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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON-LINE WITH ULTRAVIOLET/VISIBLE SPECTROSCOPY IN FOOD SCIENCE

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The aim of the present review was to describe the application of diode-array detectors for the identification of HPLC-separated chemical compounds on the basis of their UV/Vis spectra.

Diode-array detectors serve to identify compounds containing conjugated double bonds, especially aromatic compounds, and to evaluate their purity. This group of compounds includes: peptides and proteins (in that case aromatic amino acid residues are chromophores), anthocyanins and other flavonoids, other phenolic compounds, carotenoids and other dyes.

The most simple strategy of interpreting UV/Vis spectra is to determine the location of absorbance maximum in zero order spectra (such a strategy is usually applied in dye analysis). The absorbance ratio at different wavelengths was used to identify and investigate structural changes in proteins and peptides. Chemometrical analysis of zero order spectra provides a basis for evaluating peak purity and choosing the background spectrum. Derivatives of spectra may be characterised *via* determination of convexity intervals, location of minima of second derivative, calculation of amplitudes of second and third derivatives or similarity indices between derivatives of individual spectra.

The possibilities offered by UV/Vis spectroscopy cover, among others, discrimination between spectra of proteins with different contents of aromatic amino acids (mainly tyrosine and tryptophan), as well as discrimination between spectra of anthocyanins containing the same chromophore but different glycosidic moieties.

INTRODUCTION

High-performance liquid chromatography on-line with UV/Vis spectroscopy (HPLC-DAD) is the most simple and the most popular of hyphenated analytical techniques based on chromatography. It is commonly used together with mass spectrometry (as HPLC-MS) or other spectroscopic methods, *e.g.* infrared spectroscopy (as HPLC-IR) or nuclear magnetic resonance (as HPLC-NMR). Many diode array detectors (DAD) enabling the acquisition of spectra of separated fractions in real time are now available on the market [Riordon, 2000]. HPLC-DAD equipment is relatively inexpensive, compared with equipment for other hyphenated chromatographic techniques.

Most of the applications of diode array detectors are restricted to the selection of a narrow wavelength range as recommended for quantitative analysis [Lendi & Meyer, 2005]. Retention time remains the most widely used discriminant for analyte identification. In contrast, UV-Vis spectra serve as a tool for analyte identification. An example of increasing significance of HPLC-DAD is the work by Ford and co-workers [2005]. These authors proposed HPLC-DAD as a reference method in an experiment aimed at developing other methods for caffeine determination in beverages. Many reviews deal with the application of chromatographic methods in food analysis, and numerous original articles describe results obtained by the HPLC-DAD technique. However, no review focuses on the interpretation of UV/Vis spectra of food components, obtained with this technique.

This paper presents examples of the applications of diode array detectors for the identification of compounds found in foods.

CHEMICAL COMPOUNDS IDENTIFIED USING HPLC--DAD

Diode array detectors serve as a tool for the identification of compounds containing conjugated double bonds, mainly aromatic compounds, and the evaluation of their purity. Application of DAD enables identification of desired compound after chromatographic separation if its spectrum differs significantly from the spectra of other compounds present in the analysed system. Most works describing UV and/or Vis spectra acquired using DAD concern phenolic compounds, *e.g.* anthocyanins and other flavonoids.

UV spectra acquired via diode array detectors are used for the identification of proteins and peptides. In that case tryptophan, tyrosine and phenylalanine residues are chro-

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TABLE 1. Food compounds analysed using HPLC-DAD.

Group of compounds	Examples of publications
Proteins and peptides	[Grego *et al.*, 1986; Palladino & Cohen, 1990; Young & Gorenstein, 1994; Perrin *et al.*, 1995, 1996; Zhao *et al.*, 1996; Bishop *et al.*, 1996; Bartolomé *et al.*, 1997; Macaud *et al.*, 1999; Dziuba *et al.*, 2001, 2002; Wang & Lucey 2003; Minkiewicz *et al.*, 2003a; Minkiewicz, 2004; Darewicz *et al.*, 2005a, b; Mikulíkova *et al.*, 2005]
Anthocyanins	[Hong & Wrolstad, 1990; de Pascual Teresa *et al.*, 2002; Kucharska & Oszmiański, 2002; Minkiewicz *et al.*, 2004; Longo & Vasapollo, 2005]
Other flavonoids	[Caristi *et al.*, 2003; Vallejo *et al.*, 2004; Łuczkiewicz *et al.*, 2004; Slimestad *et al.*, 2005]
Other phenolic compounds	[Karamać *et al.*, 2004; Siriwoharn & Wrolstad, 2004; Seger *et al.*, 2005; Kerem *et al.*, 2005; Kowalski & Kowalska, 2005]
Carotenoids	[Azevedo-Meleiro & Rodríguez-Amaya, 2004; Cortés *et al.*, 2004; Quin *et al.*, 2005]
Other colourants	[de Villiers *et al.*, 2003]
Water-soluble vitamins	[Heudi *et al.*, 2005]
Preservatives	[Saad *et al.*, 2005]
Mycotoxins	[Puel *et al.*, 2005]

mophores. Also carotenoids, aromatic mycotoxins, and synthetic dyes are identified using HPLC-DAD. Examples of DAD applications for the identification of food compounds are presented in Table 1. This Table provides only a few representative examples of DAD applications. Many articles have been published on the analysis of phenolic compounds. We paid special attention to proteins and peptides, because due to certain analytical problems these groups of compounds require the most sophisticated strategies of spectra interpretation.

STRATEGIES USED FOR THE INTERPRETATION OF UV/Vis SPECTRA

Strategies of interpreting UV/Vis spectra acquired using DAD are summarised in Table 2. The calculation of the wavelength corresponding to the absorbance maximum is the most simple among protocols of the processing of zero order spectra. The absorbance maximum is one of the key elements of colourant characteristics.

The absorbance ratio at different wavelengths was used as a tool for investigating protein structure changes [Wu *et al.*, 1986; Bishop *et al.*, 1996], as well as for protein and peptide identification [Dziuba *et al.*, 2002, 2003; Darewicz *et al.*, 2005a, b]. The application of such a strategy does not require diode array detectors. The absorbance ratio at the wavelengths of 280 and 210–240 nm can be recommended. The first one is a measure of the aromatic amino acid content [Pace *et al.*, 1995; Moffatt *et al.*, 2000], but it may also depend on the tertiary and quaternary structure of proteins. Absorbance within the range below 240 nm depends on the number of all amino acid residues in the sample [Moffatt *et al.*, 2000]. Solvents containing water, acetonitrile and trifluoroacetic acid show no significant absorbance at the wavelength of 280 nm. Visser *et al.* [1991] have designed a solvent system allowing to obtain an approximately horizontal baseline at the wavelength of 220 nm. The possibility to obtain the horizontal baseline depends on the quality of the reagents used, *e.g.* water [Mabic *et al.*, 2005]. The application of the absorbance ratio has two major advantages: possibility to analyse substances at low concentrations, and high precision [Dziuba *et al.*, 2003]. The disadvantage of this identification strategy is low robustness [Darewicz *et al.*, 2005a]. Robustness has been recently [Cuadros-Rodrguez *et al.*, 2005] understood as the capacity of an analytical procedure to remain unaffected by small, but deliberately introduced variation in method parameters. The differences

TABLE 2. Strategies of UV/Vis spectroscopy-based identification of substances separated *via* HPLC.

Strategy	Examples of publications
Determination of absorbance maximum location in zero order spectra	[Hong & Wrolstad, 1990; Bartolomé *et al.*, 1997; Azevedo-Meleiro & Rodriguez-Amaya, 2004; Kerem *et al.*, 2005; Slimestad *et al.*, 2005; Kowalski & Kowalska, 2005]
Calculation of the absorbance ratio at various wavelengths in zero order spectra	[Wu *et al.*, 1986, Bishop *et al.*, 1996; Dziuba *et al.*, 2002, 2003; Darewicz *et al.,* 2005a, b]
Chemometrical analysis of zero order spectra	[Young & Gorenstein, 1994; Macaud *et al.*, 1999; Kuczyńska & Walczak, 2002; Lindholm *et al.*, 2003; Papadoyannis & Gika, 2004, Boelens *et al.*, 2004]
Determination of convexity intervals on the basis of the first derivatives of UV spectra	[Bartolomé *et al.*, 1997]
Determination of minima and/or zero crossing location in the second derivatives of UV spectra	[Grego *et al.*, 1986; Palladino & Cohen, 1990; Dziuba *et al.*, 2003]
Calculation of amplitudes of derivatives of UV spectra at selected wavelengths	[Perrin *et al.*, 1995, 1996; Zhao *et al.*, 1996; Dziuba *et al.*, 2001, 2002]
Calculation of parameters including amplitudes of the second derivative of a spectrum and absorbance at selected wavelength in zero order spectrum	[Darewicz *et al.*, 2005a, b]
Identification of peptides on the basis of retention times obtained *via* RP-HPLC and parameters describing the first and second derivatives of UV spectra	[Perrin *et al.*, 1996]
Chemometrical analysis of UV spectra derivatives	[Minkiewicz *et al.*, 2003, 2004; Minkiewicz, 2004]
Chemometrical analysis including characteristics of chromatograms at selected wavelength and spectra of individual peaks	[Yan *et al.*, 2005]

in the A_{280}/A_{220} ratio calculated for bovine β-casein described by Darewicz *et al.* [2005a] were caused by chromatogram-to-chromatogram variation in the automatically calculated baseline. The Lambert-Beer law was not fulfilled at the wavelength of 220 nm for high protein concentration, either.

Methods classified as chemometrical are also applied for the interpretation of UV/Vis spectra. Chemometrics is defined as the science of relating measurements made on a chemical system or process to the state of the system *via* the application of mathematical or statistical methods [Tyszkiewicz & Tyszkiewicz, 2004]. Chemometrical methods for processing and interpreting spectra, aimed at the identification of compounds, evaluation of their purity and background selection include, among others, principal component analysis, calculation of the so-called match angle, *i.e.* the angle between spectra expressed as vectors in multidimensional space, and calculation of the similarity index between spectra (cosine of the angle between spectra expressed as vectors in multidimensional space) [Young & Gorenstein, 1994; Macaud *et al.*, 1999; Kuczyńska & Walczak, 2002; Lindholm *et al.*, 2003; Papadoyannis & Gika, 2004, Boelens *et al.*, 2004]. Yan *et al.* [2005] have recently described an example of chemometrical analysis including both a chromatographic pattern acquired at selected wavelengths and spectra of individual peaks. These authors have shown that two-dimensional chemometrical analysis is a more powerful analytical tool than the analysis based on chromatographic patterns only.

The analysis of proteins and peptides *via* UV spectroscopy poses certain problems due to the fact that all of them (except for chromoproteins) contain the same chromophores: tryptophan, tyrosine and phenylalanine residues. Their zero order spectra are usually too similar to be an adequate tool for identification [Dziuba *et al.*, 2001]. Derivative UV spectroscopy is more recommended for this purpose. The applications, advantages and limitations of derivative UV/Vis spectroscopy have been reviewed in several publications [Kuś *et al.*, 1996; Bosch Ojeda & Sanchez Rojas, 2004; Karpińska, 2004; El-Sayed & El-Salem, 2005]. The application of UV spectra derivatives in studies on protein structure changes has been reviewed by Lange & Balny [2002]. The spectra are characterised by convexity intervals calculated on the basis of the first derivative of a spectrum [Bartolom *et al.*, 1997], the location of minima of the second derivative of spectrum [Grego *et al.*, 1986; Palladino & Cohen, 1990], calculation of amplitudes in the second or fourth derivatives of spectra [Perrin *et al.*, 1995, 1996; Zhao *et al.*, 1996; Dziuba *et al.*, 2001, 2002] and calculation of similarity indices (SI) between standard spectra and the spectra of the substances analysed [Minkiewicz *et al.*, 2003, 2004; Minkiewicz, 2004]. Darewicz *et al.* [2005a b] have proposed parameters including amplitudes in the second derivative of a UV spectrum and absorbance within the wavelength range of 220–240 nm for the identification of bovine β-casein and its fragments. The amplitudes of the second and first derivatives of UV spectra may serve to determine the aromatic amino acid molar ratio in short peptides [Perrin *et al.*, 1995; Zhao *et al.*, 1996]. In the case of proteins the shape of UV spectra derivatives may depend also on tertiary and/or quaternary structure. Changes in amplitudes in the second or fourth derivatives of spectra are recommended to detect changes in these structures [Lange & Balny, 2002]. Changes in protein structures during sample preparation, adsorption and desorption from the surface of stationary phase have not been examined in detail yet.

Another strategy used for analyte identification involves the construction of a library containing spectra or derivatives of spectra of groups of compounds (*e.g.* libraries of UV spectra and derivatives of UV spectra of major milk proteins described by Minkiewicz *et al.* [2003]). Chemometrical interpretation is understood as matching the spectrum or spectrum derivative of analyte with the spectra or spectra derivatives of standards from the library, *e.g.* on the basis of similarity indices. A computer program arranges spectra or spectra derivatives from the library in the order from the highest to the lowest index of similarity to the spectrum of the substance identified. The chemometrical interpretation of spectra provides higher robustness than amplitudes of spectra at selected wavelengths. Robustness is an important property taken into account while evaluating various qualitative analysis methods [van Zoonen *et al.*, 1999; Cuadros-Rodrguez *et al.*, 2005]. On the other hand, amplitudes at selected wavelengths seem to be a better tool for detecting changes in the composition of chromatographic peaks or structure of components (*e.g.* tertiary structure of proteins) than the procedure involving analysis of spectra within the whole wavelength range. Bara-Herczegh and co-workers [2002] have arrived at a similar conclusion when comparing chemometrical analysis of chromatograms of a peptide mixture from cheese and analysis of changes in the areas of selected peaks from the same chromatograms.

Taking into account the above findings, chemometrical analysis allows discriminating between derivatives of UV spectra of compounds with closely related structures, *e.g.* cyanidin-3-galactoside and cyanidin-3-arabinoside, two compounds containing the same chromophore (cyanidin residue) and different sugar residues [Minkiewicz *et al.*, 2004].

EXAMPLES OF INTERPRETATION OF UV SPECTRA ACQUIRED USING A DIODE-ARRAY DETECTOR

Chromatograms of peptides obtained *via* casein hydrolysis by chymosin are presented in Figure 1. The comparison of absorbance at the wavelengths of 220 and 280 nm in Figure 1 shows the problem occurring during protein and peptide analysis. This problem is caused by a relatively low content of aromatic amino acids, especially tyrosine and tryptophan [Otaki *et al.*, 2005] and hence low absorbance at the latter wavelength [Muehlenkamp & Warthesen, 1996; Moffatt *et al.*, 2000; Dziuba *et al.*, 2002, 2003; Shan *et al.*, 2005]. In Figure 1 only one major component (C-terminal β-casein fragment) forms a peak clearly visible at 280 nm. The dominant components of peptide mixtures are fractions of macropeptide (C-terminal κ-casein fragment) [Minkiewicz *et al.*, 2000]. This peptide does not contain aromatic amino acids. Apart from the macropeptide, the major components of peptide mixtures are the N-terminal fragment of α_{s1}-casein and the C-terminal fragment of β-casein.

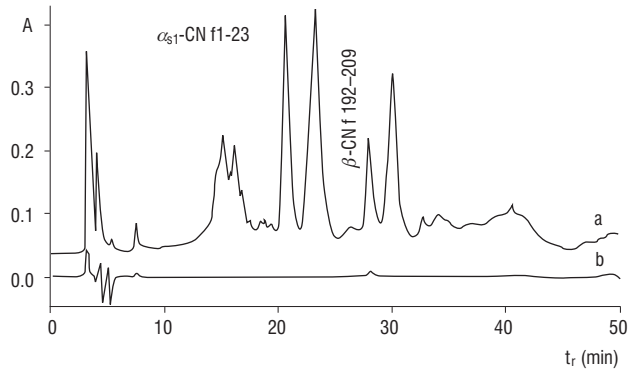

FIGURE 1. Chromatogram of products of bovine casein hydrolysis by chymosin: (a) chromatogram recorded at the wavelength of 220 nm; (b) chromatogram recorded at the wavelength of 280 nm. Peaks whose second derivatives of spectra are presented in Figure 2 are indicated. The composition of the peptide mixture has been described by Minkiewicz *et al*. [2000]. The chromatogram has been obtained *via* RP-HPLC using solvents containing acetonitrile, water and trifluoroacetic acid according to the protocol described by Dziuba *et al*. [2003].

The second derivatives of their UV spectra are presented in Figure 2. The N-terminal fragment of α_{s1}-casein contains one phenylalanine residue. In the second derivative of its UV spectrum three minima within the range of 250–266 nm correspond to absorbance of this amino acid residue. The minimum at the wavelength of 256±2 nm (258 nm in Figure 2) is used as an indicator of the presence of phenylalanine [Grego *et al.*, 1986]. This minimum is also used to measure the phenylalanine content and the ratio of tryptophan or tyrosine to phenylalanine [Perrin *et al.*, 1995; Zhao *et al.*, 1996]. The C-terminal β-casein fragment contains one phenylalanine residue and one tyrosine residue. Minima corresponding to the phenylalanine absorbance occur at the same wavelength as in the second derivative of the UV spectrum of the α_{s1}-casein fragment. Two minima correspond to tyrosine absorbance: main (or primary) at the wavelength of 283±2 nm [Grego *et al.*, 1986] and the so-called secondary minimum of tyrosine absorbance [Palladino & Cohen, 1990] at the wavelength of 276±2 nm. An unusual fraction has been found in the peptide fraction obtained *via* enzymatic hydrolysis of micellar casein systems

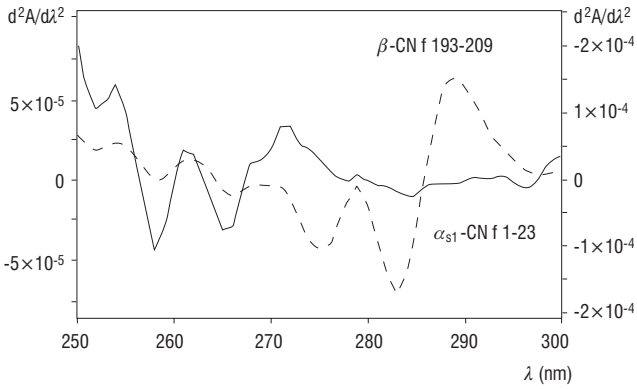

FIGURE 2. Second derivatives of UV spectra of the products of bovine casein hydrolysis by chymosin: fragment 1–23 of α_{s1}-casein (solid line and left scale) and fragment 193–209 of β-casein (dashed line and right scale). Derivatives of spectra are normalised to show differences in their shape as recommended by Papadoyannis & Gika [2004].

stabilised by dephosphorylated and glycated β-casein. This fraction contained substance whose second derivative of UV spectrum possessed one minimum at the wavelength of 265 nm [Dziuba *et al.*, 2003]. This substance could be an aromatic product of the Maillard reaction. Reactions of reducing sugars with proteins lead to the formation of many aromatic compounds [Yayalayan, 1997]. Roig & Thomas [2003] have described the product of sugar degradation revealing absorbance maximum at 268 nm.

A chromatogram of whey proteins is presented in Figure 3. The major components of whey protein fractions have been identified as α-lactalbumin and β-lactoglobulin. This agrees with literature data on bovine milk protein composition [Farrell *et al.*, 2004]. Zero order spectra, the second and third derivatives of α-lactalbumin and β-lactoglobulin UV spectra within the wavelength range of 270–300 nm are presented in Figure 4. The components of mobile phase reveal no detectable absorbance within this wavelength range. Additional background correction is thus not necessary [Dziuba *et al.*, 2001]. Similarity between two spectra may be expressed, *e.g.* by a similarity index [Papadoyannis & Gika, 2004]. The similarity index depends on the shape of spectra, but is independent of the concentration of analyte and standard. The shape of zero order spectra presented in Figure 4 is very similar. The similarity index between them is 0.999. The value of SI may vary from -1.000 to 1.000. The latter value corresponds to identical spectra. The second derivatives of spectra (Figure 4 b) are typical of tryptophan-rich proteins. They have their minimum at the wavelength of 2902 nm [Grego *et al.*, 1986], and their maximum overlaps with the secondary minimum of tyrosine absorbance. The last minimum is visible only in the second derivatives of spectra of peptides or proteins containing a low amount of tryptophan or no tryptophan at all.

The second and third derivatives of UV spectra of the

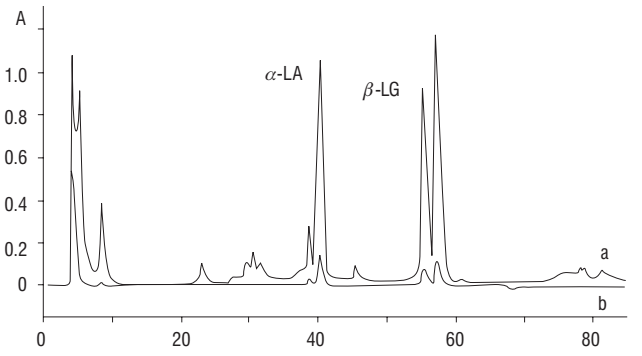

FIGURE 3. Chromatogram of whey obtained during the production of Gouda-type cheese: (a) chromatogram recorded at the wavelength of 220 nm; (b) chromatogram recorded at the wavelength of 280 nm. Peaks whose spectra and derivatives of spectra are presented in Figure 4 are indicated as follows: α-LA – α-lactalbumin; β-LG – β-lactoglobulin. The chromatogram has been obtained *via* RP-HPLC using solvents containing acetonitrile, water and trifluoroacetic acid according to the protocol published by Dziuba *et al.* [2004] using modified gradient: start: 25% of B solvent; 31% B after 10 min; 37% B after 17 min; 37% B after 25 min; 40% B after 50 min; 48% B after 60 min. The solvents A and B contained acetonitrile, water and trifuoroacetic acid at the ratio: 100:900:1 and 900:100:0.7, respectively [Visser *et al.*, 1991]. The proteins were not reduced.

substances analysed (Figure 4 b, c) reveal bigger difference than zero order spectra. The similarity index between the third derivatives of UV spectra of α-LA and β-LG does not exceed 0.95 and permits correct identification. The similarity indices between the third derivatives of UV spectra of non-reduced, reduced or thermally denatured α-LA, as well as the same protein from whey protein concentrate, kefir or acidophilous milk and the standard α-LA spectrum (acquired for reduced protein according to Minkiewicz *et al.* [2003]) were at least 0.981. The similarity index between analogous samples of β-LG and the standard spectrum of this protein was at least 0.973 [Minkiewicz, 2004]. The lowest similarity indices (below 0.990) were obtained for proteins at low concentrations (absorbance at 280 nm below 0.02). α-Lactalbumin and β-lactoglobulin are easy to discriminate on the basis of derivatives of UV spectra due to significant difference in the Trp/Tyr molar ratio (1.00 and 0.50 for α-LA and β-LG, respectively). Problems with the identification of these proteins occur if they are not well separated. Their peaks may overlap when α-LA and β-LG are separated after reduction. An example of such a situation has been described by Minkiewicz *et al.* [2003].

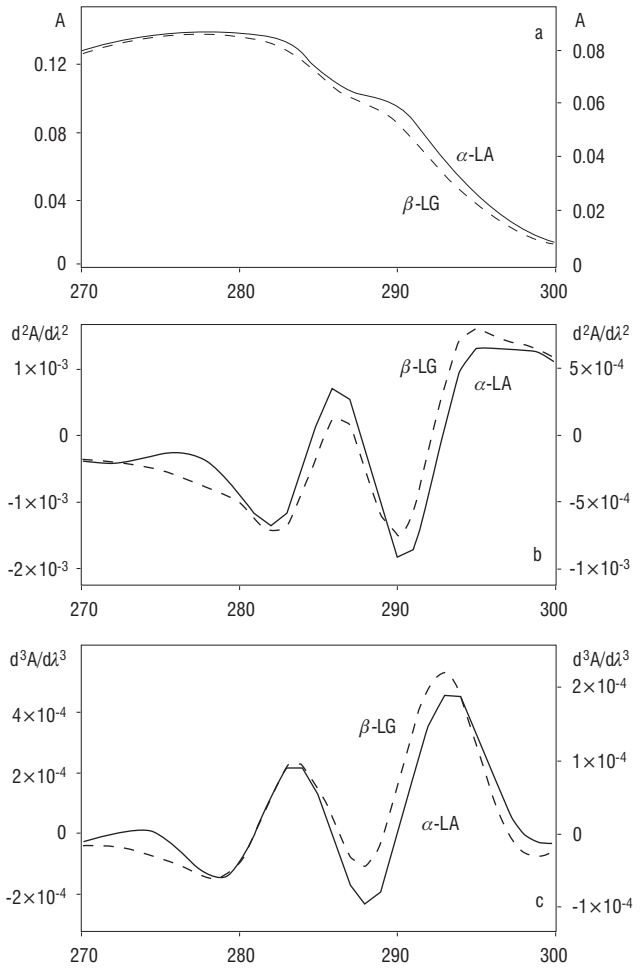

FIGURE 4. a) Zero order spectra of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) from the chromatogram presented in Figure 3; b) second derivatives of spectra of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG); c) third derivatives of spectra of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG); Solid lines and left scales – α-lactalbumin; dashed lines and right scales – β-lactoglobulin. Spectra and derivatives of spectra are normalised (see legend to Figure 2).

Bovine κ-casein may serve as an example of a protein difficult to identify, in contrast to the whey proteins described above. There are problems with the identification of this protein because its molar Trp/Tyr (1/9) is close to the analogous ratio in bovine serum albumin (1/10), and α_{s2}-casein (1/6). κ-Casein does not form compact globular structures, but aggregates containing up to 15 chains linked *via* disulphide bonds [Farrell *et al.*, 2004]. Aggregation affects UV spectra of protein. The reduced protein can be identified on the basis of the second or third derivative of UV spectrum. The 100% sensitivity of identification is achieved in both cases [Minkiewicz, 2004]. The sensitivity is defined here as a ratio of the number of true positive identifications to the sum of the number of true positive identifications and the number of false negative identifications [Pulido *et al.*, 2003; Ellison & Fearn, 2005]. In the case of κ-casein aggregates the sensitivity of identification based on the first, second and third derivatives of spectra was 0.0 (all results were false negative, *i.e.* in all cases κ-casein was incorrectly identified as some other protein), 89.6 and 95.8%, respectively (48 spectra analysed) [Minkiewicz, 2004]. Differences between spectra of reduced and oxidised κ-casein may be caused by changes of solvent accessibility of aromatic resdues [Lange & Balny, 2002], absorbance of disulphide bonds [Pace *et al.*, 1995] or changes in light scattering [Moffatt *et al.*, 2000]. Results described by Minkiewicz [2004] suggest that among the derivatives of UV spectra the third derivative is the best tool for analyte identification. This finding is consistent with the results obtained for anthocyanins [Minkiewicz *et al.*, 2004], showing that zero order spectra, the first, fourth and fifth derivatives of UV spectra provide lower sensitivity than the second and third ones. An additional criterion of order of derivative selection is the statistical significance between values of the similarity index [Minkiewicz *et al.*, 2004]. In our experiments we used as a criterion of statistical significance of the average difference between the first (the highest) and second similarity index calculated for all spectra [Minkiewicz *et al.*, 2003] or the statistical significance between the first and second similarity index calculated for individual compounds [Minkiewicz *et al.*, 2004]. Now we can recommend the second criterion as consistent with the universal criteria based on the number of true and false results of identification [Pulido *et al.*, 2003; Ellison & Fearn, 2005].

LIMITATIONS OF UV/Vis SPECTROSCOPY AS A METHOD FOR THE IDENTIFICATION OF CHEMICAL COMPOUNDS SEPARATED USING HPLC

The upper limit of concentration at which UV/Vis spectroscopy may be applied as a tool for substance identification is the concentration that enables to fulfill the Lambert-Beer law within the whole wavelength range used in the experiment. An example of spectra acquired using too high analyte concentrations has been presented by Darewicz *et al.* [2005a]. A mathematical problem referred to as numerical instability occurs within a low concentration range. Diode array detectors enable obtaining differential spectra, *i.e.* results of background subtraction from spectra. The resulting differential spectrum intensity decreases with

the analyte concentration, whereas the error of measurement does not decrease. At low concentrations the relative error becomes too high to obtain reproducible spectra and derivatives of spectra. An example of the influence of a decrease in concentration on the shape of the second derivative of a UV spectrum has been presented by Dziuba *et al.* [2001]. In the case of minor components of protein or peptide mixtures, identification on the basis of UV spectra may require an increase in the sample amount, leading to column overloading and hence to chromatogram quality deterioration [Dolan, 2005]. The range of concentrations providing applicable UV spectra should be determined individually for a particular detector and software.

Another significant problem has been pointed out by Karpińska [2004]. The derivatives of the UV/Vis spectrum of the same substance, calculated numerically using different programs, are not identical. The importance of this problem for all methods used in analytical chemistry has been highlighted by Cuadros-Rodrguez *et al.* [2005]. For instance, the possible cause for differences may be associated with the commonly used algorithm of derivative approximation by the least square method [Savitzky & Golay, 1964]. The value of the curve derivative (*e.g.* the derivative of a UV/Vis spectrum) may depend on the number of data points used for calculation by a computer program.

Another example of susceptibility of UV spectra derivatives to changes in calculation procedures has been described by Minkiewicz and co-workers [2003]. The second derivative of a spectrum was calculated with or without intermediate display of the first derivative. The first one was smoother as compared with the second one. Two spectra of the same protein, from the same chromatogram obtained at the same retention time (in the paper by Minkiewicz *et al.* [2003] – bovine α-lactalbumin) differed markedly, although from the mathematical point of view they should be identical. Susceptibility to changes in computation protocols is a common problem associated with numerical calculations [Peitgen *et al.*, 1992].

Spectra processing programs offered by individual companies may differ in details of computation protocols. The results obtained using such programs are not always comparable. Differences in the background spectrum definition may serve as an example. The Class-Vp program (Shimadzu) uses the spectrum acquired at the start of chromatographic run or the spectrum from a solvent chromatogram acquired at the retention time corresponding to the retention time of analyte. The Chemstation program (Hewlett-Packard – recently Agilent Technologies) used in HP1050 assemblies uses the average spectrum from the valley preceding and following the peak as a background. Three of the above spectra are identical only if baseline separation is achieved and solvent does not show detectable absorbance in the desired wavelength range during data acquisition. Another example of the effect of the background on the spectrum has been given by Boelens *et al.* [2004].

Taking into account the limitations described above, the only way of substance identification using UV/Vis spectra is to compare them with the standard spectrum obtained under identical conditions and processed *via* the same protocol. Otherwise it is necessary to make sure that the changes in procedure do not lead to detectable changes in the spectrum.

FINAL REMARKS

UV/Vis spectra may be a useful tool for the identification of many substances separated using high-performance liquid chromatography. In common opinion they enable only to discriminate between various chromophores, but their real potential is much higher. Further development of this method requires the elaboration of validation procedures according to recent tendencies in qualitative analysis described in review articles in the journal: "Trends in Analytical Chemistry" [Ellison & Fearn, 2005; Crdenas & Valcrcel, 2005; Lendl & Karlberg, 2005; Milman, 2005; Ros & Tllez, 2005; Trullols *et al.*, 2005; Simonet, 2005]. Design of standard computational procedures enabling a direct comparison of spectra and derivatives of spectra processed using different software seems to be an important task. Solving this problem would enhance method-validating interlaboratory tests and contribute to building libraries of UV/Vis spectra and their derivatives, which would be available on the Internet. It is also necessary to draw up a list of factors affecting the shape of spectra, and factors that allow to leave them unchanged.

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ZASTOSOWANIE WYSOKOSPRAWNEJ CHROMATOGRAFII CIECZOWEJ W POŁĄCZENIU ZE SPEKTROSKOPIĄ W NADFIOLECIE I ŚWIETLE WIDZIALNYM W BADANIACH ŻYWNOŚCI

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Celem niniejszego przeglądu jest omówienie zastosowań detektorów fotodiodowych, związanych z identyfikacją związków chemicznych na podstawie widm UV-Vis.

Detektory fotodiodowe służą do identyfikacji i oceny czystości związków chemicznych zawierających sprzężone wiązania podwójne, głównie związków aromatycznych. Do związków zawierających takie chromofory należą: peptydy i białka (chromoforami są w tym przypadku reszty aminokwasów aromatycznych), antocyjany i inne flawonoidy, inne związki fenolowe, karotenoidy i inne barwniki.

Najprostszym sposobem interpretacji widm UV-Vis jest obliczenie maksimum absorbancji widma zerowego rzędu (taki sposób interpretacji widm jest stosowany przy charakterystyce barwników). Stosunek absorbancji przy różnych długościach fali był stosowany jako parametr identyfikacji i badania zmian struktury peptydów i białek. Chemometryczna analiza widm zerowego rzędu służy do oceny czystości pików chromatograficznych lub doboru widma tła. W analizie peptydów i białek skuteczną strategią jest zastosowanie pochodnych widm. Pochodne widm mogą być charakteryzowane poprzez obliczanie przedziałów wypukłości na podstawie pierwszej pochodnej widma, położenia minimów drugiej pochodnej, amplitud drugiej i czwartej pochodnej lub wskaźników podobieństwa między pochodnymi widm.

Możliwości spektroskopii UV-Vis obejmują między innymi rozróżnianie widm UV peptydów i białek różniących się zawartością aminokwasów aromatycznych (głównie tyrozyny i tryptofanu) oraz rozróżnianie widm UV antocyjanów zawierających taki sam chromofor i różniących się strukturą reszty glikozydowej.