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Approaches towards the quantitative analysis of peptides and proteins by reversed-phase high-performance liquid chromatography in the absence of a pure reference sample

Frank Moffatt*, Paul Senkans, Dean Ricketts[1]

Zeneca Agrochemicals, Jealotts Hill Research Station, Bracknell, Berks RG42 6ET, UK

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Abstract

A reversed-phase HPLC protocol for the quantitative analysis of peptides and proteins is presented. It is applicable to purified samples and potentially to crude biological extracts. The key feature is that an analytically pure reference sample of the analyte is not required because the extinction coefficient for the UV absorbance at 280 nm can be accurately estimated from the amino acid sequence. The concentration of a protein can therefore be calculated from the peak area relative to an internal standard. Sources of error and limitations of the method are systematically considered. Tryptophan containing peptides gave closer agreement to expected values than those with only tyrosine. It was found that analogous, previously used methods could not be directly applied to lower wavelengths. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

It is beyond the scope of this introduction to review the vast number ways in which proteins can be quantified [2] except to note that the majority require a calibration using an analytically pure reference sample of the analyte, which may often not be available in the first instance. Our aim was to devise a convenient and reliable protocol for the quantitative analysis that would be applicable to impure proteins without the need for a purified reference sample. Nowadays, molecular biology provides protein sequences from DNA sequences so the necessary information for such calculations is readily available.

The determination of protein concentration by spectrophotometric means is widely practiced in biochemical laboratories. Absorptivity, $A_{280}^{1\%}$ values [1] absorbance at 280 nm, 1% (w/v) solution, path length of 1 cm are relied upon for the analysis of proteins at concentrations of 20–3000 $\mu g\ ml^{-1}$. The limiting concentrations for the avoidance of interference from common biochemical media are known. Specific corrections can be made for nucleic acids [1,2]. The method [2] is direct and rapid but a major disadvantage is that the analyte must be sufficiently pure.

Wetlaufer [3] described how the extinction coeffi-

*Corresponding author. Tel.: +44-134-441-4689; fax: +44-134-441-3677.

E-mail addresses: Frank.Moffatt@aguk.zeneca.com (F. Moffatt), Dean.Ricketts@aguk.zeneca.com (D. Ricketts).

[1]Co-corresponding author.

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cient of proteins may be regarded as the sum of the individual contributions of the amino acids. It has also been pointed out [4] that measurement of absorbance is straightforward, but conversely the determination of concentration (based upon the extinction coefficient) is not. Nevertheless, Edelhoch [5] calculated the extinction coefficients of proteins at 280 nm from the tryptophan and tyrosine content. Gill and Von Hippel [6] proposed slightly revised values and also included the contribution of cystine. Mach et al. [7] produced average values for the extinction coefficients of tryptophan and tyrosine in proteins themselves but the most comprehensive evaluation is the Pace et al. [8] analysis of 116 proteins which gave an average deviation of ±3.8% from the Edelhoch method. Thus, for any protein the extinction coefficient at 280 nm, may be calculated [8]:

$$\epsilon_{pro280} = (n_W\, 5500) + (n_Y\, 1490) + (n_{S\text{-}S}\, 125) \quad (1)$$

where n is the number of each group per protein molecule, and the subscripts W, Y and S-S denote tryptophan, tyrosine and cystine, respectively.

Analogous formulae may be constructed for lower wavelengths (adapted from Buck et al. [9]):

$$\epsilon_{pro214} = (n_{AA} - 1 + n_N + n_Q)\, 2846 + n_F\, 7200 + n_H\, 6309 + n_W\, 22\,735 + n_Y\, 5755 \quad (2)$$

$$\epsilon_{pro205} = (n_{AA} - 1 + n_N + n_Q)\, 2400 + n_F\, 8600 + n_H\, 5200 + n_W\, 20\,400 + n_Y\, 6080 \quad (3)$$

where the subscript AA represents all amino acids, and N, Q, F and H denote the usual single letter amino acid codes.

Alternative assays for proteins using UV absorbance differences that may compensate for the presence of non-proteinacious components, have been reviewed [10]. The wavelengths selected by various workers include A_{215}–A_{225} [11,12], A_{224}–A_{233} [13], A_{230}–A_{260} [14], and A_{235}–A_{280} [15]. These approaches were not followed in this investigation because the use of a high resolution separation technique achieves purification that in principle renders such compensation redundant.

A further possibility is use of low wavelengths with corrections for tryptophan and tyrosine content (from measurement at 280 nm) as described for 205 nm by Scopes [16]. The limitations of the Scopes method and of the ratio methods listed above were outlined by van Irsel et al. [17]. Silvestre et al. [18] analyzed casein hydrolysates by size-exclusion LC with detection at the wavelengths 300, 280 and 230 nm. This allowed for substraction of the individual contributions of tryptophan and tyrosine to the absorbance (peak area) at 230 nm. The corrected fraction (of peak) area at 230 nm was shown to be proportional to the amount of amino acids present so that the proportion in molar terms of peptides as a function of size could be determined. These approaches were not pursued because if measurements can be performed at 280 nm this is itself sufficient for the determination of protein concentration.

It is common in HPLC to quantify proteins by constructing a standard curve. Zhu et al. [19] found that the sensitivity by UV absorbance at 215 nm was about ten times that at 280 nm for bovine serum albumin. A recent, typical example, is the analysis of tear proteins [20]. The key concept behind our investigation, namely protein analysis in the absence of an analytically pure reference sample of the analyte, was exemplified by the work of Eberlain [21], in which the response of LC–UV detection at 215 and 277 nm was calculated from spectroscopic data. However, the method is rather cumbersome for routine use.

LC detection, using the native fluorescence of tryptophan or fluorescent tags can provide higher sensitivity than UV absorbance. Unfortunately, the quantum yield for the fluorescence of tryptophan can vary from 0.00 to 0.35 through quenching by up to four different mechanisms [22]. Therefore, the fluorescence response of a protein cannot be readily predicted, for which reason UV-detection was chosen for this study.

The approach described in this paper for the determination of the concentration of a protein (c_{Pro}) is based upon the calculated extinction coefficient (ϵ_{pro}) and peak areas by HPLC:

$$c_{pro} = c_{cal}(\epsilon_{cal280}/\epsilon_{pro280})(A_{pro280}/A_{cal280}) \quad (4)$$

where A is the peak area, the subscripts pro and cal refer to the protein and the calibrant, respectively, for absorbance at 280 nm, ϵ_{pro} is calculated using Eq. (1).

Table 1
Comparison of experimentally determined purity with the supplier's specification for a range of tryptophan containing peptides and proteins

Compound	Observed, % peptide	Expected, % peptide	Observed, % of expected
Lys–Trp–Lys	76	79	96
α-Lactalbumin	91	85	107
D-Lys–Tyr–D-Trp–D-Trp–Phe	84	79	106
pGlu–Lys–Trp–Ala–Pro	90	~95	95
Lysozyme	99	94	105
β-Ala–Trp–Met–Asp–Phe	86	91	95
Trp–Met–Asp–Phe	88	91	97
pGlu–Ser–Leu–Arg–Trp	88	89	99
Average (RSD, %)		100 (5.4)	

This expression is derived by equating the path length of the UV detection cell in terms of the Beer–Lambert parameters for the protein and tryptophan, and recognising that in HPLC the absorbance must be integrated over time (i.e. peak area). The principle of using absorbance in this manner is thus well supported by the prior work cited. Surprisingly, we have found no applications of this principle in LC or CE. This study illustrates how the method may be applied in practice. Particular complications are addressed, with emphasis upon experimental design.

2. Experimental

2.1. Chemicals and solvents

Deionised water was obtained from a Milli-Q water purification system [Millipore, Watford, UK]. All solvents were of HPLC grade (Romil, Cambridge, UK), and were passed through a 0.22 μm nylon membrane filter [Millipore, UK] prior to use as the mobile phase for HPLC analysis. Substances supplied by Sigma–Aldrich (Poole, UK) included: ethylenediamine tetracetic acid disodium salt, guanidine hydrochloride, guanidine thiocyanate, lysozyme (chick), ribonuclease A (bovine pancreas), trifluoroacetic acid (TFA) (≥99%), D-tryptophan, other proteins and peptides listed in Tables 1 and 2. α-Dithioreitol was obtained from Pierce and Warriner (Chester, UK). The plant defensin, *Rs*-AFP1 [23] was obtained from an in-house source (S. Attenborough, Zeneca Agrochemicals, Jealotts Hill Research Station, Bracknell, UK).

2.2. Sample preparation

The protein or peptide (1–5 mg) was dissolved in aqueous guanidine thiocyanate (2.4 *M*, 1–5 ml). Some hydrophobic peptides required additional acetonitrile for dissolution. In general, peptides of >20 amino acids were found to be soluble in the guanidine thiocyanate alone. Peptides were diluted to the following concentrations: 1–5 amino acids (10 or 50 $\mu g\ ml^{-1}$), 6–50 amino acids (100 or 250 $\mu g\ ml^{-1}$), >50 amino acids (1 $mg\ ml^{-1}$). Solutions

Table 2
Comparison of experimentally determined purity with the supplier's specification for a range of tyrosine containing peptides and proteins

Compound	Found, % peptide	Expected, % peptide	Found, % of expected
Thr–Tyr–Ser	77	92	84
Gly–Gly–Thr–Arg	68	80	85
Thr–Gly–Gly–Phe–Met	73	83	88
des-Asp angiotensin I human	71	82	87
Average (RSD)		86 (2.1)	

of D-tryptophan (10 or 100 μg ml^{-1}) in aqueous guanidine thiocyanate (2.4 mol l^{-1}) were prepared weekly and were stored in a refrigerator when not in use. Samples were analyzed by HPLC within 24 h.

2.3. HPLC conditions

An HP1100 Series HPLC instrument (Hewlett-Packard, Bracknell, UK) was used, consisting of a degasser, binary pump, autosampler, column temperature control compartment and a UV detector. Detection was by UV absorbance at a wavelength of 280 nm and a bandwidth of 2 nm. Reversed-phase HPLC was performed on a Jupiter column (15 cm × 4.6 mm I.D., C_{18} bonded to silica particles of diameter 5 μm and pore size 30 nm) (Phenomenex, Cheshire, UK). Analyses were performed at a column temperature of 40°C and a flow-rate of 1.0 ml min.$^{-1}$. The following gradient elution profiles were used: Solvent A, 0.1% (v/v) TFA in water, Solvent B, 0.085% (v/v) TFA in acetonitrile. The scouting conditions, for substances of unknown elution properties were 5–80% B over 30 min. Gradients were adjusted to provide resolution of tryptophan from the analyte and generally started at 5 or 10% B followed by an increase in the range of 1–3% B per minute. The sample injection volume was 10 μl in all cases. The calibrant, D-tryptophan (10 μl injection volume, 10 or 100 μg ml^{-1} in 2.4 *M* aqueous guanidine thiocyanate), was injected with the sample via an injection program.

2.4. Reduction of proteins with α-dithiothreitol

The protein (ca. 1 mg) was dissolved in "GET" buffer (1 ml, 6 *M* guanidine hydrochloride, 8 m*M* EDTA, 0.5 *M* Tris, pH 8.6). α-Dithiothreitol (40 mol equiv., 65 m*M*, in "GET" buffer) was added at room temperature. The resulting mixture was analyzed by HPLC within 0.5–1.5 h.

3. Results and discussion

To measure the concentration of a protein according to Eq. (4), a calibrant is required. Tryptophan was selected because it is readily available in pure form and has a known extinction coefficient (ϵ_{cal} = 5630, under acid conditions [24]). Reversed-phase HPLC analyses were performed using a solvent gradient owing to the steep adsorption isotherms associated with proteins in this mode of LC [25]. TFA was added to the mobile phase in order to obtain good chromatography [26]. Many proteins and peptides are isolated as salts of the basic amino acid residues [27] histidine (pK_a 6–7), arginine (pK_a 12) and lysine (pK_a 10.4–11.1). Commercial sources were used that provided peptide, counter-ion, salt and solvent composition data. The calibrant was kept in a separate vial to the sample but equal volumes were injected in each run via an injection program. All analytes were well resolved from tryptophan by reversed-phase HPLC (e.g. Figs. 1 and 2). The observed purity of the test analytes (from Eq. (4)),

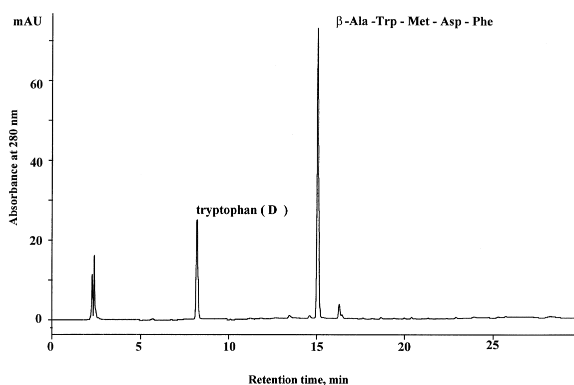

Fig. 1. Quantitation of a peptide by RP-HPLC–UV_{280} – typical chromatogram.

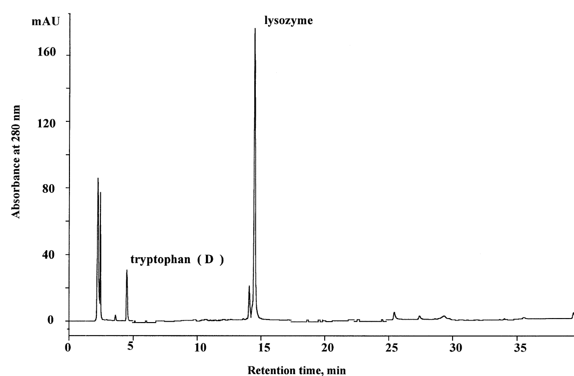

Fig. 2. Quantitation of a protein by RP-HPLC–UV_{280} – typical chromatogram.

were compared with the expected values (supplier's data). Excellent agreement was found, the overall average agreement for tryptophan containing samples was 100% (relative standard deviation (RSD) 5.1%) (Table 1).

One possible source of inaccuracy is the attenuation of light in the solution phase through light scattering owing to turbidity, which becomes more significant as the size of the proteins increases – the Tyndall effect [28]. This obeys a Beer–Lambert type of relationship:

$$\ln I/I_0 = \tau l \quad (5)$$

where τ is the turbidity of the solution, l is the path length.

The full expression for the turbidity includes the concentration, relative molecular mass and the wavelength of light. Turbidity can be used for the quantitation of proteins [29] but in this paper the concern is to assess the impact upon "apparent" UV absorbance values. Mach et al. [30,31] have proposed that turbidity can be accounted for by measuring absorbance readings at 320 and 350 nm, on the assumption that there is no interfering absorbance at these wavelengths, and adjusting the 280 nm absorbance accordingly:

$$A_{\lambda} = 10^{(m+1)\log A_{320} - m \log A_{350}} \quad (6)$$

where $m = 64.32 - 25.67$, $\log \lambda = 1.5$ at 280 nm, λ is the wavelength of light.

We found that peptides/proteins of molecular mass 5000–6000 exhibited light scattering of around 1% by HPLC. The impact of light scattering depends upon the size of protein, but need only be considered for realization of the highest accuracy in the case of large or aggregated proteins.

Sample handling is an important practical issue because there is a high potential for proteins to adhere to LC vials and the LC system. The stabilization of proteins in solution, to avoid non-specific binding or sticking to surfaces, is a classical problem addressed both in the biochemical literature [32,33] and in the context of capillary electrophoresis [34,35]. The "sticking problem" is the outcome of diverse mechanisms but we have found that in many cases dissolving samples in guanidine thiocyanate (2.4 mol l^{-1}) provides reliable stabilization. The choice of this salt follows the empirical rules of Hofmeister [27] for the stabilization by anions and cations of proteins in solution. It is advisable to assess the risk of losses through adsorption, for example, by dipping pipettes and LC tubing into a dilute solution of the analyte and by transfer of the protein solution between two and three vials. Attenuation of LC signal intensity after dipping or transfer is an unambiguous indicator of loss. Consequently, the consistent use of one source of glass or polypropylene, pipettes and vials, and one type of LC tubing are recommended. Additives for protein stabilization may only be effective up to the point that the sample reaches the head of the HPLC column. Losses from the column onwards can be diagnosed via a linearity check. Deviation from linearity, or sudden loss of signal, shows that sticking is significant. A difference in the proportions of analyte and calibrant lost within the LC system would produce systematic error. It has been a regular observation, that the first one to two injections can give spurious results. This is attributable to the blocking of irreversible binding sites. Sufficient replication is therefore also recommended.

In order to adapt the A_{280} method to separation techniques such as LC or CE, it is necessary to establish the effect of mobile phase composition upon the chromophores. The absorbance of tyrosine undergoes significant shifts [27] at higher pH (associated with the pK_a of the phenolic group). Organic solvents can also alter the pK_a of both acid and basic groups by as much several pH units in aqueous acetonitrile mobile phases [36,37]. The tryptophan chromophore has been found to be unaffected by the presence of urea or guanidine in aqueous solution, but had a 9% higher extinction coefficient in 1-propanol compared with water [8] but others have claimed that denaturing agents may cause hypsochromic and hypochromic effects [38]. In any event spectral differences may arise though a combination of intra- and intermolecular effects upon ionisable groups that influence the chromophore, as discussed elsewhere [39]. It has often been found that reversed-phase HPLC denatures proteins [40], giving rise to greater exposure of hydrophobic amino acids which include tryptophan and tyrosine, to the solvent medium. This would reduce any potential influences of the local structural environment within folded

proteins, upon the chromophore. By working under a controlled pH remote to the pK_a of the amino acids this source of variation could be eliminated. A UV spectrophotometer was used to determine the effect of a range of HPLC mobile phases containing water (TFA, 0.1%) and acetonitrile (TFA, 0.085%) upon proteins, peptides and D-tryptophan. These tests showed that the proportions of these mobile phases did not affect the responses at 280 and 205 nm relative to that in water.

Measurements at 280 nm impose a limitation because tryptophan has the lowest average natural abundance of any amino acid. In some cases the absorbance will depend upon tyrosine alone. Commercially available peptides containing tyrosine but not tryptophan consistently gave a value that was about 14% lower than the expected value (Table 2). Further study is required to establish whether this is due to inaccuracy in the specification of the purity of the sample or another source.

Attempts to apply Eqs. (2) and (3) using 205 and 214 nm data proved unreliable (Table 3). The explanation for this was considered. The UV absorbance of peptides has been shown [41] to be proportional to the number of peptide bonds at 200 nm. However, in the case of proteins, absorbance has been seen to be highly dependent upon the intramolecular environment with alpha helices showing attenuation of up to 40% of the absorbance found in extended forms [42]. Evidence of such influences is provided by the observation that enzymatic digests of globular proteins produce hypochromic and hypsochromic shifts by as much as 10–15% which was attributed to the removal of conformational contributions [38]. To study this possibility, three proteins were reduced. The completeness of the reductions reported herein were confirmed by electrospray mass spectrometry. The reduced forms all eluted at longer retention times by reversed-phase LC (Fig. 3). Comparison of the ratios of peak areas at selected wavelengths shows significant differences in the absorbance of the reduced and non-reduced forms of three proteins (Fig. 4). These changes cannot simply be accounted for by the loss of the cystine linkages. It must therefore be advantageous to minimize effects upon the chromophore that are not purely due to amino acid composition in the development of lower wavelength methods. Reduction of cystines and denaturation would contribute to this. A further refinement of this strategy would be to convert the

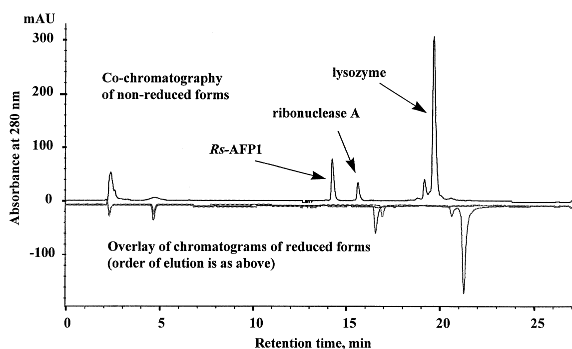

Fig. 3. The effect of reduction of cystine linkages upon the chromatography of selected proteins. Conditions: 5–60% acetonitrile (0.085% TFA) water (0.1% TFA) in over 55 min.

Table 3
Comparative of values for the quantification of peptides at several wavelengths

Compound	214 nm[a]	205 nm
β-Ala–Trp–Met–Asp–Phe	62.8	94.2
pGLu–Lys–Trp–Ala–Pro	68.7	103.8
Insulin B	44.0	118.9
Angiotensin I human	68.8	27.5
Thr–Tyr–Ser	73.6	44.5

[a] Values are percentages relative to the 280 nm value.

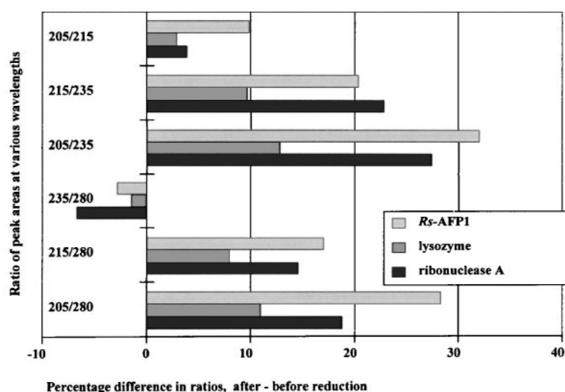

Fig. 4. Percentage changes in absorbance ratios upon reduction of selected proteins.

reduced protein to smaller fragments by enzymatic digestion, however this would introduce an extra source of inaccuracy.

The A_{280}method can be used on a "rule of thumb" basis for the quantification of proteins of unspecified amino acid composition [33] (a 1 mg ml^{-1} solution is estimated to have an absorbance of 1 in a 1 cm path length cuvette). A similar approximation can be made to the LC method described in this paper. Taking the occurrence in proteins [43] of tryptophan, tyrosine and cysteine as 1.3 and 3.2, 1.7 molar percentages, respectively, the relative response of the average protein would be about 0.04 relative to tryptophan in HPLC (by peak area at 280 nm, after correcting abundance to a mass basis). However, the associated errors would be large because of the dependency upon two amino acids of relatively low and variable abundance and does not therefore play to the main strengths of the method.

In summing up, the protocol outlined in this paper enables the determination of the concentration of a protein in solution, using only microlitre volumes, at concentrations in the microgram–milligram per millilitre range using standard 4.6 mm I.D. RP columns running at a mobile phase flow-rate of 1 ml min^{-1}. This is sufficient for many bio-efficacy tests and analysis of matrix samples.

Quantitation by mass of a solid protein by the method described herein, is more sample demanding being limited by weighing accuracy. In our experience, with ordinary four or five figure balances, 1–10 mg of solid protein is required. For protein characterization, where high accuracy may be required, concentrations of about 1 mg ml^{-1} for a protein of say 50 amino acids or more are recommended. For such characterization applications the procedure offers advantages over commonly used methods [2]. It is faster and more direct than amino acid analysis; faster and less labor intensive than radiolabelling, immunoassay, enzyme-linked immunosorbent assay (ELISA) or enzyme kinetic assays. It is also simpler and more accurate than typical bio-assays and total protein assays of say Bradford or Lowry, all of which, in any event, require more calibration steps using an analytically pure sample of the analyte for reference purposes. Finally, the method is applicable in principle, to impure samples in biological matrices.

4. Conclusions

(1) Quantitation of peptides and proteins in solution by RP-HPLC with detection by UV absorbance at 280 nm relative to a calibrant is both highly convenient and accurate.

(2) The protocol is limited to tyrosine and tryptophan containing substances and may be less accurate for non-tryptophan containing analytes.

(3) An analytically pure reference sample of the analyte is not required.

(4) Good experimental practices include consideration of errors due to non-specific binding of proteins and the significance of light scattering in the case of larger proteins.

(5) Changes to the methodology that would require validation include the use of solvents other than water–acetonitrile, and non-acidic pH values.

(6) Use of absorbance at wavelengths below 280 nm is complex and would require considerably more validation than that provided in the current literature.

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