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A tandem MS precursor-ion scan approach to identify variable covalent modification of albumin Cys34: a new tool for studying vascular carbonylation

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We developed a liquid chromatography electrospray ionisation multi-stage mass spectrometry (LC-ESI-MS/MS) approach based on precursor-ion scanning and evaluated it to characterize the covalent modifications of Cys34 human serum albumin (HSA) caused by oxidative stress and reactive carbonyl species (RCS) adduction. HSA was isolated and digested enzymatically to generate a suitable-length peptide (LQQCPF) containing the modified tag residue. The resulting LQQCPF peptides were identified by LC-ESI-MS/MS in precursor-ion scan mode and further characterized in product-ion scan mode. The product ions for precursor-ion scanning were selected by studying the MS/MS fragmentation of a series of LQQCPF derivatives containing Cys34 modified with different α,β-unsaturated aldehydes and di and ketoaldehydes. We used a Boolean logic to enhance the specificity of the method: this reconstitutes a virtual current trace (vCT) showing the peaks in the three precursor-ion scans, marked by the same parent ion. The method was first evaluated to identify and characterize the Cys34 covalent adducts of HSA incubated with 4-hydroxy-hexenal, 4-hydroxy-*trans*-2-nonenal (HNE) and acrolein (ACR). Then we studied the Cys34 modification of human plasma incubated with mildly oxidized low-density lipoproteins (LDL), and the method easily identified the LQQCPF adducts with HNE and ACR. In other experiments, plasma was oxidized by 2,2′-azobis(2-amidinopropane) HCl (AAPH) or by Fe^{2+}/H_2O_2. In both conditions, the sulfinic derivative of LQQCPF was identified and characterized, indicating that the method is suitable not only for studying RCS-modified albumin, but also to check the oxidative state of Cys34 as a marker of oxidative damage. Copyright © 2008 John Wiley & Sons, Ltd.

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KEYWORDS: oxidative stress; human serum albumin; reactive carbonyl species; Cys34; LC-ESI-MS/MS analysis; precursor-ion scan

INTRODUCTION

Reactive carbonyl species (RCS) are important cytotoxic mediators that affect cell function by signaling to the nucleus, up-regulating redox-sensitive transcription factors, and inducing irreversible structural modifications of biomolecules.[1–3] Among the RCS formed in physiopathological conditions, the α,β-unsaturated aldehydes [4-hydroxy-*trans*-2-nonenal (HNE), acrolein (ACR), and 4-hydroxy-2*E*-hexenal (HHE)], and the dialdehydes [malondialdehyde (MDA) and glyoxal (GO)] are the most abundant and toxic lipid-derived compounds, generated through β-cleavage of hydroperoxides from ω-6 polyunsaturated fatty acids (arachidonic and linoleic acid).[4]

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RCS are generally considered to be involved in several pathologies, such as cardiovascular and neurodegenerative diseases, but it is not clear whether they are agents of cause or effect.[5] However, this important issue needs to be clarified in order to consider RCS as drug targets or as biomarkers or predictors of human diseases. A specific and sensitive analytical approach is needed to identify and characterize RCS and the corresponding adducts with proteins in different physiopathological conditions in an accessible fluid such as plasma. Currently plasma RCS is mainly analyzed by measuring either: (1) free aldehydes, using a hyphenated technique (i.e. HPLC-MS or GC-MS) or (2) the RCS adducts with reactive and nucleophilic macromolecules, using immunological techniques.[6,7] Both approaches have been widely applied to give an indication of systemic and tissue carbonylation, but they cannot identify and characterize unknown aldehydes in free or adducted form, or known RCS when

no suitable standards (chromatographic analyses) or specific antibodies (immunological detection) are available, or are not taken into account. However, an analytical strategy to map a wide range of known and unknown RCS is needed to understand how they are involved in different oxidative-based disorders, and to formulate an appropriate pharmacological strategy (the choice of the carbonyl-quenching compounds depends on the chemical class of RCS involved).[4]

The present study was therefore designed to establish a specific and sensitive analytical approach to identify and characterize electrophilic RCS covalently linked to an endogenous target protein, without requiring external standards.

Conventional proteomic approaches indicate albumin as the main target of carbonylation in serum.[8,9] Albumin can be carbonylated by several mechanisms, such as direct oxidative reactions sustained by reactive oxygen species (ROS) or covalent adduction induced by electrophilic compounds such as HNE and ACR.[10] Proteomic and mass spectrometric (MS) investigations[11–13] have found that albumin is a reactive target protein for RCS in plasma, acting as an endogenous target/quencher of RCS; this strongly suggests that RCS in plasma are mainly delivered as human serum albumin (HSA) adducts.

The high reactivity of HSA towards electrophilic RCS[12,14] is due to several accessible nucleophilic residues, mainly Cys34, followed by His146 and Lys199.[12,13] Thus, considering HSA Cys34 as the main target of carbonylation in human serum, the MS strategy proposed here seeks to identify covalent modifications induced by oxidative/carbonylation damage.

Cys34 is a sensitive residue for various posttranslational modifications besides carbonylation, including S-nitrosylation, disulfide formation and oxidation to sulfenic, sulfinic and sulfonic derivatives. These have all attracted interest on account of their correlations with physiopathological conditions such as intrauterine growth restriction and renal disease (Cys34 cysteinylation and homocysteinylation),[15,16] oxidative stress (Cys34 oxidation),[17] and glomerulosclerosis (Cys34 sulphonation).[18] A MS method to identify and characterize all the Cys34 covalent modifications would be valuable not only for studying RCS-modified albumin, but also to check the oxidative state of Cys34 as a marker of oxidative damage.

We describe an MS strategy for rapid identification and characterization of Cys34-covalent modifications, based on the precursor-ion scanning technique. This gives rapid confirmation of targeted compounds, or nontargeted detection of compounds with a common moiety, and has been widely used in drug discovery and development[19] and *in vitro/in vivo* drug-metabolism studies.[20–22] We have employed this approach for rapid identification of free and protein-bound histidine (His) residues modified by RCS in urine from Zucker-obese rats; in this nondiabetic animal model characterized by obesity and hyperlipidemia, RCS formation plays a key role in the development of renal and cardiac dysfunction.[23]

EXPERIMENTAL

Chemicals

Custom-synthetized LQQCPF peptide, with 90% purity was supplied by Sigma–Aldrich (Milan Italy). HHE and 4-hydroxy-non-2-enal diethylacetal (HNE-DEA) were generous gifts from E. Santaniello (University of Milan, Italy). HNE was prepared by hydrolysis of HNE-DEA with 1 mM HCl (1 h at room temperature) and titrated by UV spectroscopy (λ_{max} 224 nm; ε 13 750 l mol^{-1} cm^{-1}). ACR, crotonaldehyde (CRO), GO, methylglyoxal (MGO), and nonenal (NONE) were purchased from Fluka (Buchs, Switzerland). MDA was prepared by acid hydrolysis of MDA bis (diethyl acetal) (Sigma–Aldrich, Milan, Italy). Sequence-grade modified trypsin was obtained from Promega (Milan, Italy) and chymotrypsin from Roche Diagnostics S.p.A. (Monza, Italy); iodoacetamide, (±)-*threo*-1,4-dimercapto-2,3-butanediol (DTT) from Sigma-Aldrich (Milan, Italy). LC-grade and analytical-grade organic solvents were from Merck (Bracco, Milan, Italy). LC-grade water (18 mΩ) was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) was purchased from Wako (Società Italiana Chimici, Rome, Italy). All other reagents were of analytical grade.

Preparation of LQQCPF-RCS adducts and ESI-MS direct infusion analysis

LQQCPF (final concentration 200 μM) was incubated with 2 mM of aldehyde (HNE, HHE, CRO, GO, MGO, NONE, ACR or MDA) in 1 mM phosphate buffer (pH 7.4) and incubated for 24 h at 37 °C. The peptide adducts were reduced by incubation with $NaBH_4$ (final concentration 5 mM) for 60 min at 37 °C, followed by a desalting step using a Supelco Discovery 1 ml/100 mg DSC-18 column (Sigma–Aldrich Milan, Italy). Samples of reduced or not-reduced adducts (50 μl) were diluted 1 : 10 with CH_3CN : H_2O : HCOOH 50 : 50 : 0.1 vol/vol/vol; these were analyzed by ESI-MS (direct infusion), at a flow rate of 5 μl min^{-1} on a TSQ Quantum Ultra (Thermo Finnigan, Milan, Italy), in the following operating conditions: capillary temperature 270 °C; ionization voltage 5 kV; capillary voltage 12.5 V. The flow rate of the nebulizer gas (nitrogen) was 0.5 l/min. MS/MS analyses were done at collisional energies of 20, 30, 40, and 50 V.

LC-ESI-MS analysis of LQQCPF-RCS adducts in precursor-ion scan mode

A mixture containing native LQQCPF and the reduced form of the corresponding Cys34 adducts with HNE, HHE, CRO, GO, MGO, NONE, ACR, and MDA (25 μM for each analyte) was prepared in 1 mM phosphate buffer and diluted 1 : 1 with CH_3CN : H_2O : HCOOH 50 : 50 : 0.1 vol/vol/vol. Samples (50 μl) were analyzed by LC-ESI-MS in the following conditions: separation by reversed-phase elution with an Agilent Zorbax SB-C18 column (4 mm i.d., particle size 3.5 μm) (CPS Analitica, Milan, Italy) protected by an Agilent Zorbax R-P guard column thermostated at 25 °C. An 80-min linear gradient from 100% A (0.1% HCOOH in water) to 60% B (CH_3CN : H_2O : HCOOH; 90 : 10 : 0.1, v/v/v) was followed by 20 min equilibration to elute the peptide pool resulting from enzymatic digestion; the mobile phase was delivered

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at a flow rate of 0.2 ml min^{-1}. Samples were desalted and concentrated online using an Opti-Lynx C18 (40 μm) trap cartridge (El-Chimie srl, Bresso, Milan, Italy) installed in the Rheodyne 7010 injection valve loop (Rheodyne, USA).

ESI-MS analyses were done using a triple quadrupole TSQ Quantum Ultra (ThermoQuest, Milan, Italy); the ESI source was set in the positive-ion mode, under the following conditions: capillary temperature 270 °C; spray voltage 4.5 kV; capillary voltage 12.5 V. The flow rate of the nebulizer gas (nitrogen) was 5 l min^{-1}.

Two MS runs were done for each sample: one in data-dependent-scan mode, permitting dynamic exclusion under the following conditions: repeat count 1, repeat duration 0.5 min, exclusion duration 2 min. The second run was in precursor-ion scan mode, selecting the following product ions: m/z 242.1, 263.1 and 370.2 (collisional voltage 40 V). A mass unit resolution and scan time of 1 s were used in both quadrupoles Q1 and Q3, and the Q1 scan range was set at m/z 300–1500.

The adducts were then characterized in product-ion scan mode in the following conditions: Q1 peak width 0.7, scan time 1 s, collision energy 30 V, scan range 50–1500 m/z.

Preparation of albumin–RCS adducts

Blood samples from six healthy normolipidemic volunteers aged between 25 and 30 years (mean HSA content 570 ± 34 μM) were collected in citrate-containing tubes after an overnight fast. Plasma was separated by centrifugation at 2000 g for 20 min (4 °C) and 500-μl aliquots were stored in liquid nitrogen until use. Albumin (HSA) was isolated using the Montage Albumin Deplete Kit (Millipore, Milan, Italy) and eluted with 1 M NaCl.[24] (see supplementary material for direct infusion MS spectra of isolated and commercial HSA). HSA concentration was measured by absorbance at 279 nm (E 1% 1 cm = 5.31). Samples were then desalted on Microcon YM30 centrifugal filter devices (Millipore, Milan, Italy) by rinsing with distilled water, and diluted with 20 mM phosphate buffer saline (PBS, pH 7.4) to a final concentration of 20 μM. HNE, HHE or ACR was added at a 1 : 10 HSA : RCS final molar ratio. After 120 min incubation at 37 °C, samples were incubated at 37 °C for another 60 min with $NaBH_4$ (final concentration 5 mM), then desalted by Microcon YM30 filter devices as described above, lyophilized and stored in liquid nitrogen until required for digestion.

Isolation of LDL, oxidation, and plasma incubation

Low-density lipoproteins (LDL) from human-pooled sera were isolated by ultracentrifugation according to Havel *et al.*[25] and dialyzed against PBS containing 0.01% EDTA at 4 °C. Mildly oxidized LDL were obtained by incubating metmyoglobin/H_2O_2 (18/27 μM) in PBS at 37 °C for 2 h, as described by Vieira *et al.*[26] followed by extensive dialysis against PBS at 4 °C. LDL oxidation was evaluated by measuring lipid hydroperoxides, thiobarbituric reactive substances (TBARS), and HNE content.[27] Lipid peroxidation levels ranged between 55 and 74 nmol lipid hydroperoxides/mg apoB, between 5 and 10 nmol TBARS/mg apoB, and between 15 and 20 nmol HNE mg/apoB, very close to values already reported.[26] OxLDL were incubated with human plasma for 2 h in a thermostated shaker water-bath at 37 °C and at a final concentration of 1 mg apoB/ml plasma. At the end of incubation HSA was isolated as described above.

Radical-induced oxidation of human plasma

Radical-induced oxidation of human plasma was started by the radical initiator AAPH or using Fe^{2+}/H_2O_2, as already described.[28,29] Briefly, plasma aliquots (250 μl) were spiked with 25 μl of 0.5 M AAPH solution or 25 μl of H_2O_2 (1 M) and 25 μl of $FeSO_4$ (2 mM). After overnight incubation at 37 °C, BHT was added to stop the radical reaction, and HSA was isolated by affinity chromatography, desalted and lyophilized as above.

Albumin enzymatic digestion and analysis by LC-ESI-MS in precursor-ion mode

Lyophilized albumin (420 μg) was dissolved in 100 μl of 100 mM Tris-HCl (pH 7.8) solution containing 6 M urea, DTT (5 μl of 200 mM solution), and heated at 60 °C for 60 min. Trypsin and chymotrypsin digestion was then done as previously described.[11] The digested samples were treated with TCA (final concentration 10%), centrifuged at 18 000 × g for 10 min and 50 μl of the supernatants were injected into a quaternary pump HPLC system (Surveyor LC system, ThermoQuest, Milan, Italy). LC-ESI-MS analyses were done in data-dependent-scan mode with precursor-ion scanning as reported above.

RESULTS AND DISCUSSION

The approach proposed, summarized in Scheme 1, involves the isolation and enzymatic digestion of HSA, to generate a suitable-length peptide containing the Cys34 residue (LQQCPF) and the corresponding adducted derivatives, which are identified by LC-ESI-MS/MS in precursor-ion scan mode, then characterized in product-ion scan mode. We used LQQCPF peptides adducted by a series of different RCS or albumin exposed to selected α,β-unsaturated aldehydes, which were then employed to identify and characterize the covalent modification of Cys34 in human plasma incubated with mildly oxidized LDL, and with different radical inducers to promote lipid peroxidation.

Development of the method: ESI-MS properties of RCS-LQQCPF adducts

The LQQCPF peptide obtained by trypsin and chymotrypsin digestion had previously been identified as a suitable tag for covalently modified HSA.[12,13] In developing the new precursor-ion scanning approach, the first step was to select, from among its most abundant and stable products, the ions arising from a fragmentation pathway not involving the target residue, such as the b and y ions which are respectively before and next to Cys34.

These were selected by a two-step approach. First, on the basis of the theoretical fragmentation pattern, we generated a list of potential product ions not containing the Cys34 residue, namely y1 (m/z 166.1), y2 (m/z 263.1), b2 (m/z 242.1), b2-NH_3 (m/z 225.1), b3 (m/z 370.2), b3-NH_3 (m/z 353.2), the retro-Michael fragment arising from the neutral loss of the RCS at m/z 735.3 (RM) and the immonium ions

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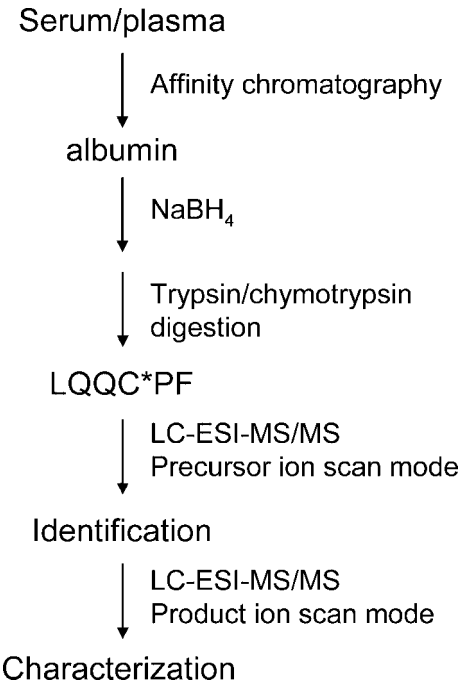

Scheme 1. Experimental procedure for the identification and characterization of the variable covalent modifications of Cys34 human serum albumin.

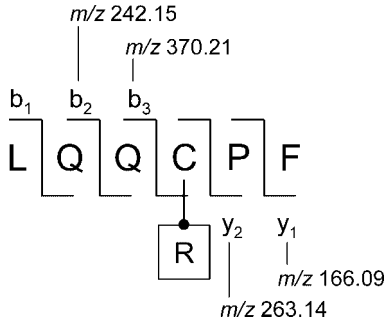

Figure 1. Theoretical fragmentation pattern of the hexapeptide LQQCPF, obtained by trypsin/chymotrypsin digestion of HSA. R indicates the variable covalent modification.

of Leu (m/z 86.0), Gln (m/z 101.1), Cys (m/z 76.0), Pro (m/z 70.1), and Phe (m/z 120.1) (Fig. 1).

The second step involves selection of potential product ions that are abundant, stable and diagnostic for both native and adducted LQQCPF, and whose formation is independent of the moiety linked to the Cys34 residue. We therefore prepared a series of LQQCPF adducts using various RCS, in this case α,β-unsaturated aldehydes, with different side chains (HNE, HHE, ACR, CRO, NONE), dialdehydes (GO and MDA) and ketoaldehyde (MGO). The covalent products were identified and characterized by ESI-MS and ESI-MS/MS analysis (direct infusion analysis, positive-ion mode). As an example, Fig. 2 shows the ESI-MS spectra in positive-ion mode of LQQCPF incubated with and without HHE, dissolved in phosphate buffer and diluted with the infusion medium; the spectrum is dominated by the ion at m/z 735.4 $[M + H]^+$, accompanied by the corresponding cationized species at m/z 757.4 $[M + Na]^+$ (Fig. 2(A)). The spectrum for the reaction mixture containing LQQCPF incubated for 24 h with HHE is characterized by the $[M + H]^+$ of the unmodified LQQCPF, and also the ion at m/z 849.5, attributed to the $[M + H]^+$ of the Michael adduct with HHE (confirmed by MS/MS analysis) (Fig. 2(B)). The ions at m/z 871.5 corresponds to the cationized adduct, $[M + Na]^+$. Owing to the soft ionization afforded by ESI, it is unlikely, however, that m/z 831.5 belongs to an $[M + H - H_2O]^+$ species. A more plausible explanation is that m/z 831.5 corresponds to $[M + H]^+$ of the Schiff-base adduct formed between the terminal amine group (of Leu) and HNE. Such a modification only occurs with the model peptide and it cannot be formed by the reaction of HSA with RCS.

Table 1 summarizes the reaction products involving the Cys34 residue. All the α,β-unsaturated aldehydes formed the corresponding Michael adducts, and ACR and CRO gave an additional cross-link with an amino propene structure, involving the terminal amino group of the peptide (data not shown). The reaction product between MDA and LQQCPF was attributed to a thio-propenal adduct through Michael adduction, followed by a dehydration step. GO and MGO formed the corresponding hemi-thioacetal derivatives.

We selected the diagnostic product ions for LC-ESI-MS/MS analysis on the basis of the relative abundances of the fragment ions at different collision energies (20, 30, 40, and 50 V) for native and modified LQQCPF. Figure 3 shows the isoplots of the relative intensities of these target product

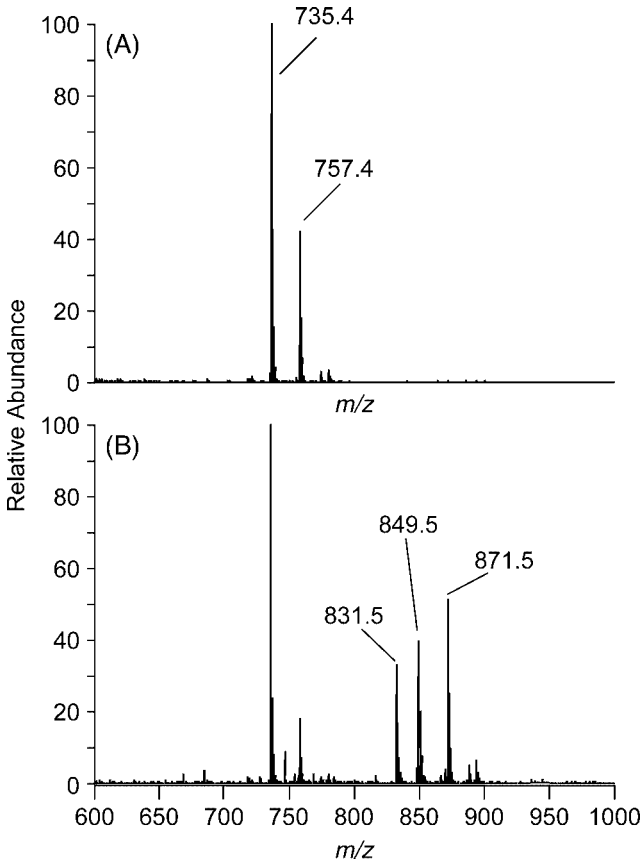

Figure 2. Positive ESI-MS spectrum (direct infusion experiment) of LQQCPF incubated for 24 h at 37 °C, without (panel A) and with (panel B) HHE. For experimental details refer the Experimental Section.

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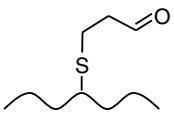
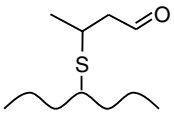
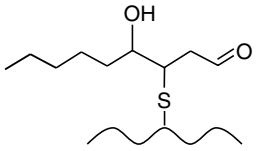
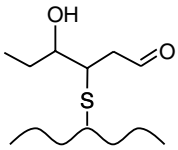
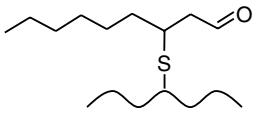
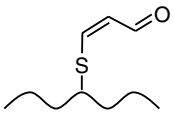
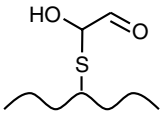
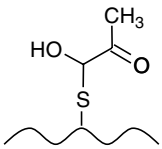
Table 1. Assigned structures and $[M + H]^+$ values of the LQQCPF adducts obtained by incubating the hexapeptide with aldehyde in a 1 : 10 molar ratio at 37 °C for 24 h. The $[M + H]^+$ of the adducts after $NaBH_4$ reduction are also shown

	LQQC*PF adduct			
Aldehyde	Structure	Abbreviation	$[M + H]^+$	$[M + H]^+$ ($NaBH_4$)
ACR		LQQC(ACR)PF	791.5	793.5
CRO		LQQC(CRO)PF	805.5	807.5
HNE		LQQC(HNE)PF	891.6	893.6
HHE		LQQC(HHE)PF	849.6	851.6
NONE		LQQC(NONE)PF	875.6	877.6
MDA		LQQC(MDA)PF	789.5	791.5
GO		LQQC(GO)PF	793.9	795.9
MGO		LQQC(MGO)PF	807.5	809.5

ions at the different collision energies. At 20 V, the only detectable product ions were the retro-Michael fragments for LQQC(HNE)PF, LQQC(HHE)PF and LQQC(MGO)PF. At 30 V, the b2 and b3 ions were clearly detectable for all the covalent adducts, but the intensity of the y ions was negligible. At 40 V, b2, b3 and y2 were prominent for all the adducts, together with the immonium ion of the Leu residue (m/z 86.1). At 50 V, the b-ion series was conserved, while the y ions had almost completely disappeared.

We then did a similar study for the LQQCPF adducts reduced with $NaBH_4$. This procedure is required to stabilize the carbonylated peptide adducts and prevent them being lost during protein digestion (DDT for 60 min at 70 °C).

ESI-MS and MS/MS direct infusion experiments confirmed, for each adduct, the quantitative reduction of the aldehyde to the alcohol group, as evidenced by the $[M + H]^+$ values listed in Table 1. The fragmentation patterns at different collision energies were superimposable on those with nonreduced LQQCPF adducts, except for the retro-Michael fragments which were not detectable at any collision energy.

From these results we identified the collision energy that gives the most abundant, stable, and diagnostic fragment ions for LQQCPF covalently modified at the Cys34 residue, regardless of the adducted moiety. From the isoplots, we selected the following product ions at a collision energy of 40 V: y2 (m/z 263.1), b2 (m/z 242.1) and b3 (m/z 370.2).

The ESI-MS/MS was then coupled to LC to analyze a mixture of native and covalently modified LQQCPF, using the product ions listed above. The total ion current (TIC) showed several peaks eluting between 60 and 95 min (Fig. 4(A)). The ion current traces in precursor-ion scanning mode were superimposable (panels B, C, D of Fig. 4), and permitted the identification of all eight LQQCPF adducts in the mixture, as confirmed by the $[M + H]^+$ of the precursor ions and the MS/MS

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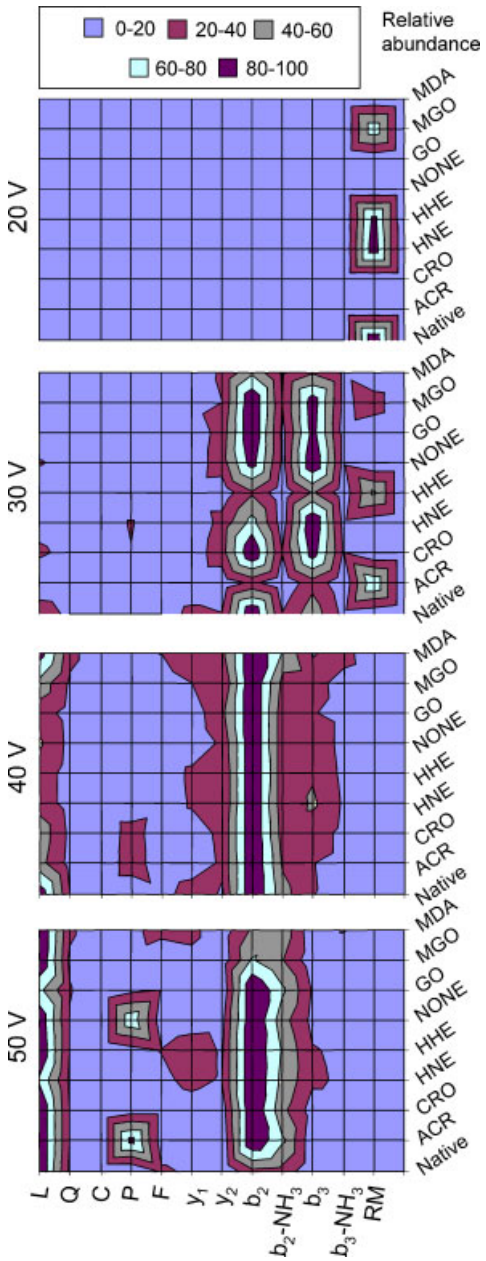

Figure 3. Isoplots showing the relative intensities of the selected-fragment ions in relation to the collisional energies (20, 30, 40, and 50 V).

spectra obtained in product-ion scan mode (data not shown).

Two additional peaks, not detectable in the precursor-ion currents, were seen in the TIC trace at 66.5 min and 69.9 min, and assigned to the LQQCPF peptides cross-linked by ACR and CRO. Structure attribution was based on direct infusion experiments (data not shown), indicating that the cross-linked peptides had different fragmentation patterns from LQQCPF adducted to Cys34, not including the b2, b3 and y2 ions.

Application of the method: LC-MS/MS analysis of covalently modified HSA

The method was applied to albumin incubated with HNE, HHE and ACR. Figure 5(A) shows the TIC for digested native HSA, with several peaks corresponding to the different peptides. The precursor-ion scan current recorded with the product ion set at m/z 242.1 (Fig. 5(B)) contains not only a peak eluting at 49.3 min relative to the carboamidomethylated LQQCPF peptide [LQQC(CAM)PF] (precursor ion at m/z 792.5) but several other peaks too. The product ions at m/z 263.1 and 370.2 (panels C and D of Fig. 5) gave similar results.

To boost the specificity of the method, we exported the data for the precursor-ion scans in Excel (Microsoft Office Professional Edition, 2003) and analyzed them using the following Boolean logic: if value1 $>$ *ts*, value2 $>$ *ts*, and value3 $>$ *ts* $\rightarrow$ value\$ $=$ value1 $+$ value2 $+$ value3 or else value\$ $= 0$, where value1, value2, value3 are the ion currents recorded at each time point, setting the product ions at m/z 242.1, 263.1, and 370.2; value\$ is the output data and *ts* is the threshold ion current (Fig. 6). Thus, for each time point, the data for the ion current of a selected precursor-ion scan is kept and summed with the data of the subsequent precursor-ion scan only when its value is higher than a certain threshold, otherwise it is discarded. The output data are reconstituted as a virtual current trace (vCT) where the peaks displayed are the sum of those present in all three precursor-ion scan traces, while those lacking in at least one trace are automatically discarded.

Figure 5(E) shows the vCT for digested native HSA, with a main peak at 49.3 min for LQQC(CAM)PF, and two minor peaks at 38.3 and 57.5 min. To confirm that the peaks identified in the vCT referred to a LQQCPF adduct, and to exclude artifacts, we examined the MS spectra for the peaks in each precursor-ion scan trace manually. Peak (a) for digested native HSA was discarded because the precursor ions were different (m/z 774.1, 723.1, and 769.5). For the same reason we erased peak (c), and peak (b) was selected, since all three precursor-ion scans showed the same precursor ion at m/z 792.5 (Fig. 5 panel F).

Figure 7 (panels A, B, C) shows the precursor-ion scan traces of HSA adducted by HNE and digested with trypsin/chymotryspin. Panel D of Fig. 5 gives the vCT, which identifies only two peaks attributed to native LQQC(CAM)PF and LQQC(HNE)PF, confirmed on the basis of the retention time (RT) and the precursor-ion values (m/z 792.5 and 893.6).

This approach was then employed successfully to study the Cys34-covalent modifications of HSA incubated with the other two α,β-unsaturated aldehydes, HHE and ACR. The Michael adducts between LQQCPF and HHE (parent ion m/z 851.6) and ACR (parent ion m/z 793.5) (data not shown) were easily identified.

As a further validation of the approach, Fig. 8 reports the vCT of a mixture containing digested HSA after incubation with HHE, HNE, ACR (5 μM final concentration for each analyte): all the peaks relative to LQQC(CAM)PF (peak a), LQQC(ACR)PF (peak b), LQQC(HHE)PF (peak c) and LQQC(HNE)PF (peak d) were easily identified and characterized.

The feasibility of the method was further confirmed in biological samples under conditions mimicking the *in vivo* generation of RCS, by studying the covalent modifications of Cys34 after incubation of human plasma with mildly

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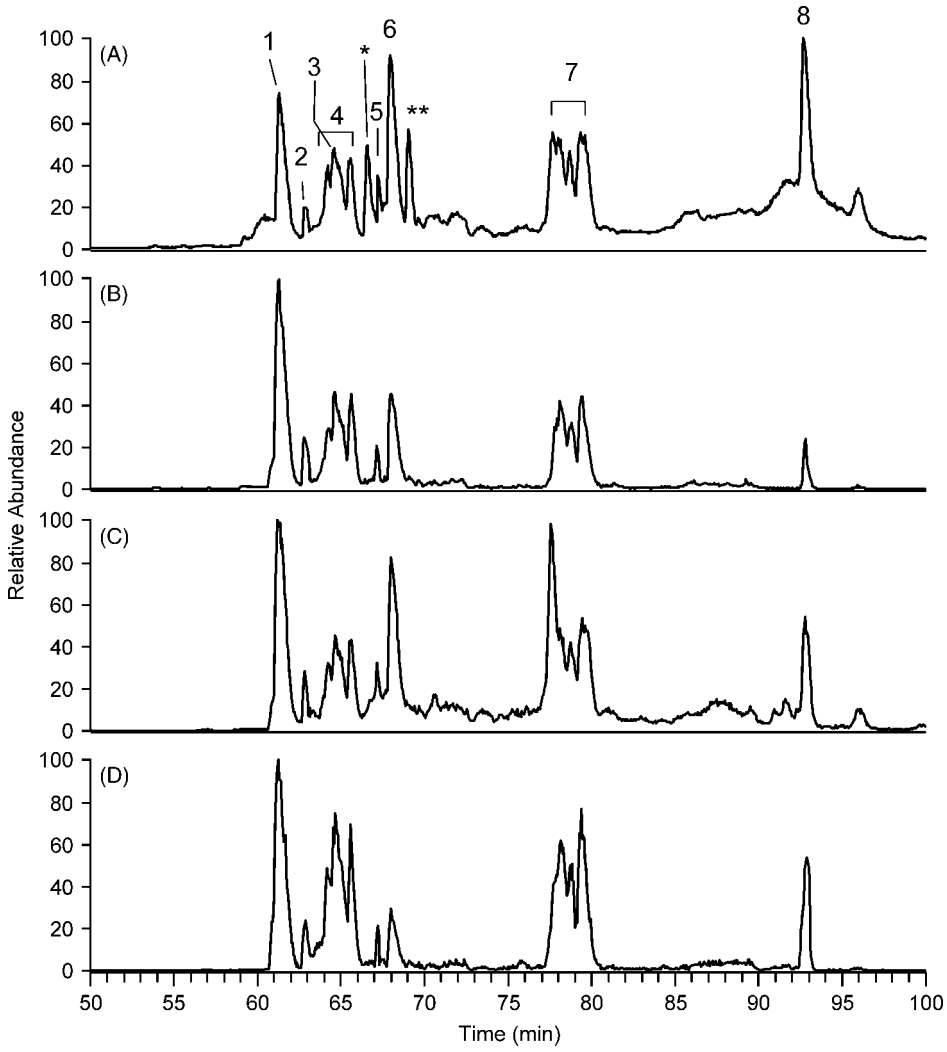

Figure 4. LC-ESI-MS/MS analysis in data-dependent-scan mode and precursor-ion scanning of a mixture of LQQCPF adducts with RCS. Panel A: total ion current (TIC) recorded in data-dependent-scan mode (scan range *m/z* 100–1500); panels B,C,D: precursor-ion scanning currents recorded setting the product ions at *m/z* 242.1, 263.1, 370.2. 1: LQQC(ACR)PF; 2: LQQC(MGO)PF; 3: LQQC(CRO)PF; 4: LQQC(HHE)PF (4 diastereoisomers); 5: LQQC(GO)PF; 6: LQQC(MDA)PF; 7: LQQC(HNE)PF (4 diastereoisomers); 8: LQQC(NONE)PF; * and ** are the LQQCPF cross-linked adducts (with amino propene structure) of ACR and CRO, respectively.

oxidized LDL. Two main peaks were identified, relative to LQQC(HNE)PF and LQQC(ACR)PF (data not shown), confirming that HNE and ACR are the main aldehydes released from oxidized LDL, as already shown using immunochemical procedures.[30,31] Several other minor, but clearly detectable peaks were also identified (currently being characterized).

The method was finally applied to human plasma exposed to a flux of radicals generated by the radical inducer AAPH or by Fe^{2+}/H_2O_2 to promote lipid peroxidation. Figure 9 (panel D) shows the vCT trace for the AAPH experiment before manual analysis of the precursor ions. There is a main peak at 49.0 min, attributed to the LQQC(CAM)PF peptide, and three other peaks. Peaks (a) and (d) were discarded because the precursor ions were different in all three ion traces (Fig. 9, panels A, B, C), but peak (b) was maintained in the final vCT (panel E), as it had a precursor ion at *m/z* 767.3.

The increase of 32 Da from LQQCPF clearly indicates thiol oxidation to the sulfinic acid derivative [LQQC(SO_2H)PF], which was confirmed by LC-ESI-MS/MS in product-ion scan mode (data not shown). No Cys34-RCS adducts were detected in this experimental model, and results were similar with HSA isolated from human plasma oxidized by Fe^{2+}/H_2O_2 (data not shown). Thiols can in fact be oxidized in a two-electron oxidation pathway beyond disulfide (RSSR) to yield sulfenic acid (RSOH) or further oxidized, in the presence of an excess of oxidants, to sulfinic (RSO_2H) or sulfonic (RSO_3H) acid derivatives.[32] In our conditions, Cys34 was almost completely consumed, forming the sulfinic acid derivative. This explains why no other LQQCPF adducts were identified, and at the same time indicates that Cys34 acts as a first defense against oxidants.

Although oxidation of Cys protein residues to sulfenic, sulfinic, and sulfonic acids by H_2O_2 has already been reported, to our knowledge there is no information on the effect of AAPH on albumin Cys34. Our data clearly show that the peroxyl radicals generated by thermal decomposition of AAPH oxidize Cys34 to sulfinic acid, through a mechanism involving formation of the thiyl radical ($RS^{\bullet}$) which can

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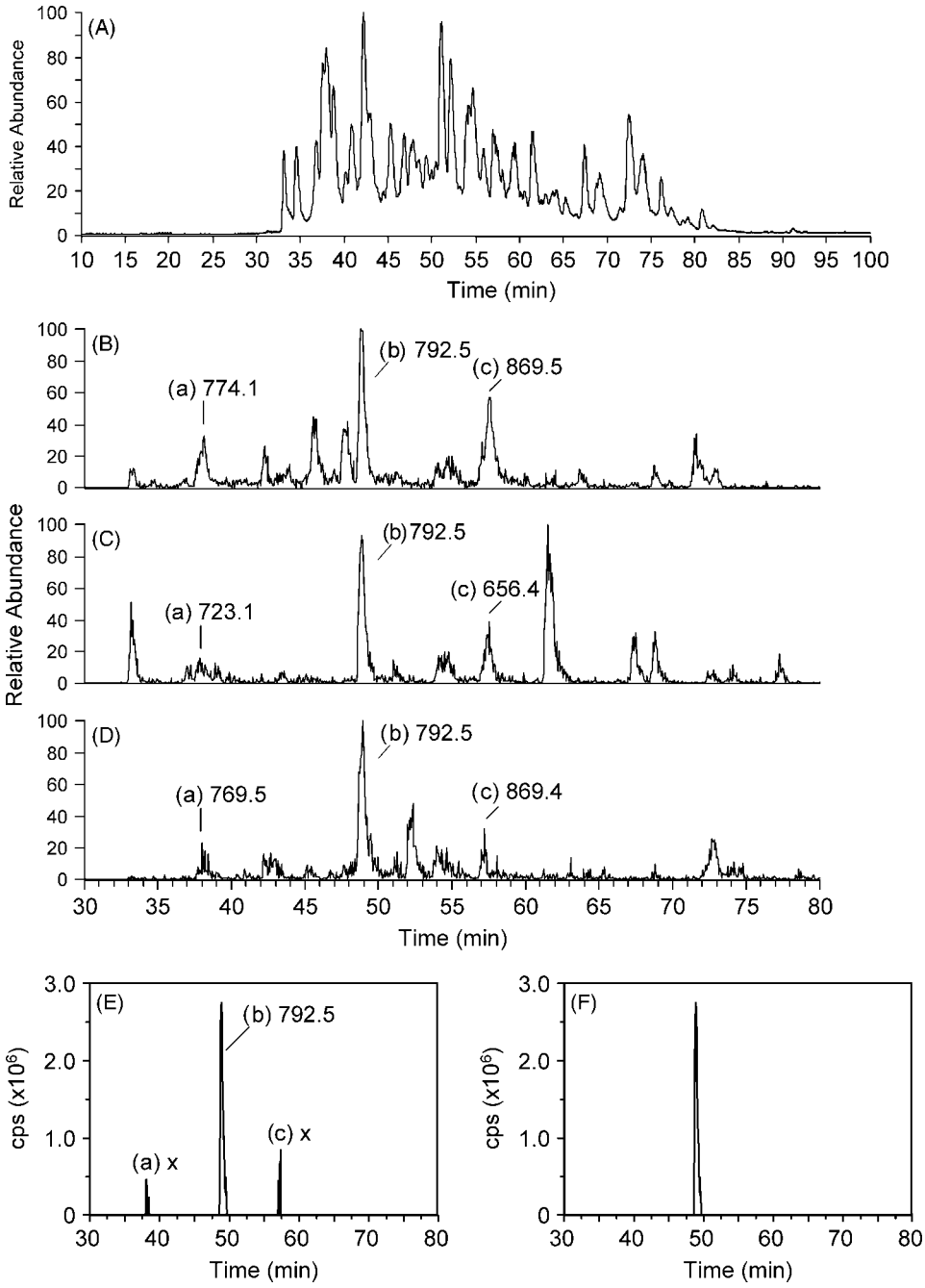

Figure 5. LC-ESI-MS/MS analysis in data-dependent-scan mode and precursor-ion scanning of trypsin/chymotrypsin digested native HSA. Panel A: TIC recorded in data-dependent-scan mode (scan range 100–1500); panels B,C,D: precursor-ion scanning currents recorded setting the product ions at *m/z* 242.1, 263.1, 370.2, respectively; panels E, F: virtual current traces (vCT) before (E) and after (F) manual examination of the precursor ions in the three precursor ion scanning currents.

trigger an oxygen-dependent chain reaction to produce sulfinyl radical (RSO•) that evolves to yield sulfenic acid and then sulfinic acid.[33] Therefore, the method described is suitable not only for studying RCS-modified albumin but also to check the oxidative state of Cys34 as a marker of oxidative damage.

CONCLUSIONS

In this study we employed LC-ESI-MS/MS in precursor-ion scan mode to identify and characterize the covalent modifications of Cys34 in HSA. This provides a valuable means of studying vascular carbonylation, since it gives detailed information on the structure of the adducted reactive aldehyde generated in the vasculature from lipid peroxidation. The method was successfully validated by identifying HNE and ACR as the main aldehydes generated by mildly oxidized LDL, as already found in immunochemical analyses.[30,31] These data confirm that LDL oxidation is a source of vascular carbonylation, and suggest that Cys34 acts as a physiological detoxifying agent in the vasculature (further studies are in progress on this aspect).

The method also gives information on the oxidative state of Cys34, demonstrated by incubating human plasma under

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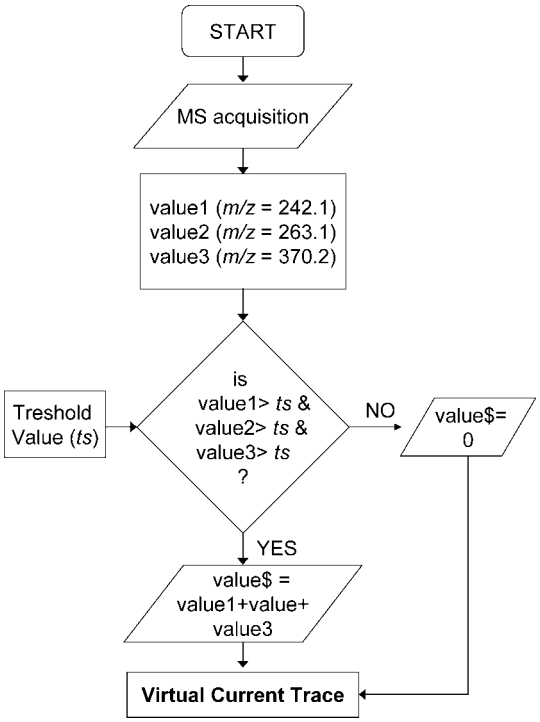

Figure 6. Flow chart of the Boolean logic used to make the method more specific. Value1, value2, value3 are the ion currents recorded for each time point by setting the product ions at *m/z* 242.1, 263.1, and 370.2; value$ is the output data.

conditions to promote lipid peroxidation. No adducted aldehydes to Cys 34 were observed in the presence of two different radical inducers. This is not surprising in view of the thiol groups' high reactivity to oxidation under drastic conditions, far removed from the *in vivo* setting.

At present we cannot exclude aldehyde adduction to other reactive nucleophilic sites on albumin, such as His146 or Lys199, as a consequence of Cys34 saturation. This study focused on Cys34 modified by RCS, but the precursor-ion scanning technique should be applicable generally to other digested tag peptides such as those containing the target His146 and Lys199 residues H*PY and LK*CASLQK. We are currently investigating this, and also testing the overall method for recognizing changes of HSA in human diseases involving systemic oxidation/carbonylation, particularly end-stage renal disease, where RCS-induced tissue damage is a known feature.

We believe that a specific and sensitive MS assay to identify and characterize RCS in physiopathological conditions would significantly improve our knowledge in the protein carbonylation field, and help reach the following goals: (1) a better understanding of the pathogenic role of RCS, (2) predicting and following the progression of the disease, (3) identifying new drug targets, and (4) demonstrating the efficacy of carbonyl sequestering agents, a new class of compounds to prevent carbonylation damage.[34] The strategy

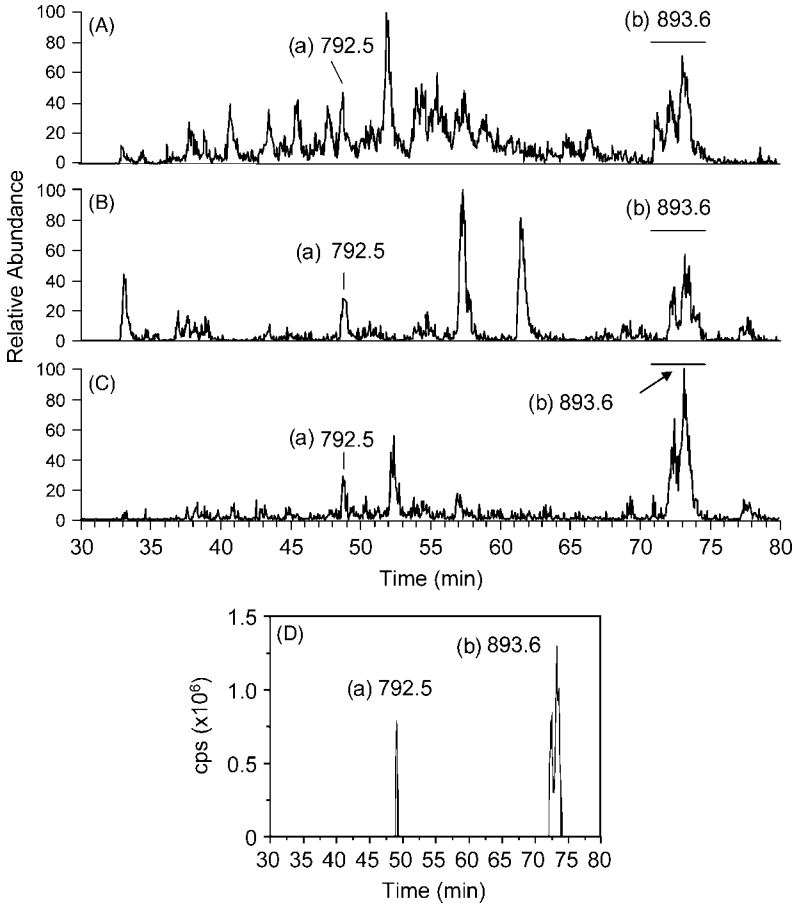

Figure 7. LC-ESI-MS/MS in precursor-ion scanning mode of HSA adducted by HNE. Panels A, B, C show the ion-scanning currents recorded setting the product ions at *m/z* 242.1, 263.1, 370.2. Panel D shows the vCT identifying two peaks, attributed to LQQC(CAM)PF (peak a) and LQQC(HNE)PF (peak b).

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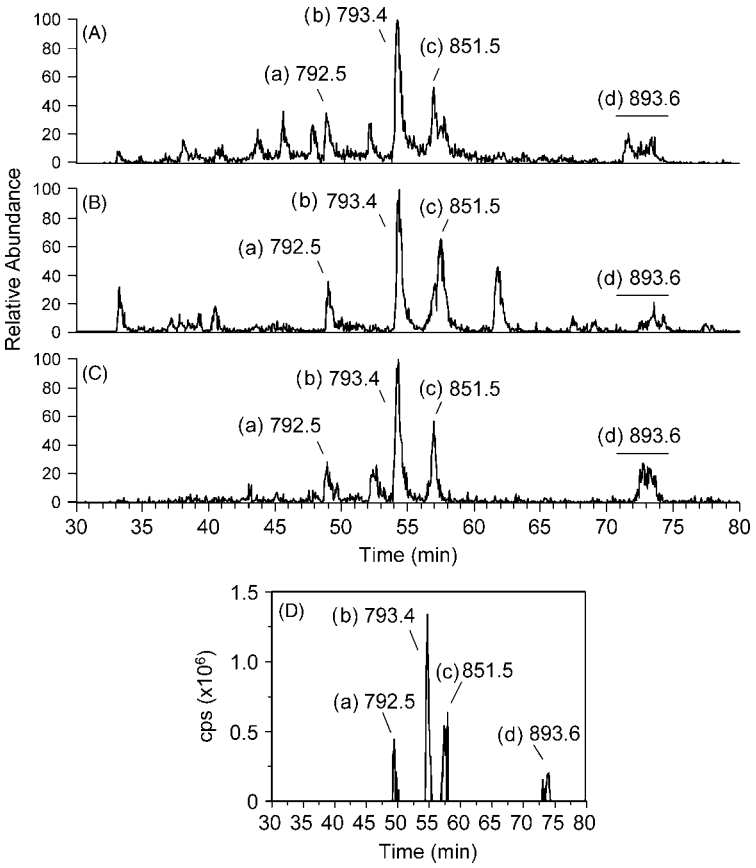

Figure 8. LC-ESI-MS/MS in precursor-ion scanning mode of a mixture containing HSA adducted by HNE, HHE, and ACR. Panels A, B, C show the ion-scanning currents recorded setting the product ions at m/z 242.1, 263.1, 370.2. Panel D shows the vCT identifying four peaks attributed to LQQC(CAM)PF (peak a), LQQC(ACR)PF (peak b), LQQC(HHE)PF (peak c) and LQQC(HNE)PF (panel d).

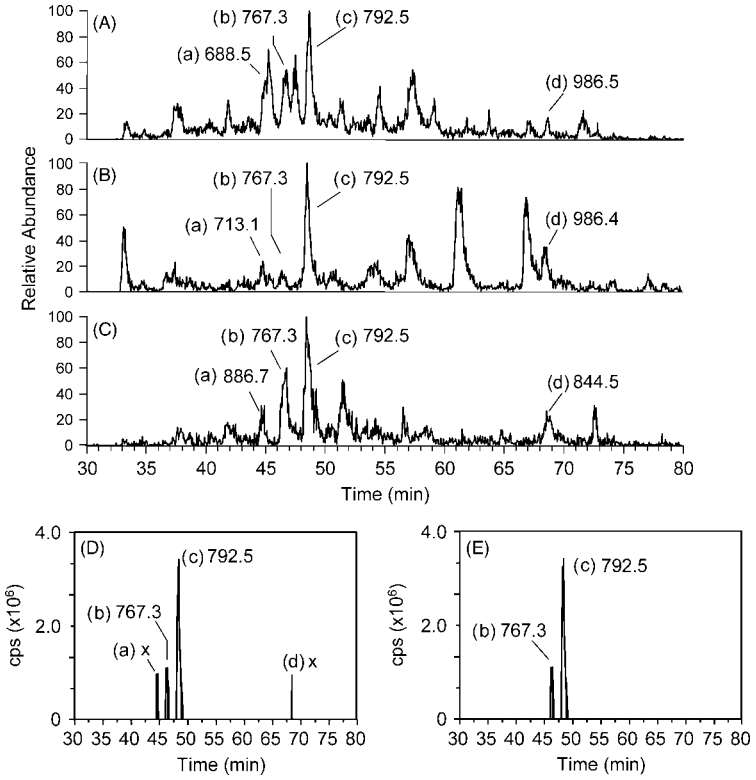

Figure 9. LC-ESI-MS/MS in precursor-ion scanning mode of human plasma oxidized by the radical inducer AAPH. Panels A, B, C show the ion- scanning currents recorded setting the product ions at *m/z* 242.1, 263.1, 370.2. Panel D shows the vCT identifying four peaks. Manual examination of the precursor ions excluded peaks a) and d) as LQQCPF adducts. The corrected vCT (panel E) shows two peaks, attributed to LQQC(CAM)PF (peak c) and LQQC(SO_2H)PF (peak b).

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presented here seems to offer the potential for reaching these goals, and will be further implemented by quantitative analysis in MRM mode, using isotopically labeled derivatives of the carbonylated tag peptides.

Thus the combined LC-MS/MS approaches (in both precursor ion and MRM mode) could well be successful in oxidative stress-related research, where detailed characterization and quantitation of covalently modified proteins is particularly important.

Supplementary material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1076-5174/suppmat/.

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