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Prediction of Molar Extinction Coefficients of Proteins and Peptides Using UV Absorption of the Constituent Amino Acids at 214 nm To Enable Quantitative Reverse Phase High-Performance Liquid Chromatography−Mass Spectrometry Analysis

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The molar extinction coefficients of 20 amino acids and the peptide bond were measured at 214 nm in the presence of acetonitrile and formic acid to enable quantitative comparison of peptides eluting from reversed-phase high-performance liquid chromatography, once identified with mass spectrometry (RP-HPLC-MS). The peptide bond has a molar extinction coefficient of 923 M^{-1} cm^{-1}. Tryptophan has a molar extinction coefficient that is ∼30 times higher than that of the peptide bond, whereas the molar extinction coefficients of phenylalanine, tyrosine, and histidine are ∼six times higher than that of the peptide bond. Proline, as an individual amino acid, has a negligible molar extinction coefficient. However, when present in the peptide chain (except at the N terminus), it absorbs ∼three times more than a peptide bond. Methionine has a similar molar extinction coefficient as the peptide bond, while all other amino acids have much lower molar extinction coefficients. The predictability of the molar extinction coefficients of proteins and peptides, calculated by the amino acid composition and the number of peptide bonds present, was validated using several proteins and peptides. Most of the measured and calculated molar extinction coefficients were in good agreement, which shows that it is possible to compare peptides analyzed by RP-HPLC-MS in a quantitative way. This method enables a quantitative analysis of all peptides present in hydrolysates once identified with RP-HPLC-MS.

KEYWORDS: Molar extinction coefficient; molar absorption coefficient; UV absorbance; proteins; peptides; amino acids

INTRODUCTION

Food-derived peptides may have a positive impact on body functions or conditions and ultimately influence human health (*1*−*4*). In addition, peptides may also exert technofunctional properties (e.g., solubility and gelation) in foods. Using reversed-phase high-performance liquid chromatography mass spectrometry (RP-HPLC-MS), the sequence of peptides present in protein hydrolysates can be easily obtained when the sequence of the parental proteins is known (*5*, *6*). Besides knowing the sequences of peptides, it is also important to know the quantities of the peptides of interest present in a peptide mixture.

Quantification of all peptides present in a hydrolysate can be carried out via the determination of the peptide bonds as these are present in all peptides. The π electrons in the peptide bond are to some extent delocalized over three atoms: the peptide nitrogen, carbon, and oxygen. At low wavelengths (180−220 nm), the $\pi \rightarrow \pi^*$ transition in the peptide bond absorbs light (*7*). This absorbance can be used to estimate the relative amounts of peptides present in a hydrolysate. However, quantification based on the absorbance at 214 nm is not a common method, since in this wavelength region the absorption of the peptide bond is also conformation-dependent (*8*). Furthermore, besides the peptide bond, several amino acid residues are reported to contribute significantly to the absorption (*9*, *10*). Moreover, the absorbance of peptides is also pH-dependent due to the absorption of the carboxylic acid group, which depends on whether the carboxyl group is protonated or not. This results in a variation of the absorbance around the pK_a (∼pH 3) of the free carboxylic acid in the peptides (*11*). This effect plays the most dominant role in dipeptides and decreases with increasing peptide length (*12*, *13*).

Because of the complexity of absorption at 214 nm, researchers are, in general, skeptic toward quantification of proteins at this wavelength. However, for peptides devoid of tryptophan or tyrosine, there is no good alternative (*14*). As explained above, the complexity of the absorbance is much higher as

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Table 1. Molar Extinction Coefficients at 214 nm (M^{-1} cm^{-1}) of Amino Acids and Peptide Bonds as Found in Literature and Software

	GPMAW (*16*)	Buck (*15*)	Saidel (*9*)[a]	Wetlaufer (*10*)[a]
peptide bond	1000	2846[c]	–[e]	–[e]
tyrosine	5000	5755	5450	6100
histidine	5000	6309	5700	5550
phenylalanine	5000	7208	5050	5800
tryptophan	33000	22735	32250	31950
asparagine	~0[b]	2846[d]	140	–[e]
glutamine	~0[b]	2846[d]	140	–[e]
cysteine	1000	~0[b]	275	210
methionine	1000	~0[b]	1050	1100
proline	~0[b]	~0[b]	25	–[e]

[a] Manually deduced from wavelength scans given in the article. [b] Regarded not to have a significant influence on the absorption at 214 nm. [c] Determined as the difference in extinction coefficient at 214 nm between N-acetylphenylalanine and phenylalanine. [d] Regarded to be the same as the peptide bond. [e] Not determined.

compared to the absorbance at 280 nm, resulting in a higher expected error at 214 nm. Nevertheless, this has to be accepted due to the absence of good alternatives, for example, during HPLC analysis. The high complexity of the absorbance at 214 nm is probably the reason why, to our knowledge, no research is performed to validate whether the absorbance of peptides at 214 nm can be predicted based on its amino acid composition. However, the rapid development of RP-HPLC-MS in the last years enables such an approach and the increase in research on (bioactive) peptides requires such an approach. To quantify the amounts of all peptides present in protein hydrolysates, the molar extinction coefficients of the individual peptides have to be known. In literature, several, somewhat conflicting data have been reported. Buck and co-workers (*15*) and GPMAW software (*16*) both calculate the molar extinction coefficients of a protein/peptide at 214 nm as the sum of the number of peptide bonds and the sum of the number of each type of amino acid multiplied by their individual molar extinction coefficients (ϵ). The molar extinction coefficients used by Buck and co-workers (*15*) and GPMAW (*16*) can be found in **Table 1**. In the GPMAW software (*16*), no reference or experimental data are provided for the molar extinction coefficient of the amino acids and the peptide bond. In addition in **Table 1**, the molar extinction coefficients of individual amino acids, manually deduced from the 200−230 nm wavelength scans given by Saidel and co-workers (*9*) and Wetlaufer (*10*), are shown as well. The values of Saidel and co-workers (*9*) and Wetlaufer (*10*) were not aimed at representing the absorbances of individual amino acids in a peptide chain. Therefore, in these studies, no value is presented for the absorbance of the peptide bond. In general, it can be seen that tryptophan, histidine, phenylalanine, and tyrosine are strong contributors to the absorbance at 214 nm. The values reported for these amino acids agree rather well with each other. However, there are large differences between the four studies with respect to the contributions of the peptide bond, asparagine, glutamine, cysteine, and methionine.

Characterization of protein hydrolysates using RP-HPLC-MS would be improved if, next to peptide identification, its quantity can also be easily deduced from the RP-HPLC chromatograms. This can be performed if a quick and relatively easy method to quantify the amounts of individual peptides present based on their UV absorbances is available. A method as presented by Buck and co-workers (*15*) can be applied. However, one might doubt whether molar extinction coefficients and in particular those of asparagine and glutamine used in that work are correct. In this respect, the values, although manually deduced, from the work of Saidel and co-workers (*9*) and from Wetlaufer (*10*) might be more correct. Another issue is the presence of proline. Proline, as a free amino acid, is reported to have a low molar extinction coefficient of ~25 M^{-1} cm^{-1} (*9*) or not regarded as being a major absorbing group in proteins and peptides (*15*, *16*). However, because of the cyclic nature of the three-carbon side chain to the nitrogen of the peptide backbone, proline might show different absorption properties when present in a peptide chain (except when present at the N terminus).

In the present study, we, therefore, measured the molar extinction coefficients of all 20 amino acids in the presence of 20% (v/v) acetonitrile and 0.1% (v/v) formic acid (~pH 3). This condition is similar to conditions during RP-HPLC-MS analysis. The objective of this study was to validate whether the molar extinction coefficient at 214 nm of a peptide can be primarily determined by its amino acid sequence. First, the individual contributions of the peptide building blocks were measured, the individual amino acids and the peptide bond. The next step is the validation of the hypothesis that the molar extinction coefficients can be rather well-predicted based on the individual contribution of the building blocks by measuring the molar extinction coefficients of several proteins and peptides.

MATERIALS AND METHODS

Materials and Chemicals. Amino acids were all purchased from Fluka (Buchs, Switzerland): alanine (A; product number 5130), arginine (R; 11040), asparagine (N; 11150), aspartic acid (D; 1190), cysteine (C; 30090), glutamine (Q; 49420), glutamic acid (E; 49450), glycine (G; 50050), histidine (H; 53370), isoleucine (I; 58880), leucine (L; 61820), lysine (K; 62930), methionine (M; 64320), phenylalanine (F; 78020), proline (P; 81710), serine (S; 84960), threonine (T; 89180), tryptophan (W; 93660), tyrosine (Y; 93830), and valine (V; 94620).

Gly-Gly-Gly (H-3355), Gly-Gly-Pro (H-3470), Gly-Pro-Gly (H-9745), Pro-Gly-Gly (M-1730), Gly-Tyr-Gly (GYG; H-3670), RGDS (H-1155), YGGFLRR [Dynorphyn A (1-7); H-2660], GPRP (H-2935), and RPPGFSP [Bradikinin (1-7); H-1955] were obtained from Bachem (Heidelberg, Germany). RPPGFSPFR (Bradikinin; B-3259) was obtained from Sigma (St. Louis, MO). RINKKIEK, EQLSTSEENSK, and YIPIQYVLSR, with a purity of ≥95% as determined by RP-HPLC, were obtained from Ansynth (Ansynth Service BV, Roosendaal, The Netherlands).

Polylysine (P2658) was obtained from Sigma with a DP of ~190 as indicated by the supplier. Soy glycinin and β-conglycinin were prepared as described previously (*17*). α-Lactalbumin (bovine; L5385), β-lactoglobulin (bovine; L0130), BSA (bovine; A4503), α-casein (bovine; C6780), and lysozyme (egg white; L6876) were obtained from Sigma. All other chemicals used were of analytical grade and purchased from Sigma or Merck.

Determination of the Protein/Peptide Concentration. The nitrogen content of various proteins, peptides, or solutions thereof was determined in duplicate by the Dumas method using an NA2100 Nitrogen and Protein Analyzer (CE Instruments, Milano, Italy) according to the manufacturer's instructions. Methionine was used as a standard. The nitrogen conversion factors for the various peptides and proteins were based on their amino acid compositions. For the proteins, the amino acid compositions as found in the Swiss-Prot database (www.expasy-.org) were used. The primary accession numbers used were as follows: glycinin (P04776, P04405, P11828, P02858, and P04347), β-conglycinin (P13916, P25974, and P11827), α-lactalbumin (P00711), β-lactoglobulin (P02754), BSA (P02769), α-casein (P02663 and P02662), and lysozyme (P0698). In case more than one variety was present, they were all regarded to be present in a 1:1 ratio.

Determination of the Molar Extinction Coefficients of Peptide Building Blocks. All 20 amino acids, polylysine, and Gly-Gly-Gly, Gly-Gly-Pro, Gly-Pro-Gly, and Pro-Gly-Gly were dissolved in 20% (v/v) acetonitrile containing 0.1% (v/v) formic acid, up to an accurately known concentration of ~5 mM. Aspartic acid, glutamic acid, and

tyrosine did not fully dissolve in the solvent used. Therefore, for these amino acids, HCl was added up to 20 mM, as well as to their corresponding sample blanks. The absorbance was measured at 214 nm using a UV Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Several dilutions were prepared to obtain a calibration curve in the linear region of the spectrophotometer.

Determination of the Molar Extinction Coefficients of Proteins and Peptides. To validate whether the molar extinction coefficient of a protein or peptide can be predicted based on the amino acid composition as found in the Swiss-Prot database (www.expasy.org), the molar extinction coefficients of various proteins and peptides were analyzed. Of all proteins and peptides, the protein content was determined using the Dumas method. Peptides that were hygroscopic or present in a too low quantity to weigh accurately (GPRP, YGGFLRR, RINKKIEK, EQLSTSEENSK, YIPIQYVLSR, RPPGFSP, and RPPGFSPFR) were predissolved in water followed by determination of the peptide concentration in the solution using the Dumas method. Of each protein and peptide, a stock solution was prepared containing an accurately known concentration between 0.5 and 1.0 mg/mL in 20% (v/v) acetonitrile and 0.1% (v/v) formic acid (v/v). These stock solutions were diluted 25 and 50 times with the following four solutions: (i) 5% (v/v) acetonitrile/0.09925% (v/v) formic acid, (ii) 20% (v/v) acetonitrile/0.097% (v/v) formic acid, (iii) 35% (v/v) acetonitrile/0.09475% (v/v) formic acid, and (iv) 50% (v/v) acetonitrile/0.0925% (v/v) formic acid. The acetonitrile/formic acid ratio was chosen to mimic RP-HPLC-MS analysis in which 100% water/0.1% (v/v) formic acid was frequently (*18*) used as buffer A and 100% acetonitrile/0.085% (v/v) formic acid was used as buffer B. No higher concentrations of acetonitrile were chosen, since in general peptides elute from an RP-HPLC column before a 50% (v/v) acetonitrile concentration was reached (*5*, *19*, *20*). To determine the molar extinction coefficient at 214 nm, the absorbance was measured using a UV Shimadzu UV-1601 spectrophotometer (Shimadzu).

Alkylation of α-Lactalbumin. α-Lactalbumin, dissolved in 8 M urea and 50 mM Tris-HCl, pH 8.0, was reduced using 50 mM dithiothreitol at 40 °C for 2 h, followed by alkylation for 1 h in the dark using iodoacetamide (IAA) up to 150 mM. The pH was adjusted to 8.0 after IAA addition. After dialysis against Millipore water and freeze-drying, the alkylated, as well as the untreated, α-lactalbumins were dissolved in water containing 0.1% (v/v) trifluoroacetic acid (TFA) up to ~1 mg/mL. Subsequently, the samples were mixed in a 1:1 ratio with matrix solution [10 mg/mL sinapinic acid in 50% (v/v) acetonitrile and 0.3% (v/v) TFA] and applied onto a matrix-assisted laser desorption/ionization (MALDI) plate. MALDI-time-of-flight (TOF) MS analysis was performed to verify whether alkylation was sufficient, using an Ultraflex workstation (Bruker Daltonics, Hamburg, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was used in the positive mode. After a delayed extraction time of 340 ns, the ions were accelerated to a kinetic energy of 25 kV. The ions were detected in the linear mode. External calibration of the mass spectrometer was performed using protein calibration standard I (mass range 5000−20000 Da; Part # 206355, Bruker Daltonics).

The MALDI-TOF mass spectrum of untreated and alkylated α-lactalbumin showed a main peak at 14177.8 and 14642.1 Da, respectively (mass spectra not shown). Because of the presence of eight cysteines in α-lactalbumin, upon alkylation, an increase in mass of 464.4 Da (8 × 58.05) was expected, which was the exact difference in observed masses. Of both the untreated and the alkylated α-lactalbumin, the molar extinction coefficient was determined in duplicate using 20% (v/v) acetonitrile and 0.1% (v/v) formic acid as described above.

RESULTS

Molar Extinction Coefficients of Individual Amino Acids and the Peptide Bond. Table 2 shows the determined molar extinction coefficients (ϵ) of all individual amino acids. Relative standard deviations (RSDs) were all below 4% and, therefore, not individually presented in the table. It is clear that tryptophan has the highest molar extinction coefficient (29050 M^{-1} cm^{-1}), followed by tyrosine, phenylalanine, and histidine. All three of these amino acids have similar molar extinction coefficients just above ~5000 M^{-1} cm^{-1}. Of the sulfur-containing amino acids, methionine (980 M^{-1} cm^{-1}) has a higher absorbance than cysteine (225 M^{-1} cm^{-1}). All other amino acids have a molar extinction coefficient below 150 M^{-1} cm^{-1}.

Table 2. Molar Extinction Coefficients (ϵ) of Free Amino Acids (M^{-1} cm^{-1}) at 214 nm in 20% (v/v) Acetonitrile and 0.1% (v/v) Formic Acid

amino acid	ϵ (M^{-1} cm^{-1})[a]	amino acid	ϵ (M^{-1} cm^{-1})[a]
alanine (A)	32	leucine (L)	45
arginine (R)	102	lysine (K)	41
asparagine (N)	136	methionine (M)	980
aspartic acid (D)	58	phenylalanine (F)	5200
cysteine (C)	225	proline (P)	30
glutamine (Q)	142	serine (S)	34
glutamic acid (E)	78	threonine (T)	41
glycine (G)	21	tryptophan (W)	29050
histidine (H)	5125	tyrosine (Y)	5375
isoleucine (I)	45	valine (V)	43

[a] The relative standard deviations were all below 4%.

Table 3. Measured Molar Extinction Coefficients of the Triglycine and Tripeptides with Two Glycines and One Proline in 20% (v/v) Acetonitrile and 0.1% (v/v) Formic Acid

	ϵ (M^{-1} cm^{-1})[a]		ϵ (M^{-1} cm^{-1})[a]
Gly-Gly-Gly	1080 ± 35	Gly-Pro-Gly	3620 ± 5
Pro-Gly-Gly	950 ± 5	Gly-Gly-Pro	3880 ± 65

[a] Measured in duplicate.

Prior to MS of peptides, usually the cysteines are reduced and alkylated to prevent the formation of new disulfide bridges (*21*, *22*). To determine the effect of alkylation on the molar extinction coefficient of cysteine, α-lactalbumin was reduced and alkylated. After verifying that the alkylation was complete (material and method section), the molar extinction coefficients of the reduced and both reduced and alkylated α-lactalbumin were determined. There appeared to be no significant difference between the molar extinction coefficients of untreated and alkylated α-lactalbumin (no further data shown). Therefore, it can be concluded that alkylation does not change the molar extinction coefficient of cysteine.

To estimate the molar extinction coefficient of the peptide bond, the molar extinction coefficient of polylysine was determined using eq 1, in which n_{lysine} and $n_{peptidebonds}$ represent the number of lysine residues and peptide bonds present in the polylysine, respectively.

$$\epsilon_{peptidebond} = \frac{\epsilon_{polypeptide} - (\epsilon_{lysine} \times n_{lysine})}{n_{peptidebonds}} \quad (1)$$

The molar extinction coefficient for polylysine was determined to be 182200 ± 450 M^{-1} cm^{-1}. The molar extinction coefficient of lysine is 41 M^{-1} cm^{-1} as presented in **Table 2**. Given the fact that polylysine has a DP of 190, this results in a molar extinction coefficient for the peptide bond of 923 M^{-1} cm^{-1}.

Absorption of Proline in a Peptide Chain. Table 3 shows the molar extinction coefficients of the tripeptides triglycine and the tripeptides with two glycines and one proline, in which proline is present at the N terminus, C terminus, and in the middle of the peptide chain. It can be observed that Gly-Pro-Gly and Gly-Gly-Pro have extinction coefficients much higher that of Pro-Gly-Gly. The latter value is in the same range as that of the tripeptide Gly-Gly-Gly.

Table 4. Measured Molar Extinction Coefficients (214 nm) of Protein and Peptides Including the Relative Standard Deviation (RSD)

proteins	$\epsilon_{measured}$ (M^{-1} cm^{-1})	RSD (%)	peptides	$\epsilon_{measured}$ (M^{-1} cm^{-1})	RSD (%)
glycinin	972700	0.8	RGDS	2960	4.4
β-conglycinin	841500	1.8	RINKKIEK	7900	2.4
α-lactalbumin	292500	2.0	EQLSTSEENSK	8410	5.6
β-lactoglobulin	302900	0.5	GYG	5835	1.3
BSA	1155700	1.0	YGGFLRR	18000	4.5
α-casein	495000	3.0	YIPIQYVLSR	22000	5.2
lysozyme	258400	1.2	GPRP	8980	1.4
			RPPGFSP	23100	3.0
			RPPGFSPFR	31600	3.6

Measured Molar Extinction Coefficient of Proteins and Peptides. Table 4 shows the measured molar extinction coefficients of the several proteins and peptides analyzed. It can be clearly seen that, as expected, the proteins exhibit higher molecular extinction coefficients than peptides. The values are the average of the observed molar extinction coefficients measured in duplicate at the four different acetonitrile concentrations [5, 20, 35, and 50% (v/v)] acetonitrile and ~0.1% (v/v) formic acid. The low RSDs presented in **Table 4** show that there is not a significant influence of the acetonitrile concentration on the absorbance. In the observed variation between different acetonitrile concentrations, no trend could be observed (results not shown). These results show that when comparing peaks in one RP-HPLC chromatogram, peaks can be compared without taking into account the acetonitrile concentration at the moment of elution.

DISCUSSION

Molar Extinction Coefficients of Protein and Peptide Building Blocks. The molar extinction coefficient of the peptide bond is determined based on the absorbance of polylysine at pH 3. This polypeptide was deliberately chosen since at this pH the polypeptide is present in a random coil conformation (*23*). The molar extinction coefficient was calculated to be 923 M^{-1} cm^{-1}. This value strongly deviates from the one reported by Buck and co-workers as presented in **Table 1** (*15*). On the basis of the data of Goldfarb (*11*), the molar extinction coefficient of a peptide bond, also based on the absorbance of polylysine at 214 nm, should be ~1000 M^{-1} cm^{-1}, which is close to our findings and similar to the value used in GPMAW (*16*). Therefore, for all further calculations in this report, for the peptide bond, a molar extinction coefficient of 923 M^{-1} cm^{-1} is used.

In **Figure 1**, the measured values of protein and peptide building blocks, as presented in **Table 2**, are compared with values deduced from Saidel and co-workers (*9*), Wetlaufer (*10*), and those reported by GPMAW (*16*) and Buck and co-workers (*15*). The amino acids can be divided into four groups, based on their contributions relative to the molar extinction coefficient of the peptide bond itself. The first group has a high contribution to the absorption at 214 nm, being larger than the absorption of the peptide bond [$\epsilon_{aminoacid} > \epsilon_{peptidebond}$:Pro (not at N terminus), Trp, Tyr, His, and Phe]. The second group has a rather similar molar extinction coefficient as the peptide bond ($\epsilon_{aminoacid} \approx \epsilon_{peptidebond}$:Met). The third and fourth groups have molar extinction coefficients lower than that of the peptide bond. The molar extinction coefficient of the third group is between 10 and 100% of that of the peptide bond (10% < $\epsilon_{aminoacid}$ < 100% $\epsilon_{peptidebond}$:Arg, Asn, Gln, and Cys), whereas the fourth group has an almost negligible molar extinction coefficient, less than 10% of the extinction coefficient of the peptide bond [$\epsilon_{aminoacid}$ < 10% $\epsilon_{peptidebond}$:Pro (at N terminus), Gly, Ala, Ser, Lys, Thr, Val, Ile, Leu, Asp, and Glu]. Below, each group will be discussed individually.

Group I $\epsilon_{aminoacid} > \epsilon_{peptidebond}$. Just as presented in all references, tryptophan is the most dominant contributor to the absorbance at 214 nm. Histidine, phenylalanine, and tyrosine have measured values around 5000−6000 M^{-1} cm^{-1}, which is in the same range as the previously reported values.

As an individual amino acid, proline does not show a significant absorbance (30 M^{-1} cm^{-1}), as also shown by Saidel and co-workers (*9*). This low absorbance is probably the reason why Buck and co-workers (*15*) and GPMAW (*16*) do not regard proline to be a significant contributor to the absorbance of peptides and proteins. However, when the (secondary) amino group of proline participates in the peptide bond (Gly-Pro-Gly and Gly-Gly-Pro), the molar extinction coefficient strongly increases (**Table 3**). It should be noted that this increase is not observed when proline is present at the N terminus of the peptide. The contribution of proline to the molar extinction coefficient of a protein or a peptide, when proline is present in other positions than the N terminus, can be calculated by the difference in absorbance between Gly-Gly-Gly and the average molar extinction coefficient of Gly-Pro-Gly and Gly-Gly-Pro. This results in a molar extinction coefficient for proline of 2675 (±155) M^{-1} cm^{-1}, in which the calculation is based on the raw data from which the values presented in **Table 3** are calculated.

Group II: $\epsilon_{aminoacid} \sim \epsilon_{peptidebond}$. Methionine is the only amino acid that has a rather similar molar extinction coefficient as the peptide bond. The result found in this study is found to be in the same range as the ones deduced from Saidel and co-workers (*9*) and Wetlaufer (*10*). It is also quite similar to values used by GPMAW. In contrast, Buck and co-workers (*15*) assumed that methionine would not contribute significantly in the absorption at 214 nm.

Group III: $10\% < \epsilon_{aminoacid} < 100\% \epsilon_{peptidebond}$. Group III comprises cysteine, asparagine, glutamine, and arginine. The measured molar extinction coefficient of cysteine was similar to the absorbances deduced from Saidel and co-workers (*9*) and Wetlaufer (*10*). GPMAW (*16*) expects a higher contribution for cysteine, which is not in line with other reported values. It might be that GPMAW (*16*) deduced its values from Saidel and co-workers (*9*) but mixed up the value for cysteine with cystine. Alkylation was shown not to have a significant effect on the molar extinction coefficient of α-lactalbumin. This indicates that upon alkylation as often used in peptide analysis, the molar extinction coefficient of alkylated cysteine will remain ~230 M^{-1} cm^{-1} as presented for cysteine in **Table 2**.

Buck and co-workers (*15*) assumed that glutamine and asparagine both have the same molar extinction coefficient as the peptide bond due to the presence of the amide group. This assumption is, in our opinion, not valid. Our measured values and the values presented by Saidel and co-workers (*9*) show a much lower molar extinction coefficient of glutamine and asparagine, indicating that the assumption of Buck and co-workers (*15*) is indeed invalid.

Group IV: $\epsilon_{aminoacid} < 10\% \epsilon_{peptidebond}$. This group contains amino acids that hardly contribute to the absorbance of proteins and peptides when compared to the peptide bond. Proline, when present at the N terminus, is also present in this group.

Validation of the Molar Extinction Coefficients of Proteins and Peptides. To validate whether the molar extinction coefficient of a protein or peptide can be calculated based on the absorbance of the individual building blocks, the molar extinc-

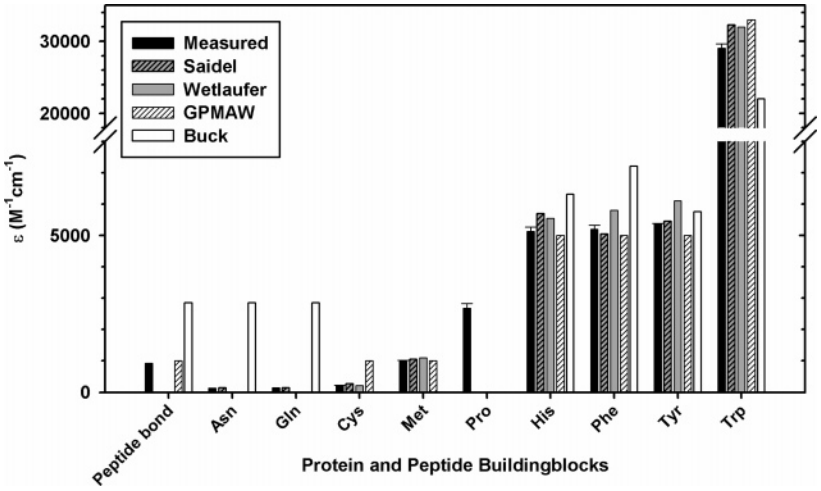

Figure 1. Molar extinction coefficients at 214 nm of measured and literature values for the protein/peptide building blocks (peptide bond and individual amino acids) as presented by Saidel and co-workers (*9*), Wetlaufer (*10*), GPMAW (*16*), and Buck and co-workers (*15*). The value for proline represents its molar extinction coefficient at the non-N terminus position.

tion coefficients of the various proteins and peptides analyzed were calculated using eq 2. This calculation is based on the assumption that the molar extinction coefficient of proteins and peptides at 214 nm is only defined by the contribution of the peptide bonds present together with the sum of the contribution of the individual amino acids present.

$$\epsilon_{\text{protein/peptide}}\,(\text{M}^{-1}\,\text{cm}^{-1}) = \epsilon_{\text{peptidebond}} \times n_{\text{peptidebonds}} + \sum_{i=1}^{20} \epsilon_{\text{aminoacid}(i)} \times n_{\text{aminoacid}(i)} \quad (2)$$

In eq 2, the $\epsilon_{\text{peptidebond}}$ is 923 M^{-1} cm^{-1} and for $\epsilon_{\text{aminoacid}}$ the values from **Table 2** are used, with the only exception that for proline 2675 M^{-1} cm^{-1} (when not present at the N terminus) is used. In **Figure 2**, the calculated molar extinction coefficients are compared with the values measured as presented in **Table 4**. In addition to this, also, the molar extinction coefficients solely based on the absorption of the peptide bonds ($\epsilon_{\text{peptidebond}} \times n_{\text{peptidebonds}}$) are presented. The difference between the measured value and the calculated value only based on the peptide bond illustrates the significant contribution of the amino acids itself to the overall absorbance.

Validation of the Molar Extinction Coefficients of Proteins. It can be observed (**Figure 2A**) that the measured values for glycinin, β-conglycinin, α-lactalbumin, and β-lactoglobulin and BSA are quite close to the calculated values (error <11%), whereas the errors for α-casein and lysozyme are higher (20 and 25%, respectively). For the calculation of the molar extinction coefficient of β-conglycinin (containing two or four GlcNAc per protein molecule; *24*, *25*), the molar extinction coefficient of GlcNAc (experimentally determined to be ~500 M^{-1} cm^{-1}) was not taken into account. The results show that the concentration of proteins can be rather well-estimated by their absorbance at 214 nm, although the error is larger than when measuring the absorbance at 280 nm. Using this wavelength, the error is reported to be below 2% (*26*). The differences between calculated and measured values might have several reasons. The presence of impurities might be one of the reasons for the large errors. In this paper, all protein and peptide preparations were regarded to be 100% pure. Another reason for a high error when measuring at 214 nm might be the influence of the protein conformation (*8*). This is difficult to take into account in the calculation. However, it should be stated that the conformation has a larger effect on the absorbance at lower wavelengths (190−200 nm) than at 214 nm (*8*).

Validation of the Molar Extinction Coefficients of Peptides. From **Figure 2B**, it can be concluded that the molar extinction coefficient can be predicted rather well for the different peptides as can be observed by the small difference between the measured and the calculated values. Only the difference between the measured and the calculated value of GYG, RPPGFSP, and RPPGFSPFR is relatively large.

When peptides do not contain amino acids that play a significant role in the absorbance at 214 nm (groups III and IV; RGDS, RINKKIEK, and EQLSTSEENSK), the molar extinction coefficient is only defined by the absorbance of the peptide bond. Therefore, for some peptides, it has a linear relation with the length of the peptide (*27*).

When tyrosine is present in the peptides (GYG, YIPIQYVLSR, and YGGFLRR), this clearly results in an increase in molar extinction coefficients and is rather well-predicted based on the amino acid composition.

The peptides that contain proline (GPRP) and proline with phenylalanine (RPPGFSP and RPPGFSPFR) clearly show a higher absorbance than only based on their peptide bonds. It shows, taking into account the relative high molar extinction coefficient of phenylalanine, the strong influence of proline on the absorption. However, for RPPGFSP and RPPGFSPFR, there is still a relative large error between the calculated and the measured molar extinction coefficient. This might indicate that the contribution of proline to the molar extinction coefficient is not yet fully understood. It might be that when prolines are present next to each other, the absorbance is strengthened as in RPPGFSP(FR), but this aspect was not studied.

The large differences observed in the molar extinction coefficients between peptides with a similar number of peptide bonds (e.g., RINKKIEK and RPPGFSP) illustrates the already mentioned necessity to find a method to estimate peptide concentrations based on their molar extinction coefficients during RP-HPLC analysis.

Table 3 shows that Gly-Gly-Gly has a molar extinction coefficient of 1080 M^{-1} cm^{-1}, whereas a value of 1909 M^{-1} cm^{-1} was expected according to eq 2 and **Table 2**. A similar observation can be made for the absorbance of GYG, for which a value of 7263 M^{-1} cm^{-1} was expected and a value of 5835 M^{-1} cm^{-1} was measured. These results indicate that for these peptides probably the peptide bond does not have a molar

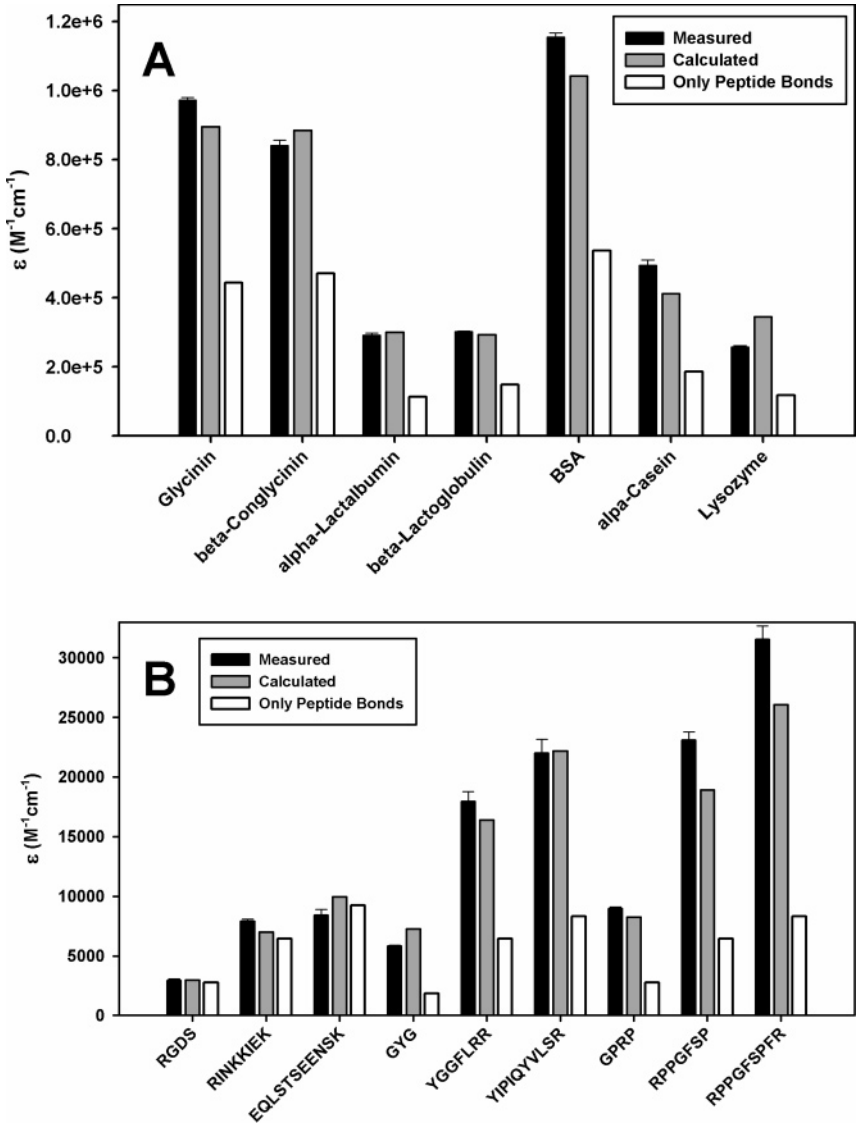

Figure 2. Measured and calculated molar extinction coefficients at 214 nm for various proteins (**A**) and peptides (**B**). Measured values are determined in the average of duplicate measurements at four different acetonitrile concentrations.

extinction coefficient of 923 M^{-1} cm^{-1} but a lower value. However, the molar extinction coefficients for RGDS, RINKKIEK, and EQLSTSEENSK show that a molar extinction coefficient of 923 M^{-1} cm^{-1} for the peptide bond is a good estimation. The reason for the lower molar extinction coefficient for Gly-Gly-Gly can be due to influence of the terminal carboxyl group. If the terminal carboxyl group is charged, this influences the absorption of the peptide bond. The absorbance is lower below the pK_a of the carboxyl group (~pH 3) than above the pK_a (*11*). Because this only counts for the C-terminal carboxyl group, its influence will decrease with increasing chain length (*12*, *13*). Also, there is no difference between the measured and the calculated molar extinction coefficient of RGDS. This shows that not for all small peptides a lower value is measured when compared to the calculated value.

It can be concluded that based on the absorbance at 214 nm and the known amino acid sequence of a peptide, the molar extinction coefficient can be predicted rather well. Therefore, **Table 5** presents molar extinction coefficients of protein and peptide building blocks to be used in RP-HPLC-MS quantification. We are aware that due to the complexity of the peptide and protein absorbance at 214 nm there is still an error in the prediction of the molar extinction coefficients. Therefore, more research with the aim to better understand the absorbance of peptides at 214 nm will be needed. Nevertheless, when using

Table 5. Molar Extinction Coefficients of Protein and Peptide Building Blocks for Calculation of the Molar Extinction Coefficients of Peptides and Proteins

building block	ϵ (M^{-1} cm^{-1})	building block	ϵ (M^{-1} cm^{-1})
group I: $\epsilon_{aminoacid} > \epsilon_{peptidebond}$			
proline (not at N terminus) (P)	2675	tyrosine (Y)	5375
histidine (H)	5125	tryptophan (W)	29050
phenylalanine (F)	5200		
group II: $\epsilon_{aminoacid} \sim \epsilon_{peptidebond}$			
peptide bond	923	methionine (M)	980
group III: 10% < $\epsilon_{aminoacid}$ < 100% $\epsilon_{peptidebond}$			
arginine (R)	102	glutamine (Q)	142
asparagine (N)	136	cysteine (C)	225
group IV: $\epsilon_{aminoacid}$ < 10% $\epsilon_{peptidebond}$			
glycine (G)	21	valine (V)	43
proline (at N terminus) (P)	30	isoleucine (I)	45
alanine (A)	32	leucine (L)	45
serine (S)	34	aspartic acid (D)	58
lysine (K)	41	glutamic acid (E)	78
threonine (T)	41		

the values presented in **Table 5**, a good estimation can be made of the peptide concentration, preventing large over- or under-estimations of peptide amounts present.

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