Antioxidants and protein oxidation

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ABSTRACT

Proteins are susceptible to oxidation by reactive oxygen species, where the type of damage induced is characteristic of the denaturing species. The induction of protein carbonyls is a widely applied biomarker, arising from primary oxidative insult. However, when applied to complex biological and pathological conditions it can be subject to interference from lipid, carbohydrate and DNA oxidation products.

More recently, interest has focused on the analysis of specific protein bound oxidised amino acids. Of the 22 amino acids, aromatic and sulphydryl containing residues have been regarded as being particularly susceptible to oxidative modification, with L-DOPA from tyrosine, ortho-tyrosine from phenylalanine; sulphoxides and disulphides from methionine and cysteine respectively; and kynurenines from tryptophan. Latterly, the identification of valine and leucine hydroxides, reduced from hydroperoxide intermediates, has been described and applied.

In order to examine the nature of oxidative damage and protective efficacy of antioxidants the markers must be thoroughly evaluated for dosimetry *in vitro* following damage by specific radical species. Antioxidant protection against formation of the biomarker should be demonstrated *in vitro*. Quantification of biomarkers in proteins from normal subjects should be within the limits of detection of any analytical procedure. Further to this, the techniques for isolation and hydrolysis of specific proteins should demonstrate that *in vitro* oxidation is minimised. There is a need for the development of standards for quality assurance material to standardise procedures between laboratories.

At present, antioxidant effects on protein oxidation *in vivo* are limited, and include animal studies, where dietary antioxidants have been reported to reduce dityrosine formation during rat exercise training. Two studies on humans have been reported this year. The further application of these methods to human studies is indicated, where the quality of the determinations will be enhanced through inter-laboratory validation.

INTRODUCTION

Proteins constitute more than 50% of the dry weight of cells, and as such can be considered important targets for the effects of oxidising species. The rate of reaction of amino acids with radiolytically generated •OH, is of the same order of magnitude as PUFA and DNA bases [1], indicating that proximity to site of oxidant generation and the presence of bound metal ions are the major determinants of intracellular targets for oxidation.

The earliest work on the oxidation of a biologically important protein was on the effects of selected radicals generated by radiolysis on the enzyme, lysozyme [2]. Both the thiocyanate radical (a selective modifier of tryptophan) and •OH were found to inactivate the enzyme, implying that tryptophan residues are essential for biological activity, now well established from classical enzymology. Similarly, for α-1-antitrypsin, it was found that modification of a single methionine residue at position 358 rendered the protein inactive [3]. Subsequent hydrolysis and amino acid analysis revealed the presence of methionine sulphoxide. This was one of the first pieces of evidence linking amino acid oxidation to denaturation of proteins and loss of function.

The process of protein oxidation frequently introduces new functional groups, such as hydroxyls and carbonyl, which contribute to altered function and turnover. Improved characterisation of the effects of protein oxidation has identified a spectrum of secondary effects including fragmentation, crosslinking and unfolding, which may accelerate or hinder proteolytic and proteasome-mediated turnover, according to the severity of oxidative damage [4].

The effects of reactive oxygen species are not always deleterious; collagenase can be released from its latent form following oxidation by hypochlorous acid, and also by a metal ion dependent product of $O_2^{-\cdot}$ [5,6]. Furthermore, the DNA binding of the transcription factor, AP-1, is modulated via intracellular redox status; the reduction of a single cystine in the DNA binding domain is necessary for the binding of the AP-1 heterodimer [7]. Many modified amino acids have the capacity to exert distant effects, away from the initial site of oxidative attack, and as such, are likely to play an important role in signal transduction processes. The differential induction of MAP-kinases elicited in epithelial cells by peroxynitrite is associated with nitrotyrosine formation, where selenium supplementation attenuated both formation of the nitrated protein and signalling [8].

As the role of ROS as intracellular signalling molecules becomes further elucidated, the importance of oxidised protein moieties as effectors of these pathways can be characterised. The stability of certain oxidised proteins may convey memory in the cells of previous oxidative attack, thereby facilitating a more rapid response to further oxidative insult. From our increasing power to resolve and characterise oxidised proteins, in association with significant advances in cell biology the importance of protein oxidation will begin to be realised.

PROTEIN OXIDATION IN PATHOLOGY

Within the literature, there are many published reports of the presence of elevated protein oxidation products in the "ageing-related" diseases. These include; (1) cataractogenesis, where the opacity of the lens may be due to UV oxidised tryptophan residue accumulation within lens crystallin proteins[9]. These proteins are removed by reticulocytic processes and proteosomes;
(2) Atherosclerosis, where oxidation of the apolipoprotein B moiety on LDL alters receptor recognition[10]. The resultant non-regulated path of LDL uptake is implicated in foam cell formation;
(3) Diabetes, where elevated plasma glucose levels are believed to contribute to protein bound glucose derived oxidising species[11]. These may be important in basement membrane damage to proteins such as collagen, thereby contributing to diabetic complications;
(4) Rheumatoid arthritis, where oxidative modification to IgG has been suggested to lead to a self-perpetuating inflammatory lesion within the affected joint[12]; and
(5) Neurodegenerative diseases, such as Alzheimer's disease, where altered amyloid precursor protein processing result in a peptide capable of generating oxidants, and elevated levels of oxidised protein are reported[13].

Each pathology differs in both the source of oxidising species and the target protein, indicating the random nature of radical attack, governed only by protein size and concentration in the local region of oxidative stress.

An observable increase in reported levels of biomarkers of protein oxidation may arise from either enhanced oxidative stress and/or decreased antioxidant status. Many studies have undertaken analysis of antioxidant capacity in disease. These are summarised in Table 1. This evidence lends credence to the hypothesis that antioxidant insufficiency may accelerate the onset ageing-related diseases. Whilst clinical and epidemiological studies have indicated that

antioxidant nutrients may be effective in disease management, the analysis of their capacity to reduce *in vivo* oxidative damage has barely been touched [14]. The establishment of appropriate biomarkers of protein oxidation will aid analysis of antioxidant efficacy *in vivo*.

BIOLOGICAL SOURCES OF OXIDANT STRESS RELEVANT TO PROTEIN OXIDATION

The superoxide anion radical is considered the primary oxygen radical species formed from a single electron reduction of molecular oxygen. In physiological and pathophysiological systems it can be generated from a variety of different sources. The energy emitted by UV light is insufficient to cause photolysis of water. Rather UV mediates its oxidative damage through direct excitation of susceptible amino acids, such as tryptophan, in the presence of molecular oxygen. Subsequent charge transfer to dissolved oxygen in the aqueous environment results in elevated H_2O_2 concentrations in the immediate environment of the protein.

The mitochondrial respiratory chain carries pairs of reducing equivalents to molecular oxygen, however, leakage of single electrons occurs, which is accentuated during physiological stress and increases during ageing [33]. Mitochondria are implicated in generation of oxidative stress within ageing neurones [34].

The initial bactericidal defence launched against foreign material and invading pathogens is the respiratory burst. First identified in polymorphs but now widely reported in other cell types, a membrane bound NADPH oxidase catalyses the production of $O_2^{-\cdot}$ from molecular oxygen, at the expense of NADPH. Azurophilic granules within PMNs, enriched in myeloperoxidase, catalyse the formation of hypochlorous acid from hydrogen peroxide and chloride. Eosinophils generate a related hypohalous acid from bromide [35]. PMNLs may generate up to 100μmol H_2O_2 per minute at an inflammatory site, and have been implicated in both the pathophysiology of rheumatoid arthritis and atherosclerosis[10,12]. Whilst these species are not radicals *per se*, they are oxidising species, whose biological effects can be limited by antioxidants and are therefore worthy of study.

Protein bound glucose can undergo autoxidative glycosylation, whereby the autoxidation of sugar via dicarbonyl residues generates superoxide radicals adjacent to the protein backbone. Free sugars may also undergo autoxidation in the presence of catalytic metal ions. Elevated plasma concentrations of glucose in diabetes are believed to contribute to enhanced oxidative

stress through these processes although the relative importance of the two pathways remains to be elucidated [11].

The production of nitric oxide (NO) by endothelial and neuronal cells is determined by the activity of distinct isoforms of nitric oxide synthase. The effects of NO in the nervous system and within the vasculature are attenuated by O_2^- . The product of this interaction, peroxynitrite, is a potent oxidising species [36].

Biologically important transition metal ions such as copper and iron redox cycle in the presence of superoxide anion and H_2O_2 to generate the short lived highly reactive hydroxyl radical through Fenton Chemistry. The spectrum of oxidised products formed from •OH damage is broad, and encompasses both aromatic and aliphatic amino acid modifications [4].

CHEMISTRY OF AMINO ACID OXIDATION

Generally, those amino acids capable of delocalising charge are more susceptible to oxidant attack, such as amino acids containing aromatic and sulphur side chains. However, carbonyls are ubiquitous products of oxidation arising from many amino acids in addition to sugars and lipids [37]. Both oxo-acids and aldehydes are formed following oxidative attack, having either the same number or one carbon atom less than the original amino acid [38].

Any study of the formation of carbonyls in a physiological system cannot differentiate between those formed through addition of other oxidised products (e.g. glucose, malondialdehyde [39]) and protein oxidation itself, and thus should be considered as a broad marker of oxidation [37]. Cao and Cutler have extensively investigated the determination of protein carbonyls in tissue, and conclude that the method of DNPH derivatisation is valid only for the study of purified proteins [40].

In contrast, formation of hydroperoxides and hydroxides by the addition of hydroxyl radical in the presence of oxygen, across the side chains of amino acids is relatively specific to aliphatic amino acid side chains. Extensive characterisation of the three isomeric forms of valine and leucine hydroxides has been undertaken by Fu et al [41,42].

The postulated reactions mediating formation of protein (Pr) hydroperoxides and hydroxides following •OH attack are shown in equations 1-6.

1. $PrH + \bullet OH \longrightarrow Pr\bullet + H_2O$

2. $Pr\bullet + O_2$ —— $PrOO\bullet$

3. $PrOO\bullet + e^-$ —— $PrOO^-$

4. $PrOO^- + H^+$ ——PrOOH (hydroperoxide)

5. PrOOH + RH — $PrO\bullet + H_2O + R\bullet$ with possibility of further reaction

6. $PrO\bullet$ + RH ——— PrOH (hydroxide) + $R\bullet$ with possibility of further reaction

Histidine is frequently involved in the co-ordination of metal ions, and thus is a target for metal catalysed oxidation [38]. Generation of 2-oxohistidine is observed during in vitro LDL oxidation, which is inhibitable by vitamin C [43].

The free thiol group of cysteine readily undergoes reversible oxidation to form a disulphide, which can be "repaired " in the presence of a thiol donor such as glutathione. Further oxidation leads to an irreversible oxidation to cysteic acid [44].

The biological significance of methionine oxidation is exemplified by the existence of a specific repair enzyme present in many cell types, methionine sulphoxide reductase. Repair of the initial sulphur radical formed by •OH attack, can also be achieved *in vitro,* through aqueous antioxidants. Whilst methionine sulphoxide was one of the first biologically relevant products of oxidative attack, there is no simple analytical procedure and hence it has not been widely examined. In vitro studies of HDL oxidation have confirmed the early formation of methionine sulphoxide, and furthermore, this is protected by alpha-tocopherol [45].

The light energy emitted by UV radiation can be directly absorbed by tryptophan, producing an excited intermediate. The radical can be dissipated though transfer to dissolved oxygen (to yield $O_2^{-\cdot}$) by antioxidants or through charge transfer; the movement of an unpaired electron along a peptide or protein backbone to a susceptible amino acid of lower redox potential.

Thus, in any given protein, the target of attack may not be the ultimate site of damage. In the absence of charge transfer or repair, tryptophan undergoes a ring opening following •OH attack to yield predominantly N-formyl kynurenine and kynurenine[46].

Tyrosine dimerisation has been proposed to contribute to the aggregation of proteins frequently observed on oxidation. However, since oxygen and antioxidants rapidly repair the phenoxyl radical intermediate, it is only likely to be a significant product in metalloproteins, where the intermediate is stabilised through change in redox status of the associated transition

metal [47]. There are a number of reports demonstrating the generation of protein bound L-DOPA from tyrosine in a dose dependent fashion following •OH attack [48,49]. L-DOPA is a relatively long-lived species which can confer reducing activity in its environment. The nucleophilic non-radical oxidant, HOCl, is particularly reactive with tyrosine, inducing the characteristic chlorotyrosine. A further ring addition product of tyrosine, nitrotyrosine, is formed from attack by nitrating species including nitric oxide, peroxynitrite and reactions between hypochlorite and nitrogen containing species [reviewed in 4].

Hydroxylation of phenylalanine is a typical reaction of hydroxyl radicals, where the resultant tyrosine is hydroxylated at the ortho-, para- or meta- position, and is therefore distinct from native tyrosine.

It is evident from the body of evidence presented, that a "fingerprint" of the oxidising species present *in vivo*, should be attainable by adopting a broad spectrum of biomarkers. Several groups have undertaken studies examining the prevalence of some of the protein oxidation products described above, in disease, and these are summarised in Table 3.

For an oxidised amino acid to be presented as a marker of biological oxidation *in vivo*, it must meet several criteria. Firstly, the biomarker should be generated *in vitro* in a dose dependent manner following exposure to oxidative stress. In addition, it should be a stable product that is not destroyed during sample processing.

Ideally, the formation of the biomarker should be attenuated in vitro by antioxidants. For example, the myoglobin peroxyl radical can be scavenged effectively by ascorbate and vitamin E [57]. However, Stadtman and Berlett [58] demonstrated that Fenton chemistry induced amino acid oxidation could not be protected by antioxidants, indicating a localised site-specific generation of ROS, not available for scavenging by the bulk phase. Recent data from our laboratory investigating the protective effects of antioxidants C and E against formation of L-DOPA and kynurenine induced by metal catalysed oxidation of IgG have shown the effects of ascorbate to be dose dependent (unpublished observations). At concentrations of 100μM, both antioxidants afforded protection against induction of L-DOPA by metal catalysed oxidation. However, a significant induction of L-DOPA occurred in the presence of ascorbic acid (300μM) either alone or in the presence of copper and hydrogen peroxide. This suggests that ascorbate is behaving as a pro-oxidant. However, ascorbate did not induce formation of kynurenine, nor was

any significant protection against kynurenine formation afforded by either vitamin C or E between 30 and 300μM. This suggests that either the intermediate(s) in the pathway to the formation of kynurenine are “caged” and inaccessible to protective factors, or that the intermediates have higher redox potentials than the scavenging species.

Finally, the biomarker should be detectable in normal plasma within the limits of detection of the assay, where the presence of the oxidised product can arise only from non-enzymatic, oxidative processes. Normally, the measurement of oxidised amino acids within protein backbones suffices, as modified amino acids are not incorporated into newly synthesised proteins, whereas products such as L-DOPA may arise as freely from enzymatic degradation of tyrosine.

EFFECTS OF DIETARY ANTIOXIDANTS ON IN VIVO PROTEIN OXIDATION

The use of protein biomarkers as indices of biological protection afforded by dietary antioxidants is in its infancy. We have recently completed a study where, for the first time, a protective effect against protein oxidation following long term supplementation with 400mg vitamin C per day is observed [59]. In a dual phase study, 40 normal subjects initially received 400mg/day vitamin C for 15 weeks following a 5-week placebo period. This was followed by a randomised double blind phase for a further 10 weeks. Immunoglobulin carbonyl content was measured at five weekly intervals and vitamin C supplementation was found to significantly reduce the degree of oxidation after 10 weeks ($p<0.01$), with an overall negative correlation being observed between IgG carbonyl content and ascorbate concentration ($r=-0.1452$, $p=0.0187$). However, after five weeks supplementation, no reduction in carbonyl content was observed and a positive correlation was observed between ascorbate concentration and IgG carbonyl content ($p=0.0275$). These results are consistent with the hypothesis that dietary antioxidants can modulate the extent of plasma protein oxidation. In a smaller study, where 5 subjects received weekly increasing doses of fruit juice over 4 weeks to investigate quercetin antioxidant activity, an increase in plasma levels of 2-amino-adipic semialdehyde was observed [60]. However, erythrocyte levels remained unaffected by dietary quercetin in fruit juice over the duration of the study. Both of these studies indicate that protein biomarkers are sensitive to

antioxidant intervention, where dose and duration of supplementation and protein target may yield further information.

NON-ANTIOXIDANT EFFECTS OF ANTIOXIDANTS

The dietary vitamin C is well known for its Janus face; behaving on the one hand as an antioxidant, and on the other as a pro-oxidant. This has been demonstrated *in vitro*, where ascorbic acid is an effector of oxidative damage, by serving as a reducing agent [61]. Similarly, the formation of the alpha-tocopheryl radical has been suggested to contribute to damaged proteins within atherosclerotic plaques [53]. The efficacy of an antioxidant in scavenging radical intermediates is likely to depend on the absolute concentration of antioxidant at the site of stress and the species of radical generated [62]. Such effects can be more closely analysed through the evaluation of several biomarkers within different proteins.

CURRENT ANALYTICAL PROCEDURES

The methodology applied to the measurement of physiological protein oxidation varies from immunodetection by ELISA or Western blot, to analytical HPLC, capillary electrophoresis and GC-MS. Several antibodies are available for the detection of nitrated tyrosine and the dinitrophenyl hydrazine carbonyl product. These have been recently reviewed by the author [63].

The measurement of protein carbonyls has traditionally depended on the derivatisation of carbonyl groups by dintrophenylhydrazine, with spectrophotometric determination at 360nm of the resultant phenylhydrazone. Partial characterisation of the spectrum of oxidised proteins has been achieved by gel filtration HPLC of the derivatised product. More recent analyses have focused on the use of a monoclonal antibody raised to the hydrazone, with qualitative analysis by Western blot [64]. We have developed an ELISA procedure for quantitative analysis of carbonyl content on specific proteins, which has identified a protective effect of long term supplementation with 400mg vitamin C per day [59,65].

The determination of oxidised amino acids within proteins requires an initial hydrolysis step, to cleave the proteins of interest. The method of hydrolysis is dependent on the endpoint analysis; for example tryptophan and related moieties are destroyed by acid hydrolysis (Table 4).

The hydrolysis step is an area of concern with regard to the possibility of artefactual induction in vitro of oxidised amino acids. Steps should be undertaken to minimise oxidation,

such as through the addition of mannitol. In addition, the presence of lipids in plasma samples can contribute to significant secondary modification, such that delipidation should be undertaken in the early stages of sample preparation.

Protein hydroperoxides are degraded by either enzymatic or acid hydrolysis, and can only be successfully measured in low MW plasma filtrates, where the quenching effect of larger proteins is removed [42]. In addition, they are rapidly broken down in the presence of metal ions [66]. Hydroperoxides can be effectively repaired by antioxidants in vitro, which makes them attractive potential biomarkers. However, for the reasons stated above it is not possible to detect hydroperoxides by chemiluminescence within a quenching environment. Instead, hydroperoxides can be effectively reduced to hydroxides post-extraction, which are stable, long-lived intermediates in protein oxidation. Subsequent analysis of hydroxides has largely been restricted to measurement of leucine, valine and more recently the hydrophilic surface amino acid, lysine [41,67]. HPLC analysis requires a two step procedure; initially samples are fractionated on an amino column, followed by further chromatography of OPA derivatised products using a Zorbax ODS column, with fluorescence detection.

Several groups have undertaken the measurement of oxidised tryptophan metabolites over the past ten years [52,53]. All have utilised reverse phased chromatography, eluting the oxidised residues with increasing acetonitrile concentration. Eluent is monitored with native UV absorption and autofluorescence. Peak identity has been confirmed by spiking with authentic standard, UV and fluorescence spectra. Elevated levels of N-formyl kynurenine have been observed in cataractous lenses, IgG from patients with rheumatoid arthritis and in LDL from atherosclerosis patients [52,53,9].

Detection of the oxidised tyrosine moiety, L-DOPA, following HPLC also relies on native UV absorption (280nm) and autofluorescence (Ex280nm, Em 320nm). The formation of o and m tyrosine from phenylalanine can be observed under the same analytical conditions. In contrast, dityrosine is followed using longer wavelength fluorescence emission at 410nm. The debate as to the physiological relevance of bityrosine, is set to rage with a number of contradictory reports in the literature [13,68]. However, as alluded to in the previous section, the propensity to form bi-tyrosine is likely to be dependent on the structure of the protein under attack.

3-nitrotyrosine can be quantified by ELISA or HPLC using either UV/fluorescence or electrochemical detection [69]. The extra-virgin olive oil antioxidant, hydroxytyrosol inhibits in

vitro generation of nitrotyrosine by peroxynitrite [70]. However, Whiteman and Halliwell have demonstrated the reduction of nitrotyrosine by hypohalous acid, thus questioning the validity of nitrotyrosine as a biomarker [71]. This possibility for destruction by other oxidants may account for some of the ambiguities existing in the determination of nitrotyrosine in atherosclerotic plaques. The significance of tyrosine modification has been extensively reviewed by Crow [72].

Based on these criteria, and the observations of unequivocal elevation in disease, the likely candidates are isolated protein carbonyls (accepting that these may arise from secondary processes), hydroxides, kynurenines, L-DOPA, ortho-tyrosine and chlorotyrosine, whilst the validity of nitro-tyrosine has been questioned [71].

STANDARDISATION

There is a significant requirement for standardisation in the many protocols currently adopted in different laboratories world-wide. This can be provided through the preparation of a quality assurance material containing the native and oxidised amino acids of interest, of at least 10kDa, which can be isolated by acid precipitation. Such a material could be processed through protein extraction procedures and used to confirm minimal *in vitro* oxidation. In addition, different protein hydrolysis methods must be examined, to assess efficiency, yield and potential for artefactual induction of oxidised residues. We have demonstrated the stability of protein carbonyls on storage for 3 months at –80^{o}C. Furthermore, we and others [44,45,48] have confirmed that the protein hydrolysis and extraction procedures used do not induce oxidative damage by incorporating tyrosine and tryptophan into hydrolytic procedures. Such validations are vital before analysis of large numbers of samples in a supplementation trial, or indeed, in any clinical study. There is also a need for appropriate internal standard material, which is not subject to oxidative variation. Again, this may be addressed through the quality assurance procedures.

CONCLUSION

Analysis of protein oxidation has finally come of age. Together with the advances in proteomics, our understanding of the differential susceptibility of proteins to oxidative insult, will be further enhanced. This paper has highlighted the advantages of adopting several biomarkers for studying *in vivo* oxidation of proteins and these methods should now be carefully

applied using quality assurance material, to controlled studies of antioxidant intake, to evaluate the significance of dietary antioxidants in preventing physiological oxidative changes.

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TABLE 1; Antioxidant status in diseases associated with increased protein oxidation

Antioxidant	Cataractous lenses	Atherosclerosis	Diabetes mellitus	Rheumatoid arthritis	Alzheimer's disease
Ascorbic acid	Mild cataract have 88μmol/100mg tissue compared to Advanced cataracts – 50μmol/100g [15]	Reduction in plasma vitamin C hypertensives with elevated LDL oxidation [20]	Reduced plasma vitamin C in diabetes [reviewed in 25].	Loss of ascorbate, majority of plasma ascorbate found as dehydroascorbate [28]	Significantly reduced in AD brain $p<0.001$ [31]
Alpha – tocopherol	4.4μmol/kg lens tissue compared with 33μmol/ml of plasma [16].	Decrease in red cell but not plasma vitamin E [21]			Significantly reduced in AD brain $p<0.01$ [31]
Glutathione and related enzymes	Reductions in GSH and GSH synthetase [17,18]. Increase in GSSG [18].	GSH peroxidase reduced to 65% of normals. Loss of GSH reductase activity [22].	Significantly lower GSH compared to controls [26]		Pathogenesis
Catalase	No loss of catalase [19]	Reduction in neutrophil catalase in hyperlipo-proteinemics [23]		Low levels in synovial fluid [29]	
Superoxide dismutase	Loss of SOD with increasing opacity of lens [19].	MnSOD reduced to 25% of control [24] CuZn not affected	SOD increased [27]	Low activity in synovial fluid and plasma [29,30]	

TABLE 2; Biologically important redox couples.

Redox couple	E^o/V
RS• H^+ / RSH	1.0
Trp• H^+/ Trp	0.98
$Tyro^-$• H^+ / TyrOH	0.65

TABLE 3; The presence of potential biomarkers of protein oxidation in disease

Potential biomarker of protein oxidation	Evidence for accumulation in disease	Selected references
Carbonyls	Ageing, Werner's syndrome Diabetes (eye)	50 51
Hydroxides	Leucine (2) OH in cataractous lens Valine (1) OH and Leucine (2) OH in atherosclerotic plaques	41 48
N-formyl kynurenine and kynurenine	Rheumatoid arthritis LDL from atherosclerosis	52 53
Dityrosine	Aged brain Atherosclerotic plaque	13 48
L-DOPA	Atherosclerotic plaque Cataract	48 49
O and M tyrosine	Atherosclerotic plaque	48
Nitro-tyrosine	Colon cancer Coeliac disease	54 55
Chloro-tyrosine	LDL from atherosclerotic intima	56

TABLE 4; Hydrolysis of proteins for amino acid analysis

Hydrolysis Procedure	Analyte
Gas phase hydrolysis; 5% mercaptoacetic acid, 1% w/v phenol in 6M HCl	Leucine and valine hydroxide [41]
Pronase E, 24 hours	Tryptophan metabolites [52,53]