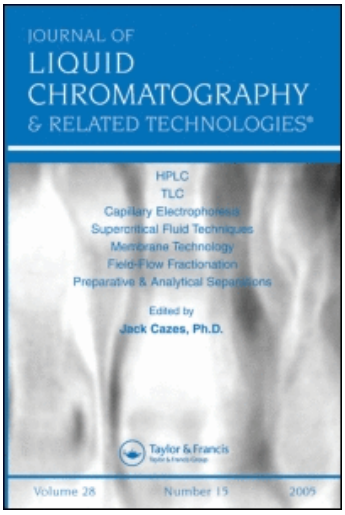

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RP-HPLC PEPTIDE MAPPING OF PROTEINS WITH AN EVAPORATIVE LIGHT SCATTERING DETECTOR

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RP-HPLC PEPTIDE MAPPING OF PROTEINS WITH AN EVAPORATIVE LIGHT SCATTERING DETECTOR

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ABSTRACT

Evaporative light scattering detection (ELSD) is an inexpensive "universal" mode of optical detection for LC. We report here on ELSD as a useful adjunct to UV detection for the RP-HPLC peptide mapping of proteins. We use peptide mapping as a "fingerprinting" technique to detect post-translational modifications and other small changes to recombinant protein pharmaceuticals. UV absorption and ELSD modes were run in series for a RP-HPLC tryptic map of the small "model" protein, cytochrome c. We also report preliminary ELSD data for the RP-HPLC tryptic map of an IgG1 monoclonal antibody.

INTRODUCTION

Evaporative light scattering detection (ELSD)[1] employs a nebulizer (Figure 1) followed by a heated drift tube to entirely evaporate the volatile HPLC mobile phase. The resulting "cloud" of solid microparticles is then quantified by light scattering. The first commercial ELSD units became available in the early 1980's[2] and used relatively high temperatures for evaporation (70-90°C for aqueous mobile phases, for example) and were thus troublesome to operate and often showed disappointing signal-to-noise ratios. Of course, these high temperatures can also cause thermal degradation of the analyte in the

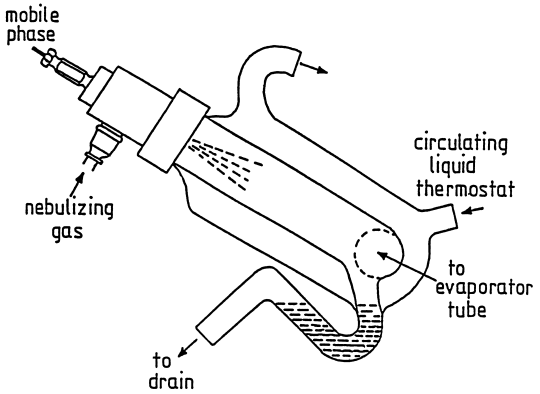

Figure 1. The cross-flow nebulizer of the Sedex 55 evaporative light scattering detector (ELSD) creates a fine aerosol mist that is evaporated in a heated drift-tube prior to entering the optical cell. The diagram is courtesy of Sedere (Vitry-Sur Seine, France).

ELSD. Modern ELSD's are vastly improved over these earlier models and can yield on-column detection limits in the low microgram to mid nanogram range. The Sedex ELSD (Figure 1), for example, condenses larger droplets in the nebulizer chamber allowing efficient evaporation of the finer droplets in the drift tube at a relatively low temperature, i.e. 40°C for aqueous mobile phases.

The main advantage (or disadvantage) of ELSD is that it can detect virtually any solute without the need for a chromophore or fluorophore. The only requirement is that the solute be less volatile than the mobile phase. Refractive index (RI) detectors are also universal but are only useful for isocratic elution. Of course, ELSD is limited in its usefulness by the requirement for volatile mobile phases only. ELSD response can be related to the mass (m) of the sample, albeit in a nonlinear fashion,

$$\text{response} = am^b$$

$$\log(\text{response}) = b \log m + \log a$$

Thus, this is sometimes referred to as a "mass detector." This relationship provides a type of "material balance" that can be useful when analyzing samples that may contain degradative impurities that lack chromophores present in the parent compound or whose relative UV extinction coefficients (response factors) are unknown.[3]

ELSD has been applied to a wide range of UV-transparent analytes including synthetic polymers,[3,4] pharmaceuticals,[3,5] fatty acids,[6,7] lipids,[8,9] amino acids,[10] carbohydrates,[11] and even metal ions.[12] We recently compared the use of ELSD to UV detection for the peptide mapping of proteins, a technique[13] that is used for studying the primary structure of proteins. This was of interest to us because ELSD is not a technique that is widely known nor used in biochemistry laboratories and we were not aware of any reported applications of ELSD to peptide mapping. We report here on the RP-HPLC/ELSD tryptic mapping of cytochrome c and an IgG1 monoclonal antibody.[14]

EXPERIMENTAL

RP-HPLC/ELSD Peptide Mapping

RP-HPLC/ELSD runs were done using a Beckman 126 binary-pump LC, a Vydac 218TP54 C_{18} column (4.6 x 250 mm) at 45°C in a thermostated column oven, and a Hewlett Packard 1050 UV/Vis detector (model 79853A) set at 215 nm was connected in series to a Sedere Sedex 55 ELSD. The nitrogen pressure to the ELSD nebulizer was set at 2.1 bar N_2 and the temperature of the drift tube at 40°C. A flow rate of 1.0 mL/min was used and mobile phases were A) 0.1% TFA/H_2O and B) 0.1% TFA/80% CH_3CN. The gradient for the tryptic map of cytochrome c in Figure 2 (30 µg injection) was 0 to 15% B in 5 min followed by 15 to 40% B in 25 min. The gradient for the tryptic map of the reduced/carboxymethylated anti-RSV antibody in Figure 4 (60 µg injection) was 0 to 50% B in 65 min. The peaks in the tryptic map of cytochome c were identified by LC/MS on a Hewlett Packard 1100 LC equipped with a binary pump, column oven, diode-array UV detector, and a model G1946A mass spectrometry detector (MSD) with electrospray ionization source. The LC/MS was run exactly as above for the ELSD runs except that a Vydac 218TP52 C_{18} (2.1 x 250 mm) at a flow rate of 0.20 mL/min was used instead of the above 4.6 mm column at 1.0 mL/min.

Tryptic Mapping of Cytochrome c

A 2.0 mg/mL solution of cytochrome c (horse heart, Sigma C-7752) in 50 mM Tris/HCl, 1 mM $CaCl_2$ (pH 8.1) was digested with trypsin (Worthington, bovine, TPCK-treated) at 100/1 wt:wt substrate/enzyme at 37°C for 4 h. Digestion was halted by acidifying to pH 2 by adding 50 µL of 1.0 M HCl per mL of digest and storing frozen at -70°C.

Tryptic Mapping of Anti-RSV Monoclonal Antibody

Anti-RSV (SmithKline Beecham) was dissolved to 2.5 mg/mL in 6.0 M guanidinium chloride, 1.2 M Tris/HCl, 2.5 mM Na_2EDTA (pH 8.4), reduced

with 50 mM dithiothreitol (DTT) for 60 min at 65°C, and then carboxymethylated with 120 mM sodium iodoacetate at RT for 40 min. The above reaction mixture was then exchanged into 0.1 M NH_4HCO_3/NH_3 (pH 8.1) buffer by use of a Bio-Rad 10DG disposable Bio-Gel P-6DG gel filtration column, digested with trypsin at 100/1 wt:wt substrate/enzyme at 37°C for 24 h, acidified to pH 2 with 1.0 M HCl, and stored at –70°C.

RESULTS AND DISCUSSION

We began our evaluation with the RP-HPLC tryptic map of a small "model" protein, cytochrome c (Figures 2 and Table 1). The tryptic map is simply an RP-HPLC chromatogram of the mixture of peptide fragments (tryptic digest) from hydrolysis of the protein catalyzed by the proteolytic enzyme, trypsin.[8] Note the massive ELSD response at 3 min for the UV-transparent buffer salts and single amino acid fragments in the "break-through" volume. The data in Figure 2 show that the ELSD signal for this assay is comparable in

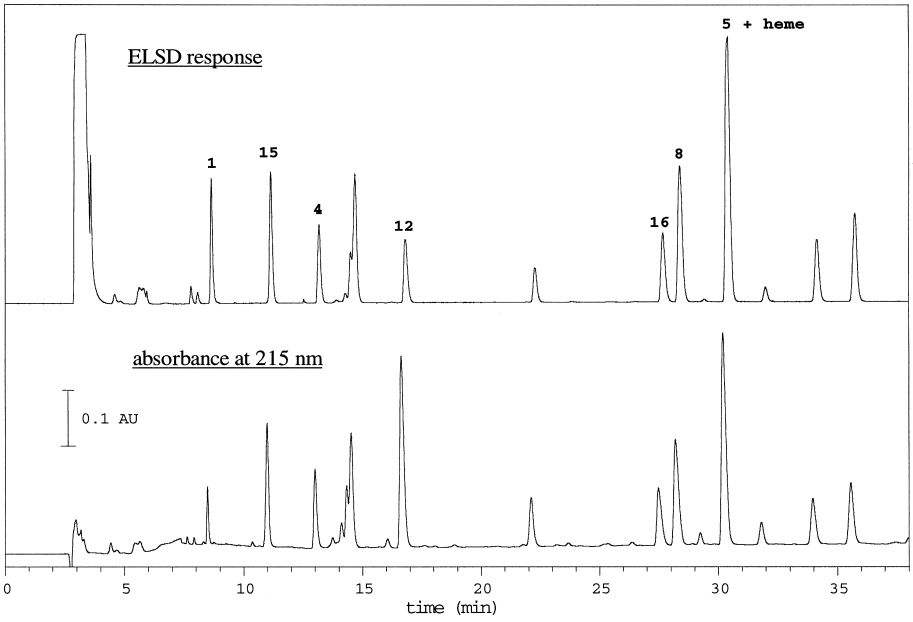

Figure 2. Comparison of UV and ELSD signals for the RP-HPLC tryptic map of cytochrome c obtained by connecting the two detectors in series. The numbered peaks in the ELSD trace correspond to numbered tryptic fragments listed in Table 1 and were identified by LC/ESI-MS.

Table 1

Theoretical Tryptic Peptides for Cytochrome c

Fragment	Residues	MW (Da)	Sequence[a]
1	1-5 (N-acetyl)	588.3	GDVEK
2	6-7	203.1	GK
3	8	146.1	K
4	9-13	633.4	IFVQK
5	14-22 (+ heme)	166.6	CAQCHTVEK
6	23-25	260.2	GGK
7	26-27	283.2	HK
8	28-38	1167.6	TGPNLHGLFG R
9	39	146.1	K
10	40-53	1469.7	TGQAPGFTYTDANK
11	54-55	260.2	NK
12	56-60	603.3	GITWK
13	61-72	1494.7	EETLMEYLENPK
14	73	146.1	K
15	74-79	677.4	YIPGT K
16	80-86	778.4	MIFAG IK
17	87	146.1	K
18	88	146.1	K
19	89-91	404.2	TER
20	92-99	963.5	EDLIAYLK
21	100	146.1	K
22	101-104	433.2	ATNE

[a] The peptide sequence, from the amino to the carboxy terminus, is listed from left to right in the one-letter code for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamin acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan. Note that the N-terminus of cytochrome c is acetylated and an iron heme prosthetic group is covalently attached to the protein (and tryptic fragment 5) via thioether linkages to the side chains of the two cysteine residues.

quality to the UV trace. The ELSD has an advantage over UV in that the base-line is generally unperturbed by changes in the mobile phase composition and quite "flat" overall. This feature of ELSD is scarcely visible for shallow gradient RP-HPLC runs, such as that in Figure 2, but can be quite significant for steep gradients and/or low sample loads, i.e. conditions that result in a deleterious rise, fall, or curvature of the UV base-line.

We have not examined the linear range for the RP-HPLC/ELSD of these tryptic peptides. The response in the low end of the range is of some concern as it appears that some of the smaller UV features in Figure 2 are not represented in the ELSD trace. The very smallest peaks are often of the most interest in peptide mapping. Note that ELSD signal falls off quickly (nonlinearly) with decreasing analyte amount (mass). This nonlinear nature of ELSD makes it virtually useless for analysis of trace residuals and other applications requiring very low limits of detection.

As shown for selected major peaks in Figure 3, the relative ELSD peak area responses do show a reasonably good apparent log-log fit to the relative masses. Since the tryptic digest is, theoretically, an equimolar mixture of peptides, the masses are directly proportional to molecular weights. Thus, for an ideal digest with 100% perfect cleavage only at the theoretical sites and 100% recovery for all peptides, all of the peak areas would lie directly on a line. For real digests, of course, there are usually differences in recoveries for the tryptic peptides due to incomplete proteolytic cleavages, minor degradation of peptides

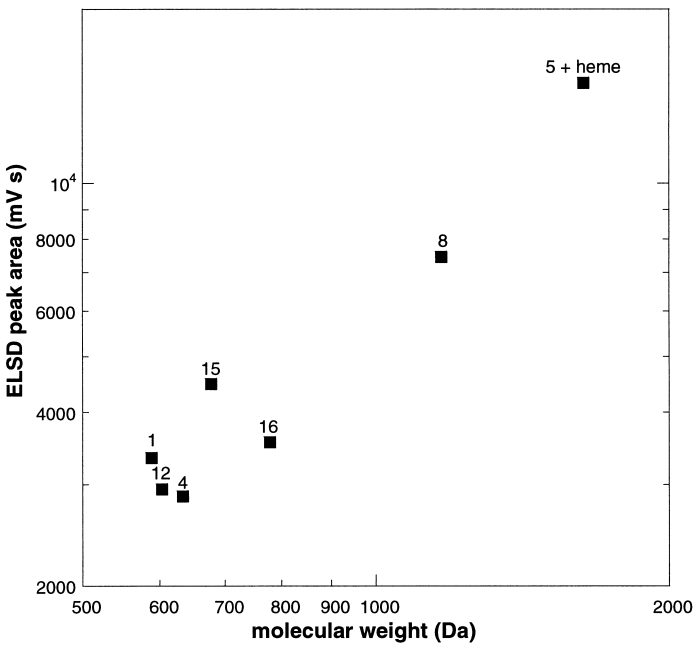

Figure 3. Apparent log-log relationship between ELSD response and molecular weight for tryptic peptides of cytochrome c. The numbered points in the plot above correspond to the numbered peaks in Figure 2.

by air-oxidation of methionine residues or deamidation of asparagine residues, and so on. Much of the deviation from linearity in Figure 3 is probably a result of these differences in recoveries for the tryptic peptides. Nonetheless, ELSD could be used as a convenient means of roughly measuring the relative recoveries of the peptides in a proteolytic digest. The simple relationship between mass and detector response makes ELSD well suited for assessing "material balance."

An interesting contrast in peak response for ELSD versus UV in Figure 2 is seen for tryptic fragment 12. The tryptophan residue in this small peptide contributes very strongly to the UV absorption at 215 nm but only modestly to the molecular weight and the ELSD signal versus other amino acids. Fragment 5 contains the heme which contributes strongly to both the UV and ELSD signals.

As shown in Figure 4, we also ran RP-HPLC/ELSD tryptic mapping for a relatively large glycoprotein, a 150 kDa IgG1 antibody.[14] The ELSD trace for this

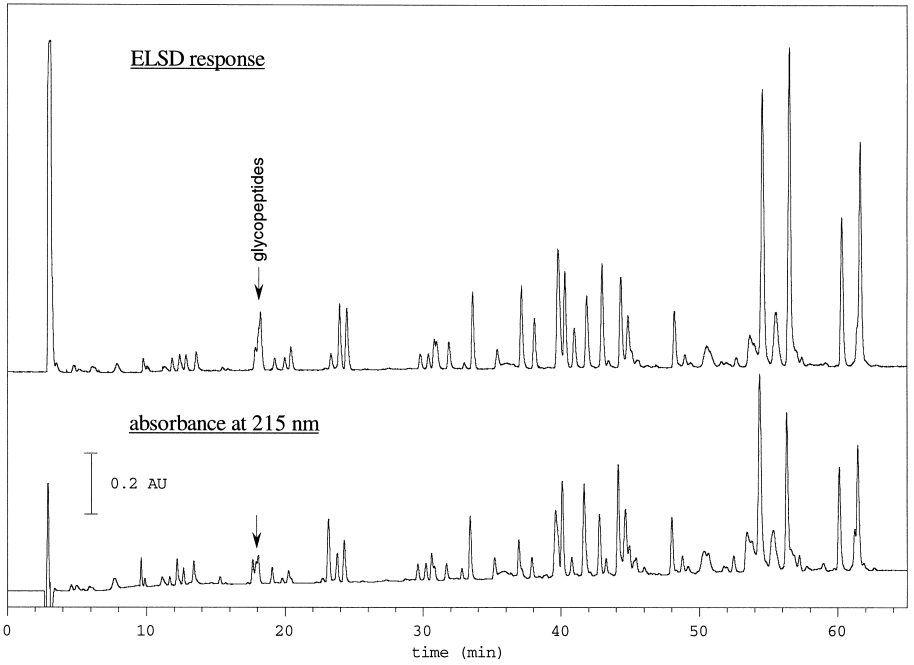

Figure 4. Comparison of UV and ELSD signals for the RP-HPLC tryptic map of a reduced/carboxymethylated IgG1 monoclonal antibody obtained by connecting the two detectors in series. Note the signal enhancement for the glycopeptides in the map by ELSD versus UV.

map is of excellent quality with respect to the UV trace. Once again, the ELSD signal intensities of the peptides are closely related to their masses and this is illustrated by the dramatic signal enhancement for the glycopeptides eluting at 18 min. The N-linked oligosaccharides attached to this peptide fragment[9] are UV-transparent but constitute over half the molecular weight for these glycopeptides.

ELSD is a moderately useful adjunct to UV detection for peptide mapping. As the above glycoprotein example suggests, ELSD may be most useful for proteins conjugated to high molecular weight poly(ethyleneglycol), which is UV-transparent. "PEGylation" can be used to increase the in vivo circulating half-life of protein drugs and thus enhance their effective potency.[15] We are interested in future studies RP-HPLC/ELSD for characterizing the PEGylation of proteins.

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