrraline	255.2	84.3	23	2-CHO-5-$HOCH_2$-pyrrole, H_2CO_2
Fluorescent AGEs	Argpyrimidine	255.3	140.3	17	$NH_2CH(CO_2H)CH_2CH{=}CH_2$
	Pentosidine	379.3	250.4	22	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$
Non-fluorescent crosslink AGEs	GOLD	327.1	198.3	21	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$
	MOLD	341.2	212.3	21	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$
	Glucosepane	429.2	382.1	38	C_2H_5O
Oxidation adducts	MetSO	166.1	102.2	14	CH_3-SOH
	Dityrosine	361.2	315.3	15	H_2CO_2
	NFK	235.8	191.2	18	H_2CO_2
	AASA	128.0	82.0	15	H_2CO_2
	GSA	114.0	68.0	15	H_2CO_2
Nitration adduct	3-NT	227.1	181.2	13	H_2CO_2

[a] For hydroimidazolones, structural isomer-1 is denoted — although for 3DG-H detection, structural isomers 3DG-H1, 3DG-H2 and 3DG-H3 are all detected. From [1].

AGEs and also some proteins in thermally processing food. This may be because high modification of arginine and lysine residues blocks cleavage sites for proteolysis and also because some glycation adducts inhibit proteases [51,53].

These procedures give acceptable analyte recoveries and reproducibility. Coefficients of variation are typically 6–10%, as expected for stable isotopic dilution analyse techniques of this type and complexity. The method was reported initially for FL and 12 AGEs, two oxidation markers and the nitration marker 3-nitrotyrosine (3-NT). Further analytes have been added since, including glucosepane and direct detection of "protein carbonyls", α-aminoadipic semialdehyde (AASA) and glutamic semialdehyde (GSA) — Table 5 and Fig. 1.

The major restriction of access to this technique is the availability of isotope-substituted standards for a comprehensive range of analytes since most are not available commercially. The overwhelming advantage of this technique is that it can provide a relatively comprehensive and quantitative analysis of protein glycation, oxidation and nitration adduct residues and free adducts using a small amount of sample (10 μg protein and 10 μl of ultrafiltrate). Overall, we obtain a fingerprint of the quantitative damage to cellular and extracellular proteins in physiological systems and related proteolytic debris — protein glycation, oxidation and nitration free adducts. This ensures that we are unlikely to overlook important types of protein damage. The levels of one or a combination of these adducts may provide a critical marker for disease diagnosis and progression monitoring. This has been applied to screening protein glycation, oxidation and nitration markers in plasma, red blood cells and leukocytes, tissue proteins, urine and cerebrospinal fluid [1,54].

3. Sources of error in mass spectrometric analysis and how to eliminate them

3.1. Mass spectrometric ionisation — in-source oxidation of amino acids and peptides and other potential co-founders

We employ positive ion mode ESI in the mass spectrometer ion source. ESI generally gives a more efficient transfer of amino acid and peptide ions from solution to vapour phase than the alternative matrix assisted laser desorption ionisation (MALDI) source. Both ESI and MALDI sources may, however, produce in-source oxidation and dehydration reactions that may produce interferences in the quantitation of oxidised and glycated proteins. In ESI, the sample source is typically maintained at 120 °C with a jet of nitrogen at 350 °C dispersing the chromatographic solvent at atmospheric pressure. In MALDI, the effective temperature of the sample lattice may increase several 100 °C from ambient [55]. It is therefore expected that oxidation and dehydration reactions may occur in ESI and MALDI ionisation sources. MALDI sources produce in-source methionine and tryptophan oxidation and disulphide exchange [56–58] — including oxidation on MALDI target plate air drying [56]. ESI produces oxidation of amino acids and peptides in corona discharge of the electrospray source [59]. This oxidation produces relatively minor oxidation of substrate amino acids and peptides but endogenous levels of oxidised forms are typically minor (<5% of unoxidized substrate) and so this represents a significant error in protein oxidation studies. The interference can be excluded by using ESI hyphenated with liquid chromatography and separating unoxidized amino acid and peptide from oxidised forms chromatographically. So that, for example, methionine is always detected with chromatographic resolution from MetSO. Similarly, FL residues may degrade to CML in the ESI source and FL should be resolved chromatographically for CML for LC-MS/MS detection. Chromatographic resolution of oxidation and glycation adducts from precursor amino acids and amino acid derivatives which may degrade to them is essential to avoid ESI in-source artefacts. This was not done in early application of LC-MS/MS with octdecyl silica columns giving short and similar retention times of analytes and related unmodified amino acids [60]. In proteomics and peptide mapping studies to locate hotspot sites of oxidation in proteins, the unoxidized peptide precursor should be resolved chromatographically from the oxidised peptide to ensure avoidance of in-source oxidation artefacts.

3.2. Pre-analytic processing of amino acids and peptide and protein digests — avoiding chemical derivatisation and lyophilisation

Following exhaustive enzymatic digestion of proteins and peptides or preparation of ultrafiltrates of physiological fluids, oxidised and glycated amino acids and their related unmodified amino acids are preferably detected by mass spectrometric MRM without chemical derivatisation. In early application of LC-MS/MS for amino acid analysis, and still in some clinical applications, amino acids are esterified to

facilitate positive ionisation [61] or modified on the alpha-amino group by hydroxysuccinimidyl active esters (for example, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [AQC]) [62]. In early studies with AQC derivatisation we found transfer of the methylglyoxal moiety from methylglyoxal-derived hydroimidazolone to added [$^{15}N_2$]arginine in the sample during derivatisation, indicating that even at the relatively mild derivatisation conditions for the AQC modification (pH 8.8 and 55 °C for 10 min) assay of protein damage adducts can be compromised. It is preferable to avoid chemical derivatisation because it introduces potential artefacts.

For high sensitivity detection of amino acids and their oxidised and glycated derivatives we use aqueous acetonitrile mobile phase with 0.1% TFA. This provides a low pH mobile phase that facilitates amino acid positive ionisation without esterification. The use of TFA in chromatography of tryptic peptides is avoided because of signal suppression caused by strong ion pairing between the TFA anion and the peptide cation; formic acid is often rather used [63]. For amino acids we have found rather improved sensitivity in positive ion ESI for underivatised amino acids with TFA compared to formic acid. The greater ease of formation of cations for amino acids at the lower pH of 0.1% TFA versus 0.1% formic acid (pH 2.1 versus 2.7, 3-fold ratio of [H^+]) likely more than compensates for loss of signal suppression by ion pairing.

For the detection of some protein oxidation adducts such as protein carbonyls, AASA and GSA, prior chemical reduction with sodium borohydride has been employed to reduce the analytes to 6-hydroxy-2-aminocaproic acid and 5-hydroxy-2-aminovaleric acid prior to analysis [64]. Similarly the early glycation adduct FL has been reduced to hexitol-lysine before analysis [65]. Such chemical processing requires careful implementation and validation to avoid compromising the analysis. Our recent direct estimation of AASA and GSA by LC-MS/MS without prior reduction suggests this can lead to marked overestimates of analyte.

Early studies of the reaction of methylglyoxal and other dicarbonyls with lysine and arginine residues by high resolution NMR revealed that glycation adduct product distribution can be compromised by lyophilization and reconstitution with water where dehydration reactions have been favoured [66]. It is now recognized that lyophilization can force glycation reactions. This contributes to the denaturation of pharmaceutical proteins in the presence of saccharide stabilisers — reviewed in [67]. Sample lyophilization can therefore compromise glycation adduct analysis and is preferably avoided.

3.3. Automated enzymatic hydrolysis under antioxidant and aseptic conditions

During the enzymatic hydrolysis, steps are taken to avoid oxidative degradation of protein adducts residues — addition of the antioxidant thymol and incubation under argon or carbon monoxide (the latter to exclude oxygen and inactivate heme in hemoglobin digests) [1,25]. Also, the hydrolytic enzymes undergo limited autohydrolysis and a correction has to be applied for this from hydrolysis blanks.

Exhaustive proteolysis is often thought to be inefficient but this impression may have been gained by failing to protect against bacterial growth with consequent decreased yield of amino acids. Addition of antibiotics is essential to avoid bacterial colonisation and amino acid loss. After the initial step with pepsin under acidic conditions, antibiotics were included in the enzymatic digest to prevent bacterial growth in the amino acid solution being produced [25]. In further refinement of the method we use a relatively low cost automated sample processor (PAL, CTC Analytics, Switzerland), sterile filter samples, enzymes and other reagents and perform the enzymatic hydrolysis under aseptic conditions. This has improved assay variability and sample batch-after-batch reliability and ease of training and operation for new investigators. We typically hydrolyse 100 μg protein and analyse ca. 30 μg equivalent. With the recent advance and improvement of tandem mass spectrometer sensitivity we can analysis protein oxidation and nitration adduct content routinely with *ca.* 10 μg protein.

4. Corroboration of LC-MS/MS estimates of protein oxidation and glycation damage with other techniques

Independent or orthogonal techniques to measure protein oxidation and glycation are valuable in providing estimates for corroboration with those produced by LC-MS/MS. This can add to surety of the estimation. Some characteristics of LC-MS/MS – limited sample throughput and loss of sample spatial resolution (variation of analyte concentration in different parts of a tissue sample) – are undesirable and are readily available in immunohistochemistry, for example.

4.1. Immunoassay of oxidised and glycated proteins.

Many estimates of oxidised and glycated proteins were made initially by immunoassay. Indeed, there remains a vibrant commercial market in polyclonal and monoclonal antibodies to detect oxidised and glycated proteins. Immunoassay can be adapted to high throughput techniques and by immunohistochemistry can give evidence of tissue distribution of analyte. Many problems have arisen, however, that have given misleading and erroneous results.

4.1.1. Antibody specificity

Antibodies produced for the detection of oxidised and glycated proteins are often raised by injection of a highly oxidised or glycated immunogen and the immune response is produced within an animal host which is a matrix of oxidised and glycated proteins containing many similar epitopes to which the antibody is raised. Polyclonal and even monoclonal antibodies therefore often lack the epitope affinity maturation and analytical epitope specificity required and expected. An example of this was the use of monoclonal antibody 6D12 which was considered to be specific for detection of the AGE, CML — formed mainly by the oxidative degradation of FL in glycation reactions of glucose. Later studies showed that CD12 have greater affinity for the similar epitope, Nε-(1-carboxyethyl)lysine (CEL) which is formed as a minor AGE in the glycation of proteins by methylglyoxal. Hence studies using antibody 6D12 considered to be reporting on glucose-derived AGE formation by putative estimation of CML were also and possibly in some instances rather mainly reporting on methylglyoxal-derived AGE formation. Later recognition of this has made some studies easier to now understand — such as the decreased methylglyoxal and 6D12 response we found in endothelial cell cultures with overexpression of glyoxalase 1 [68].

Inadequate antibody specificity not only produces interferences into immunoassay but it has also produces misleading data in proteomics studies. Antibodies are often used in proteomics studies to separate oxidised and glycated proteins for subsequent mass spectrometric analysis. To circumvent this problem the oxidised or glycated amino acid should be identified by high resolution mass spectrometric analysis in the antibody-captured proteins — although this critical evidence is often missing.

4.1.2. Blocking proteins

In most immunoassay procedures a large excess of blocking protein, such as serum albumin, gelatin or other, is added before and with the primary antibody to decrease non-specific binding of the primary and secondary antibodies. Blocking proteins have oxidised and glycated amino acid residues and addition of these represents inclusion of exogenous analyte to the sample. Addition of the blocking protein has been done following conventional immunoassay approaches but it is undesirable to add known or frequently unknown amount of analyte in to the assay in the blocking protein. An approach to avoid this is to use a synthetic polypeptide as the blocking protein – such as polythreonine. We employed this in a recent study of immunohistochemistry of the methylglyoxal-derived AGE in aortas of apolipoprotein E knockout

diabetic mice and background immunostaining was markedly decreased [44].

4.1.3. Other problems — high pH, heating and peroxidase inactivation

Other problems encountered in the use of immunoassay to measure oxidised and glycated proteins have been the use of extreme high pH and high temperature in pre-analytic processing. For example, FL residues degrade rapidly to form AGEs during heating at high temperatures and high pH [69,70]. Heating of proteins causes loss of amino acid residues and impairs enzymatic hydrolysis [71,72]. Heating of samples by microwave prior to immunohistochemistry [73] — the so-called heat-induced epitope retrieval technique extensively used with formalin-fixed, paraffin-embedded tissue sections [74], and boiling prior to immunoassay [75] compromised protein oxidation and glycation adduct content.

4.2. Detection of oxidation and glycation adducts by fluorescence

AGEs have been determined by "total AGE fluorescence", measuring fluorescence with excitation and emission wavelengths of 350 nm and 450 nm, respectively [76], with recent modification of this method to detect low molecular mass AGE peptides or free adducts [77,78]. These methods are compromised by several analytical problems: (i) major AGEs quantitatively are not fluorescent and therefore are not detected [1], (ii) there is significant interference from the oxidative adduct and fluorophore, and (iii) several different fluorophores contribute to the global measure of fluorescence each with a different specific fluorescence such that no quantitative calibration can be achieved [8,79–85]. There are also fluorophores formed oxidatively, such as N-formylkynurenine (NFK) [86] and dityrosine [87]. Estimates of "total AGE fluorescence" as determined currently represent a qualitative measure of damage by both glycation and oxidation adduct fluorophores — Table 6.

4.3. Detection of glycation adducts by skin autofluorescence

Recent advances in instrumentation have seen the development bed-side fluorimeter or autofluorescence readers. The instrument illuminates 1 cm^2 of skin with an excitation wavelength band of 300–420 nm (peak excitation 350 nm). Emitted light from the skin is measured over the wavelength range 300–600 nm. Autofluorescence is calculated by dividing the average light intensity emitted per nm over 420–600 nm range by the average light intensity emitted per nm over the 300–420 nm excitation range. Autofluorescence of the skin is measured 6 times over a 50 s period (every 10 s) at the volar side of the arm, ca. 10 cm below the elbow fold and at the dorsal side of the lower leg (calf). In hemodialysis patients, skin autofluorescence (SAF) correlated with skin biopsy content of pentosidine ($r = 0.75$), CML and CEL (both $r = 0.45$). Hence, measurements of SAF have been interpreted as a measurement of AGEs. The problems with this interpretation are:

(i) most AGEs are not fluorescent — particularly the quantitatively important hydroimidazolone, MG-H1, and CML and CEL;
(ii) fluorescence characteristics used in this assay are not specific for fluorescent AGEs (fluorescence of the oxidation adduct NFK, for example [88]);
(iii) fluorescence in proteins is due to multiple fluorophores which compete for the same excitation energy in the detection and hence there is no direct relationship between concentration of the adduct and fluorescence; and
(iv) main components of SAF spectra are thought to be due to nicotinamide adenine dinucleotide, flavin adenine dinucleotide, and porphyrins [89].

Despite this, SAF may have useful applications. There was increased SAF in dialysis patients which decreased after renal transplantation [90]. SAF of haemodialysis patients was an independent predictor of overall and cardiovascular mortality. Multivariate analysis revealed

Table 6
Fluorophores associated with protein damage by glycation and oxidation.

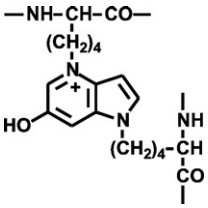
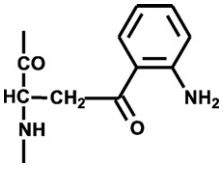
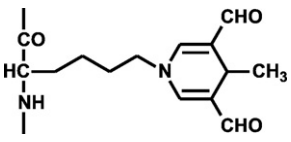
Fluorophore	Structure	$\lambda_{excitation}$, $\lambda_{emission}$	Reference
Glycation			
Pentosidine	See Fig. 1	335 nm, 385 nm	[8]
Argpyrimidine	See Fig. 1	320 nm, 385 nm	[84]
Vesperlysine A (LM-1)		345 nm, 405 nm	[85]
"AGE fluorescence"	Multiple	350 nm, 440 nm	[76]
Oxidation			
Dityrosine	See Fig. 1	283 nm, 409 nm	[87]
N-Formylkynurenine (NFK)	See Fig. 1	325 nm, 434 nm	[79]
Kynurenine		365 nm, 480 nm	[79]
2-Amino-6-(3,5-diformyl-1,4-dihydro-4-methyl-pyridin-1-yl)hexanoic acid		387 nm, 455 nm	[80]
"Fluorescent oxidation products"	Multiple fluorophores	360 nm, 430 nm	[81]
Lipofuscin	Multiple fluorophores	340 – 390 nm, 430 – 490 nm	[82,83]

Table 7
Predicted reactivity of apolipoprotein B100 of LDL towards early glycation, advanced glycation, oxidation and nitration.

Type of modification	Adduct	ApoB100 of LDL content (mmol/mol amino acid modified)	LDL adduct content (μM)	$r_{LDLmodfication\ in\ situ}$ ($\mu M day^{-1}$)
Early glycation	FL	4.17	1.9	0.44
AGE	MG-H1	0.16	0.031	0.0072
Oxidation	MetSO	13.7	1.4	0.32
Nitration	3-NT	0.0078	0.0015	0.00035

Assumptions: the rate of formation of FL, MG-H1. MetSO and 3-NT in LDL and plasma protein is equal to the rate of clearance of protein adducts, half-life of LDL is *ca.* 3 days [99] and plasma concentrations of LDL is 1.28 μM (equivalent to 3.18 mM LDL cholesterol).

that 65% of the variance in SAF was linked to independent effects of age, dialysis and renal failure duration, presence of diabetes, triglycerides levels, and C-reactive protein [91]. The relationship to disease may be complex, however. For example, although accumulation of AGEs has been linked to the development of vascular complications of diabetes — such as retinopathy, patient groups with type 1 diabetes of duration 40 years with either background, advanced and severe proliferative retinopathy showed increased SAF with increasing severity of retinopathy. The remarkable finding however, was that patients without retinopathy had the highest level of SAF [92]. Therefore, the interpretation of the SAF response is more complex than initially envisaged.

5. Role of systems biology in characterisation of protein oxidation and glycation

Despite all efforts being made to provide reliable and robust estimates of protein glycation and oxidation there always remains the possibility that one or more interfering components are present leading to over- or underestimation of protein oxidation or glycation adduct. So we need to look for independent supporting evidence, preferably bringing together all the information we know on the oxidation and glycation process concerned. This further supporting evidence of secure estimation can come from a systems biology approach: that is, by producing mathematical models of the *in situ* kinetics of analyte formation and removal with prediction of steady-state concentrations of analytes to provide independent support for accuracy of estimates and also physiological regulation of protein damage. Examples are: (i) systems modelling for prediction of methylglyoxal concentration in human plasma, and (ii) systems modelling to estimate *in situ* rates of glycation oxidation and nitration of LDL in plasma *in vivo*. These have been described elsewhere [42,93] and will be briefly summarised here.

5.1. Systems models in protein oxidation and glycation research. Example 1. Prediction of methylglyoxal concentration in human plasma

For the reaction of MG with plasma protein and the flux of methylglyoxal-derived AGEs, the following kinetic scheme applies: Methylglyoxal + plasma protein → plasma protein AGEs → AGE clearance (by protein turnover). The concentration of the major methylglyoxal-derived AGE in plasma protein, MG-H1, in healthy human subjects, $[MG\text{-}H1]_{plasma,}$ is 0.92 mmol/mol arg [13] or 16.8 μM. The rate of formation of MG-H1 in plasma protein, r_1, is given by the equation:

$$r_1 = k[MG]_{plasma} \cdot [\text{Plasma protein}]$$

Assuming a constant concentration of plasma protein,

$$r_1 = k_{obs}[MG]_{plasma}$$

where k_{obs} is the observed pseudo first order rate constant for the reaction of methylglyoxal with plasma protein. Assuming most of the MG-H1 residues are on albumin with a half-life of 19 days [94] (equivalent to an elimination rate constant k_{elim} of 0.036 day^{-1}), the clearance of MG-H1 residues from plasma, r_2, is given by the equation:

$$r_2 = k_{elim}[MG - H1]_{plasma}$$

At the steady-state, $r_1 = r_2$ or $r_1 = k_{obs}[MG]_{plasma} = k_{elim}[MG\text{-}H1]_{plasma}$, which rearranged gives

$$[MG]_{plasma} = (k_{elim}/k_{obs})[MG{-}H1]_{plasma}$$

The value of k_{elim} is known and the value of $[MG\text{-}H1]_{plasma}$ has been estimated. We also determined k_{obs} by addition of 1 μM radiolabelled [^{14}C]methylglyoxal in human plasma *ex vivo* without significant dilution; k_{obs} = 4.6 day^{-1} of the plasma [95]. Hence, we predict

$$[MG]_{plasma} = (k_{elim}/k_{obs})[MG{-}H1]_{plasma} = (0.036/4.6)\cdot 16.8\ \mu M = 0.13\ \mu M.$$

This is an approximation as MG-derived AGEs are present in proteins other than albumin in plasma with half-lives different to those of albumin. It provides support for estimates of methylglyoxal in human blood plasma as *ca.* 100 nM as being close to the true value [96–98] and markedly higher than sub-micromolar are most probably incorrect. Higher estimates are due to formation of methylglyoxal during pre-analytic processing in the biological assay matrix.

5.2. Systems models in protein oxidation and glycation research. Example 2. Prediction of rates of oxidation, glycation and nitration of LDL in human plasma in vivo

The rates of oxidation, glycation and nitration of LDL in human plasma can be predicted assuming they are equal to the rate of clearance of oxidation, glycation and nitration adducts of LDL in the steady state. The later may be calculated from the half-life $t_{1/2}$ of LDL is *ca.* 3 days [99] and the steady state concentration of oxidation, glycation and nitration adduct residues in LDL *in vivo* [42]. The outcome of these predictions is given in Table 7 where the *in situ* rates of LDL modification were deduced. The relative rates of *in situ* modification of LDL by nitration, advanced glycation (by methylglyoxal), oxidation to form MetSO residues and early glycation are: 1:21:933:1270. This suggests apolipoprotein B100 (ApoB100) of LDL is highly reactive towards oxidation and early glycation *in situ*. For early and advanced glycation by glucose and methylglyoxal, respectively, and assuming plasma concentrations of 5 mM and 100 nM of these glycating agents, the estimated rate constants for glycation of LDL under physiological conditions are: glucose glycation $k_{LDL,\ glucose}$ = 69 M^{-1} day^{-1}; and methylglyoxal glycation $k_{LDL,MG}$ = 55,000 M^{-1} day^{-1}. These were similar estimates to those obtained from glycation of LDL *in vitro* [42].

6. Closing remarks

Quantitative measurement of protein oxidation and glycation markers provides evidence of protein damage in health and disease.

Estimates of the levels of oxidation and glycation adduct residues in plasma protein provide markers of the rate of protein glycation and oxidation in the plasma compartment — also influenced by protein turnover. Estimates of rates of urinary excretion of oxidation and glycation free adducts provides indicators of total body oxidative and glycation damage — also influenced by adduct repair and contributions from absorption of oxidation and glycation free adducts from digested oxidised and glycated proteins in food. Estimates of renal clearance of oxidation and glycation free adducts provides multiple reporters of renal function which are often more sensitive to renal function decline than creatinine clearance or cystatin c measurements [13,100]. Other markers such as glycated hemoglobin provide a marker of metabolic control. Reliable and quantitative measurement of protein oxidation and glycation adduct residues and free adducts is required to gain maximum mechanistic and diagnostic information. Stable isotopic dilution analysis LC-MS/MS, with prior automated enzymatic hydrolysis for proteins, is currently the best way to achieve this. The use of this technique, and others such as immunoassay corroborated to it, will provide for robust assessment of protein oxidation and glycation markers in health and disease.

Acknowledgements

We thank current and past members of our Protein damage and System Biology research group and our collaborators for their help, efforts and fellowship.

References

[1] P.J. Thornalley, S. Battah, N. Ahmed, N. Karachalias, S. Agalou, R. Babaei-Jadidi, A. Dawnay, Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry, Biochem. J. 375 (2003) 581–592.
[2] Y. Yu, S.R. Thorpe, A.J. Jenkins, J.N. Shaw, M.A. Sochaski, D. Mcgee, C.E. Aston, T.J. Orchard, N. Silvers, Y.G. Peng, J.A. McKnight, J.W. Baynes, T.J. Lyons, Advanced glycation end-products and methionine sulphoxide in skin collagen of patients with type 1 diabetes, Diabetologia 49 (2006) 2488–2498.
[3] H. Ryberg, A.S. Soderling, P. Davidsson, K. Blennow, K. Caidahl, L.I. Persson, Cerebrospinal fluid levels of free 3-nitrotyrosine are not elevated in the majority of patients with amyotrophic lateral sclerosis or Alzheimer's disease, Neurochem. Int. 45 (2004) 57–62.
[4] N. Rabbani, P.J. Thornalley, Glycation research in amino acids: a place to call home, Amino Acids 42 (2012) 1087–1096.
[5] J.F. Day, S.R. Thorpe, J.W. Baynes, Nonenzymatically glycosylated albumin (*in vitro* preparation and isolation from normal human serum), J. Biol. Chem. 254 (1979) 595–597.
[6] N. Ahmed, D. Dobler, M. Dean, P.J. Thornalley, Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in ligand binding and esterase activity, J. Biol. Chem. 280 (2005) 5724–5732.
[7] M.U. Ahmed, S.R. Thorpe, J.W. Baynes, Identification of *N*ε-carboxymethyl-lysine as a degradation product of fructoselysine in glycated protein, J. Biol. Chem. 261 (1986) 4889–4894.
[8] D.R. Sell, V.M. Monnier, Structure elucidation of a senescence crosslink from human extracellular matrix. Implication of pentoses in the aging process. J. Biol. Chem. 264 (1989) 21597–21602.
[9] V. Witko, A.T. Nguyen, B. DescampsLatscha, Microtiter plate assay for phagocyte-derived taurine-chloramines, J. Clin. Lab. Anal. 6 (1992) 47–53.
[10] V. Witko-Sarsat, M. Friedlander, C. Capeillere-Blandin, T. Nguyen-Khoa, N.T. Nguyen, J. Zingraff, P. Jungers, B. Descamps-Latscha, Advanced oxidation protein products as a novel marker of oxidative stress in uremia, Kidney Int. 49 (1996) 1304–1313.
[11] V. WitkoSarsat, M. Friedlander, C. CapeillereBlandin, T. NguyenKhoa, N.T. Nguyen, J. Zingraff, P. Jungers, B. DescampsLatscha, Advanced oxidation protein products as a novel marker of oxidative stress in uremia, Kidney Int. 49 (1996) 1304–1313.
[12] C. Capeillere-Blandin, V. Gausson, B. Descamps-Latscha, V. Witko-Sarsat, Biochemical and spectrophotometric significance of advanced oxidized protein products, Biochim. Biophys. Acta 1689 (2004) 91–102.
[13] N. Ahmed, R. Babaei-Jadidi, S.K. Howell, P.J. Beisswenger, P.J. Thornalley, Degradation products of proteins damaged by glycation, oxidation and nitration in clinical type 1 diabetes, Diabetologia 48 (2005) 1590–1603.
[14] L. Selmeci, M. Szekely, P. Soos, L. Seres, N. Klinga, A. Geiger, G. Acsady, Human blood plasma advanced oxidation protein products (AOPP) correlates with fibrinogen levels, Free Radic. Res. 40 (2006) 952–958.
[15] P.J. Thornalley, Glycation free adduct accumulation in renal disease: the new AGE, Pediatr. Nephrol. 20 (2005) 1515–1522.
[16] N. Ahmed, P.J. Thornalley, R. Luthen, D. Haussinger, K. Sebekova, R. Schinzel, W. Voelker, A. Heidland, Processing of protein glycation, oxidation and nitrosation adducts in the liver and the effect of cirrhosis, J. Hepatol. 41 (2004) 913–919.
[17] Z. Makita, S. Radoff, E.J. Rayfield, Z. Yang, E. Skolnik, V. Delaney, E. Friedman, A. Cerami, H. Vlassara, Advanced glycosylation end products in patients with diabetic nephropathy, N. Engl. J. Med. 325 (1991) 836–842.
[18] A. Cerami, Aging of proteins and nucleic acids: what is the role of glucose? TIBS 11 (1986) 311–314.
[19] N. Verzijl, J. DeGroot, E. Oldehinkel, R.A. Bank, S.R. Thorpe, J.W. Baynes, M.T. Bayliss, J.W.J. Bijlsma, F.P.J.G. Lafeber, J.M. TeKoppele, Age-related accumulation of Maillard reaction products in human articular cartilage collagen, Biochem. J. 350 (2000) 381–387.
[20] N. Brot, H. Weissbach, Biochemistry and physiological role of methionine sulfoxide reductase in proteins, Arch. Biochem. Biophys. 253 (1983) 271–281.
[21] G. Delpierre, M.H. Rider, F. Collard, V. Stroobant, F. Vanstapel, H. Santos, E. Van Schaftingen, Identification, cloning, and heterologous expression of a mammalian fructosamine-3-kinase, Diabetes 49 (2000) 1627–1634.
[22] X. Zhang, K.F. Medzihradszhy, J. Cunningham, P.D.K. Lee, C.L. Rognerud, C.-N. Ou, P. Harmatz, H.E. Witkowska, Characterization of glycated hemoglobin in diabetic patients: usefulness of electrospray mass spectrometry in monitoring the extent and distribution of glycation, J. Chromatogr. B 759 (2001) 1–15.
[23] G. Delpierre, F. Collard, J. Fortpied, E. Van Schaftingen, Fructosamine 3-kinase is involved in an intracellular deglycation pathway in human erythrocytes, Biochem. J. 365 (2002) 801–808.
[24] M.A. Virtue, F.Q. Nuttall, J.K. Furne, M.D. Levitt, Relationship between GHb concentrauion and erythrocyte survival determined from breath carbon monoxide concentration, Diabetes Care 27 (2004) 931–935.
[25] N. Ahmed, O.K. Argirov, H.S. Minhas, C.A. Cordeiro, P.J. Thornalley, Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatisation by aminoquinolyl-N-hydroxysuccimidyl-carbamate and application to Nε-carboxymethyl-lysine- and Nε-(1-carboxyethyl) lysine-modified albumin, Biochem. J. 364 (2002) 1–14.
[26] M. Xue, N. Rabbani, H. Momiji, P. Imbasi, M.M. Anwar, N.R. Kitteringham, B.K. Park, T. Souma, T. Moriguchi, M. Yamamoto, P.J. Thornalley, Transcriptional control of glyoxalase 1 by Nrf2 provides a stress responsive defence against dicarbonyl glycation, Biochem. J. 443 (2012) 213–222.
[27] F. Collard, G. Delpierre, V. Stroobant, G. Matthijs, E. Van Schaftingen, A mammalian protein homologous to fructosamine-3-kinase is a ketosamine-3-kinase acting on psicosamines and ribulosamines but not on fructosamines, Diabetes 52 (2003) 2888–2895.
[28] N. Abello, H.A.M. Kerstjens, D.S. Postma, R. Bischoff, Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins, J. Proteome Res. 8 (2009) 3222–3238.
[29] V.I. Perez, R. Buffenstein, V. Masamsetti, S. Leonard, A.B. Salmon, J. Mele, B. Andziak, T. Yang, Y. Edrey, B. Friguet, W. Ward, A. Richardson, A. Chaudhuri, Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat, Proc. Natl. Acad. Sci. 106 (2009) 3059–3064.
[30] D. Dobler, N. Ahmed, L.J. Song, K.E. Eboigbodin, P.J. Thornalley, Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification, Diabetes 55 (2006) 1961–1969.
[31] T. Grune, T. Reinheckel, K.J.A. Davies, Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome, J. Biol. Chem. 271 (1996) 15504–15509.
[32] M. Hernebring, G. Brolen, H. Aguilaniu, H. Semb, T. Nystrom, Elimination of damaged proteins during differentiation of embryonic stem cells, Proc. Natl. Acad. Sci. 103 (2006) 7700–7705.
[33] E.J. Dudek, F. Shang, Q. Liu, P. Valverde, M. Hobbs, A. Taylor, Selectivity of the ubiquitin pathway for oxidatively modified proteins: relevance to protein precipitation diseases, FASEB J. 19 (2005) 1707–1709.
[34] A.L. Goldberg, T.N. Akopian, A.F. Kisselev, D.H. Lee, Protein degradation by the proteasome and dissection of its in vivo importance with synthetic inhibitors, Mol. Biol. Rep. 24 (1997) 69–75.
[35] H.A. Franch, S. Sooparb, J. Du, A mechanism regulating proteolysis of specific proteins during renal tubular cell growth, J. Biol. Chem. 276 (2001) 19126–19131.
[36] S. Agalou, N. Ahmed, R. Babaei-Jadidi, A. Dawnay, P.J. Thornalley, Profound mishandling of protein glycation degradation products in uremia and dialysis, J. Am. Soc. Nephrol. 16 (2005) 1471–1485.
[37] N. Rabbani, K. Sebekova, K. Sebekova Jr., A. Heidland, P.J. Thornalley, Protein glycation, oxidation and nitration free adduct accumulation after bilateral nephrectomy and ureteral ligation, Kidney Int. 72 (2007) 1113–1121.
[38] N. Rabbani, A. AntonySunil, K. Rossing, P. Rossing, L. Tarnow, H.-H. Parving, P.J. Thornalley, Effect of Irbesartan treatment on plasma and urinary protein glycation, oxidation and nitration markers in patients with type 2 diabetes and microalbuminuria, Amino Acids 42 (2011) 1627–1639.
[39] P.J. Thornalley, M. Argirova, N. Ahmed, V.M. Mann, O.K. Argirov, A. Dawnay, Mass spectrometric monitoring of albumin in uraemia, Kidney Int. 58 (2000) 2228–2234.
[40] R.N. Johnson, R.W. Easdale, M. Tatnell, J.R. Baker, Significance of variation in turnover of glycated albumin on indexes of diabetic control, Clin. Chim. Acta 198 (1991) 229–238.
[41] K. Matsumoto, K. Sano, R. Nagai, H. Suzuki, T. Kodama, M. Yoshida, S. Ueda, B. Smedsrod, S. Horiuchi, Endocytic uptake of advanced glycation end products by mouse liver sinusoidal endothelial cells is mediated by a scavenger receptor distinct from the macrophage scavenger receptor class A, Biochem. J. 352 (2000) 233–240.

[42] N. Rabbani, M. Varma Chittari, C.W. Bodmer, D. Zehnder, A. Ceriello, P.J. Thornalley, Increased glycation and oxidative damage to apolipoprotein B100 of LDL in patients with type 2 diabetes and effect of metformin, Diabetes 59 (2010) 1038–1045.
[43] C.G. Schalkwijk, M.A. Vermeer, C.D.A. Stehouwer, J. te Koppele, H.M.G. Princen, V.W.M. van Hinsbergh, Effect of methylglyoxal on the physico-chemical and biological properties of low-density lipoprotein, Biochim. Biophys. Acta 1394 (1998) 187–198.
[44] N. Rabbani, L. Godfrey, M. Xue, F. Shaheen, M. Geoffrion, R. Milne, P.J. Thornalley, Conversion of low density lipoprotein to the pro-atherogenic form by methylglyoxal with increased arterial proteoglycan binding and aortal retention, Diabetes 60 (2011) 1973–1980.
[45] A.N. Hoofnagle, J.O. Becker, M.N. Oda, G. Cavigiolio, P. Mayer, T. Vaisar, Multiple-reaction monitoring-mass spectrometric assays can accurately measure the relative protein abundance in complex mixtures, Clin. Chem. 58 (2012) 777–781.
[46] J. Pietzsch, P. Lattke, U. Julius, Oxidation of apolipoprotein B-100 in circulating LDL is related to LDL residence time: *in vivo* insights from stable-isotope studies, Arterioscler. Thromb. Vasc. Biol. 20 (2000) e63–e67.
[47] M. Hamelin, C. Borot-Laloi, B. Friguet, H. Bakala, Increased level of glycoxidation product Nε-(carboxymethyl)lysine in rat serum and urine proteins with aging: link with glycoxidative damage accumulation in kidney, Arch. Biochem. Biophys. 411 (2003) 215–222.
[48] C.C. Conrad, J.M. Talent, C.A. Malakowsky, R.W. Gracy, Post-electrophoretic identification of oxidized proteins, Biol. Proced. Online 2 (2000) 1–10.
[49] G. Sun, V.E. Anderson, Prevention of artifactual protein oxidation generated during sodium dodecyl sulfate-gel electrophoresis, Electrophoresis 25 (2004) 959–965.
[50] Z. Makita, S. Radoff, E.J. Rayfield, E.A. Friedman, A.M. Kaufman, S.M. Korbet, R.H. Barth, J.A. Winston, H. Fuh, K.R. Manogue, A. Cerami, H. Vlassara, Reactive glycosylation endproducts in diabetic uraemia and treatment of renal failure, Lancet 343 (1994) 1519–1522.
[51] N. Ahmed, P.J. Thornalley, Chromatographic assay of glycation adducts in human serum albumin glycated *in vitro* by derivatisation with aminoquinolyl-N-hydroxysuccimidyl-carbamate and intrinsic fluorescence, Biochem. J. 364 (2002) 15–24.
[52] T. Delatour, F. Fenaille, V. Parisod, J. Richoz, J. Vuichoud, P. Mottier, T. Buetler, A comparative study of proteolysis methods for the measurement of 3-nitrotyrosine residues: enzymatic digestion versus hydrochloric acid-mediated hydrolysis, J. Chromatogr. B 851 (2007) 268–276.
[53] R.E. Oste, R. Miller, H. Sjostrom, O. Noren, Effect of Maillard reaction-products on protein digestion — studies on pure compounds, J. Agric. Food Chem. 35 (1987) 938–942.
[54] N. Ahmed, U. Ahmed, P.J. Thornalley, K. Hager, G.A. Fleischer, G. Munch, Protein glycation, oxidation and nitration marker residues and free adducts of cerebrospinal fluid in Alzheimer's disease and link to cognitive impairment, J. Neurochem. 92 (2004) 255–263.
[55] K. Dreisewerd, The desorption process in MALDI, Chem. Rev. 103 (2003) 395–425.
[56] D. Suckau, A. Resemann, M. Schuerenberg, P. Hufnagel, J. Franzen, A. Holle, A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics, Anal. Bioanal. Chem. 376 (2003) 952–965.
[57] S.L. Cohen, Ozone in Ambient Air as a Source of Adventitious Oxidation. A Mass Spectrometric Study, Anal. Chem. 78 (2006) 4352–4362.
[58] L. Zhao, R.T. Almaraz, F. Xiang, J.L. Hedrick, A.H. Franz, Gas-phase scrambling of disulfide bonds during matrix-assisted laser desorption/ionization mass spectrometry analysis, J. Am. Soc. Mass Spectrom. 20 (2009) 1603–1616.
[59] B.L. Boys, M.C. Kuprowski, J.J. Noe¦êl, L. Konermann, Protein oxidative modifications during electrospray ionization: solution phase electrochemistry or corona discharge-induced radical attack? Anal. Chem. 81 (2009) 4027–4034.
[60] E. Mottaran, S.F. Stewart, R. Rolla, D. Vay, V. Cipriani, M. Moretti, M. Vidali, M. Sartori, C. Rigamonti, C.P. Day, E. Albano, Lipid peroxidation contributes to immune reactions associated with alcoholic liver disease, Free Radic. Biol. Med. 32 (2002) 38–45.
[61] D.J. Dietzen, A.L. Weindel, M.O. Carayannopoulos, M. Landt, E.T. Normansell, T.E. Reimschisel, C.H. Smith, Rapid comprehensive amino acid analysis by liquid chromatography/tandem mass spectrometry: comparison to cation exchange with post-column ninhydrin detection, Rapid Commun. Mass Spectrom. 22 (2008) 3481–3488.
[62] Z. Spacil, J. Eriksson, S. Jonasson, U. Rasmussen, L.L. Ilag, B. Bergman, Analytical protocol for identification of BMAA and DAB in biological samples, Analyst 135 (2010) 127–132.
[63] F. Kuhlmann, A. Apffel, S. Fischer, G. Goldberg, P. Goodley, Signal enhancement for gradient reverse-phase high-performance liquid chromatography-electrospray ionization mass spectrometry analysis with trifluoroacetic and other strong acid modifiers by postcolumn addition of propionic acid and isopropanol, J. Am. Soc. Mass Spectrom. 6 (1995) 1221–1225.
[64] J.R. Requena, C.-C. Chao, R.L. Levine, E.R. Stadtman, Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 68–74.
[65] K.J. Knecht, J.A. Dunn, K.F. Mcfarland, D.R. McCance, T.J. Lyons, S.R. Thorpe, J.W. Baynes, Effect of diabetes and aging on carboxymethyllysine levels in human urine, Diabetes 40 (1991) 190–196.
[66] T.W.C. Lo, M.E. Westwood, A.C. McLellan, T. Selwood, P.J. Thornalley, Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N_α-acetylarginine, N_α-acetylcysteine, N_α-acetyl-lysine, and bovine serum albumin, J. Biol. Chem. 269 (1994) 32299–32305.
[67] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (2000) 1–60.
[68] M. Shinohara, P.J. Thornalley, I. Giardino, P.J. Beisswenger, S.R. Thorpe, J. Onorato, M. Brownlee, Overexpression of glyoxalase I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycaemia-induced increases in macromolecular endocytosis, J. Clin. Invest. 101 (1998) 1142–1147.
[69] L. Li, L.P. Han, Q.Y. Fu, Y.T. Li, Z.L. Liang, J.Y. Su, B. Li, Formation and Inhibition of N-epsilon-(carboxymethyl)lysine in saccharide-lysine model systems during microwave heating, Molecules 17 (2012) 12758–12770.
[70] P.R. Smith, P.J. Thornalley, Influence of pH and phosphate ions on the kinetics of enolisation and degradation of fructosamines. Studies with the model fructosamine, Nε-1-deoxy-D-fructos-1-yl hippuryllysine, Biochem. Int. 28 (1992) 429–439.
[71] J. Bjarnaso, K.J. Carpente, Mechanisms of heat damage in proteins. 2. Chemical changes in pure proteins, Br. J. Nutr. 24 (1970) 313.
[72] S.A. Varnish, K.J. Carpenter, Mechanisms of heat damage in proteins. 6. Digestibility of individual amino-acids in heated and propionylated proteins, Br. J. Nutr. 34 (1975) 339–349.
[73] C.M. Hayashi, R. Nagai, K. Miyazaki, F. Hayase, T. Araki, T. Ono, S. Horiuchi, Conversion of Amadori products of the Maillard reaction to N-epsilon-(carboxymethyl) lysine by short-term heating: Possible detection of artifacts by immunohistochemistry, Lab. Invest. 82 (2002) 795–807.
[74] S.R. Shi, M.E. Key, K.L. Kalra, Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections, J. Histochem. Cytochem. 39 (1991) 741–748.
[75] M.P. Vitek, K. Bhattyacharya, J.M. Glendening, E. Stopa, H. Vlassara, R. Bucala, K. Manogue, A. Cerami, Advanced glycation end products contribute to amyloidosis in Alzheimer disease, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 4766–4770.
[76] V.M. Monnier, R.R. Kohn, A. Cerami, Accelerated AGE-related browning of human collagen in diabetes- mellitus, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 583–587.
[77] K. Wrobel, K. Wrobel, M. Garay-Sevilla, L.E. Nava, J.M. Malacara, Novel analytical approach to monitoring advanced glycosylation end products in human serum with on-line spectrophotometric and spectrofluorometric detection in a flow system, Clin. Chem. 43 (1997) 1563–1569.
[78] M.C. Thomas, C. Tsalamandris, R. MacIsaac, T. Medley, B. Kingwell, M.E. Cooper, G. Jerums, Low-molecular-weight AGEs are associated with GFR and anemia in patients with type 2 diabetes, Kidney Int. 66 (2004) 1167–1172.
[79] Y. Fukunaga, Y. Katsuragi, T. Izumi, F. Sakiyama, Fluorescence characteristics of kynurenine and N'-formylkynurenine. Their use as reporters of the environment of tryptophan 62 in hen egg-white lysozyme, J. Biochem. 92 (1982) 129–141.
[80] S. Yamada, S. Kumazawa, T. Ishii, T. Nakayama, K. Itakura, N. Shibata, M. Kobayashi, K. Sakai, T. Osawa, K. Uchida, Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine, J. Lipid Res. 42 (2001) 1187–1196.
[81] H. Shimasaki, Assay of fluorescent lipid-peroxidation products, Methods Enzymol. 233 (1994) 338–346.
[82] R.S. Sohal, Age Pigments, Elsevier/North Holland Biomedical Press, Amsterdam, 1981.
[83] G. Li, Y. Liao, X. Wang, S. Sheng, D. Yin, In situ estimation of the entire color and spectra of age pigment-like materials: application of a front-surface 3D-fluorescence technique, Exp. Gerontol. 41 (2006) 328–336.
[84] I.N. Shipanova, M.A. Glomb, R.H. Nagaraj, Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct, Arch. Biochem. Biophys. 344 (1997) 29–36.
[85] F. Tessier, M. Obrenovich, V.M. Monnier, Structure and mechanism of formation of human lens fluorophore LM-1. Relationship to vesperlysine a and the advanced maillard reaction in aging, diabetes, and cataractogenesis, J. Biol. Chem. 274 (1999) 20796–20804.
[86] T. Buxton, G.G. Guilbault, Fluorometric analysis for N'-formylkynurenine in plasma and urine, Clin. Chem. 20 (1974) 765–768.
[87] D.A. Malencik, S.R. Anderson, Dityrosine as a product of oxidative stress and fluorescent probe, Amino Acids 25 (2003) 233–247.
[88] N. Ahmed, P.J. Thornalley, Advanced glycation endproducts: what is their relevance to diabetic complications? Diabetes Obes. Metab. 9 (2007) 233–245.
[89] R.H. Na, I.M. Stender, L.X. Ma, H.C. Wulf, Autofluorescence spectrum of skin: component bands and body site variations, Skin Res. Technol. 6 (2000) 112–117.
[90] J.W.L. Hartog, A.P.J. De Vries, H.L. Lutgers, R. Meerwaldt, R.M. Huisman, W.J. Van Son, P.E. De Jong, A.J. Smit, Accumulation of Advanced Glycation End Products, Measured As Skin Autofluorescence, Renal Disease, NEW YORK, 2005. 299–307.
[91] R. Meerwaldt, H.L. Lutgers, T.P. Links, R. Graaff, J.W. Baynes, R.O.B. Gans, A.J. Smit, Skin autofluorescence is a strong predictor of cardiac mortality in diabetes, Diabetes Care 30 (2007) 107–112.
[92] B.N. Conway, J.D. Maynard, T.J. Orchard, Comment on: Sun et al. Protection From Retinopathy and Other Complications in Patients With Type 1 Diabetes of Extreme Duration: The Joslin 50-Year Medalist Study. Diabetes Care 2011;34: 968ΓÇô974, Diabetes Care 34 (2011) e148.
[93] N. Rabbani, P.J. Thornalley, Glyoxalase in diabetes, obesity and related disorders, Semin. Cell Dev. Biol. 22 (2011) 309–317.
[94] T. Peters, All About Albumin, Academic Press, New York, 1996.

[95] P.J. Thornalley, Dicarbonyl intermediates in the Maillard reaction, Ann. N. Y. Acad. Sci. 1043 (2005) 111–117.
[96] A.C. McLellan, P.J. Thornalley, J. Benn, P.H. Sonksen, The glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications, Clin. Sci. 87 (1994) 21–29.
[97] A. Bierhaus, T. Fleming, S. Stoyanov, A. Leffler, A. Babes, C. Neacsu, S.K. Sauer, M. Eberhardt, M. Schnolzer, F. Lasischka, W.L. Neuhuber, T.I. Kichko, I. Konrade, R. Elvert, W. Mier, V. Pirags, I.K. Lukic, M. Morcos, T. Dehmer, N. Rabbani, P.J. Thornalley, D. Edelstein, C. Nau, J. Forbes, P.M. Humpert, M. Schwaninger, D. Ziegler, D.M. Stern, M.E. Cooper, U. Haberkorn, M. Brownlee, P.W. Reeh, P.P. Nawroth, Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy, Nat. Med. 18 (2012) 926–933.
[98] P.J. Beisswenger, S.K. Howell, A. Touchette, S. Lal, B.S. Szwergold, Metformin reduces systemic methylglyoxal levels in type 2 diabetes, Diabetes 48 (1999) 198–202.
[99] T. Langer, R.I. Levy, W. Strober, Metabolism of low-density lipoprotein in familial type-H hyperlipoproteinemia, J. Clin. Invest. 51 (1972) 1528–1536.
[100] B.A. Perkins, N. Rabbani, A. Weston, L.H. Ficociello, A. Adaikalakoteswari, M. Niewczas, J. Warram, A.S. Krolewski, P. Thornalley, Serum levels of advanced glycation endproducts and other markers of protein damage in early diabetic nephropathy in type 1 diabetes, PLoS ONE 7 (2012) e35655.