

Chapter 2

Basis of Derivative Spectrophotometry

2.1 The Main Law of Light Absorption by a Substance

Photobiological processes occur under the influence of light of ultraviolet (UV), visible, and near infrared spectral regions. Generally, values of light flux intensity, I , and wavelength, λ are used in optical measurements. The frequency index $\bar{\nu}$ is also considered to characterize an absorbed light. Frequency is expressed in reciprocal seconds [c^{-1}] and presents itself as the ratio of a radiation velocity c to a wavelength λ [cm or nm]:

$$\bar{\nu} = c/\lambda = 3 \cdot 10^{17} / \lambda_{\text{nm}},$$

where $c = 3 \times 10^{10} \text{ cm} \cdot \text{c}^{-1}$ or $3 \times 10^{17} \text{ nm} \cdot \text{c}^{-1}$, the velocity of light. The frequently used index is the wavenumber $\bar{\nu}$ equal to the number of waves in 1 cm. The wavenumber is a reciprocal wavelength expressed in reciprocal centimeters:

$$[\text{cm}^{-1}] : \bar{\nu} = 1/\lambda_{\text{cm}} = 10^7/\lambda_{\text{nm}}$$

Thus, $\bar{\nu} = 40,000 \text{ cm}^{-1}$, when λ 250 nm. A number of spectrophotometers have wavenumbers on the scale dials. For example, the wavelength of the cadmium red line, as accepted by international agreement, is equal to 6438.4696 Å (angstroms). So 1 Å is 1/6438.4696 part of cadmium red line.

Only absorbed quanta can realize photochemical impact and the reader should remember that a spectral region of a photobiological process is conditioned by the absorption spectrum of a substance involved in this process.

The extinction law for actinic monochromatic light absorbed by a substance layer can be written in the exponential or logarithmic form:

$$I = I_0 \cdot 10^{-\varepsilon C d}; \quad (2.1)$$

$$\lg(I_0/I) = \varepsilon Cd, \quad (2.2)$$

where I_0 and I are light intensity before and after passing through a layer of substance, of solution, or a leaf, ε is a coefficient depending on wavelength and nature of a substance, C is a concentration of a light-absorbing substance in medium, through which light passes, and d the layer thickness (length of the light path in a cuvette, an optical cell). If the concentration of solution is expressed in moles, then ε is called the molar extinction coefficient (MEC). The value of ε is numerically equal to the optical density of solution of concentration 1 mole/L at cuvette thickness 1 cm [$\text{L} \cdot \text{mole}^{-1} \cdot \text{cm}^{-1}$]. Often solution concentration is expressed as a percentage and, instead of ε , the so-called specific extinction is used ($E_{1\text{cm}}^{1\%}$), equal numerically to an optical density of 1% substance solution at the cuvette thickness 1 cm. This value is necessary to determine and to use during instrument calibration for calculation of absolute concentration of a substance in solution.

The ratio of passing and incident light intensities is called the transmission and calculated as $T = I/I_0$. The same ratio expressed as a percentage is placed at the left scale of a recording card in Russian spectrophotometers of types SP-14 and SP-18 and some European ones. It is called “the transmission percent” and is given by $T = 100 I/I_0$.

More frequently the term “optical density” is used (D), i.e., logarithm of transmission taken with reversed sign $D = -\lg T = \varepsilon Cd$.

From Eqs. 2.1 and 2.2 it follows that, though absolute quantity of absorbed light energy is directly proportional to intensity of light flux incident to the object, the percentage (portion) of the absorbed light does not depend on it. Therefore, the effect of absorption is assessed not according to the absolute value of light energy absorption, but according to its ratio to the intensity of light incident to the object. The absorption coefficient $(I_0 - I)/I_0$ shows the percentage of absorbed light, and the transmission coefficient I/I_0 the percentage of passed light. However, the absorption coefficient is not proportional to a substance concentration. Direct proportion takes place between $\lg(I_0/I)$ and a substance concentration. The value of $\lg(I_0/I)$ is acceptable to call “the optical density of solution” (D). It is agreed to determine the optical density for 1 cm thickness of the layer (Babushkin et al. 1962; Konev and Volotovskii 1974; Lebedeva 1977). Optical density is dimensionless and is used as an absorption characteristic of a substance and for calculation of its concentration. The law of proportionality of the degree of light extinction to layer thickness and to the amount of substance, through which the light passes, was formulated by Bouguer–Lambert–Beer and we will call it Bouguer’s law, using the name of the first pioneer (Morton 1975; Bershtein and Kaminskii 1975; Brandt and Eglinton 1979; Willyams 1978; Lebedeva 1977).

If the thickness of the substance layer or solution is constant, then the dependence $D = f(C)$ is presented with a straight line passing through the origin of coordinates; moreover, tangent of slope angle α of this straight line is equal to ε .

2.1.1 *Reasons for Deviation from Bouguer's Law*

If dependence $D = f(C)$ is broken, i.e., the extinction coefficient is not constant any more but increases or decreases with increase of C , then apparent deviations from Bouguer's law can arise (Babushkin et al. 1962; Calder 1969; Samsonova and Gak 1971; Rubin 1974; Morton 1975; Bershtein and Kaminskii 1975). They are known as physicochemical reasons, conditioned by discrepancy between the C value used in calculation and the real value of colored substance concentration in solution. An operator performing analysis should remember this.

Other reason for non-Bouguer's law behavior is fluorescence of an analyzed substance. If a dissolved substance can be excited by monochromatic light and to emit fluorescence, fluorescent flux reaching a photodetector causes growth of transmitted light intensity and decrease of an experimentally determined optical density. This deviation from Bouguer's law will rise with increase of optical density and decrease with rise of amount of dissolved substance (the quenching effect) (Braude et al. 1950; Rubin 1974; Konev and Volotovskii 1974; Brandt and Eglinton 1979; Lebedeva 1977).

The reader should bear in mind that another (third, instrumental one) reason for apparent non-Bouguer's law behavior can be the result of any defect of a photodetector and of an amplifying circuit of the spectrophotometer, causing nonlinear dependence of instrument readings on light flux intensity. To check a device, an operator should routinely measure transmission of several neutral light filters or solutions of studied standard substances with known density, usually supplied with spectrophotometers (Cannon and Butterworth 1953; Calder 1969; Sverdlova 1973; Shtern and Timmonis 1974; Morton 1975; Fraifelder 1980).

We should also take into account that the most frequent instrumental reason for apparent deviations from Bouguer's law is nonmonochromaticity of light flux incident to a sample. In practice, to avoid substantial deformation of a spectral band shape and of the MEC value, ε , a spectral width of an output slit of a device should be much less than half-width of investigated band ($\Delta\bar{\nu} \ 1/2$), i.e., $S \leq 0.2 \Delta\bar{\nu} \ 1/2$. (Sverdlova 1973; Tarasov 1968; Bershtein and Kaminskii 1975).

We should not lose sight of the fact that a significant contribution to light flux heteromonomochromaticity is caused by scattered light, i.e., by polychromatic emission incident into a tray camera of a spectrophotometer as a result of different reflections, and also by scattered light in a dispersive system as a result of a not tightly closed cover of an instrument. Influence of scattered light grows with decrease of I_0 , i.e., in those spectral regions, where emission of a radiation source is small or optical density of a comparison solution is great. Because of this, spectral measurements must be practically performed in those regions of a spectrum where the absorption of an investigated substance is great enough.

The following group of reasons for apparent deviations from Bouguer's law, often neglected by an operator, is connected with distribution of an absorbent material in a volume of the analyzed object. First, absorption of the nonpolarized light by anisotropic molecules depends on their order strength. The specific

ordering of absorbent material molecules arrangement can be realized in films (Platonova et al. 1970; Popov and Smirnov 1971). An additional reason for deviations from Bouguer's law is the nonuniform distribution of absorbent material in a light beam (in a cuvette) appearing due to object nonhomogeneity. Therefore, working with homogenates of leaves, suspensions of algae, of mitochondria, of chloroplasts, or with turbid solutions, an operator should regularly shake cuvettes in order to prevent nonuniform particle distribution in them or the possibility of bubbling (Babushkin et al. 1962; Shibata et al. 1973; Morton 1975; Fraifelder 1980).

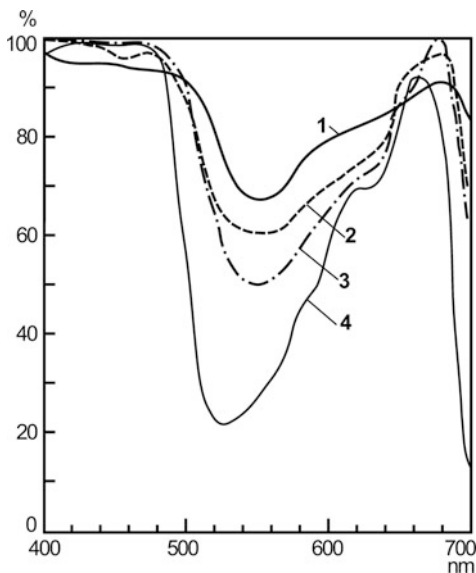
Research experience shows that three effects appear in measurements of absorption spectra of biological subjects and are most significant for errors of spectral determination: nonselective light scattering by uncolored parts of plants, selective light scattering by pigments, and the effect of "skipping" or "sieve" (Moos and Loomis 1952; Gurinovich et al. 1968).

We must not forget that biological subjects and structures not only absorb but also scatter light. So only a part of the light passing through the object reaches the instrument photoreceiver and this creates a false impression of absorption increase. Light scattering also influences the form of spectral absorption curves, causing an overall lift of the spectral curve relative to the density axis. Therefore, corrections for light diffusion in the region of the absorption band should be performed by extrapolation from the region, where true absorption is absent, usually from the region located in the long-wave part of the spectrum comparatively the investigated absorption band, for example, for chlorophyll *a* solutions – from $\lambda = 750$ nm. Correction should be made in measurements of light-scattering objects and it can be useful when the instrument is supplied with adaptations for light scattering decrease (Fig. 2.1).

When choosing experimental conditions and an object for study it is reasonable to decrease optical heterogeneity of systems (particles sizes in a system and difference between refractive indices of an incubation medium and studied particles). Practically this is achieved, on one hand, by usage of tissue homogenates and suspensions of chloroplasts and mitochondria instead of intact leaves, and also by decrease of layer thickness at sufficiently high optical density. On the other hand, addition of substances increasing the refractive index of the medium (glycerin, saccharose, and other) to the incubation medium results in object light diffusion decrease (Moos and Loomis 1952; Zaidel' et al. 1972).

Therefore the operator must find the concentration of the added substance at which the optical density of the solution in the region of minimum absorption is lowest. In the case of leaf homogenates this corresponds to 40–45% glycerin. This question is described at length in the Rabinovich's monograph (Rabinovich 1953) and books by Rubin (Rubin 1974, 1975). In performing spectra registration the reader should remember that the optical density rises because of extensive light scattering and multiple reflection of rays in a heterogeneous biological system. This increase of *D* is more prevalent in absorption minima which results in lower absorption selectivity and smoothing of the differences between absorption spectrum maxima and minima. Application of the method of derivative

Fig. 2.1 Absorption spectra: 1 fresh intact leaf of spinach; 2 isolated chloroplasts; 3 chloroplasts fragments; 4 unrefined methanol extract of pigments (Moos and Loomis 1952; Khit 1972)



spectrophotometry allows one to decrease substantially errors caused by light diffusion (see Chap. 4).

Substances absorbing light in biological systems are unevenly distributed, so one part of the ray traversing absorbing particles (chloroplasts, chromatophores) is weakened, whereas another part “passes” through the sample thickness without absorption. The phenomenon of “skipping,” resulting in admixture of actinic (acting) light, causes an optical density decrease that strongly affects the absorption maximum (Morton 1975; Fraifelder 1980).

Thus we should remember that light diffusion and the “skipping” effect, acting in opposite directions, has several effects – e.g., reduction of absorption selectivity, spectra smoothing, maxima broadening (Popov and Smirnov 1971; Rubin 1975; Lebedeva 1977; Willyams 1978). It is always necessary to take into consideration that methods of quantitative spectrophotometric analysis of mono- and multicomponent mixtures, when Bouguer’s law is valid and when substances do not chemically interact with each other, are based on the principle of additivity of optical densities formulated by Vierordt (Vierordt 1873). In accordance with this principle the optical density of a complex mixture of substances is equal to the sum of the partial optical densities (see Figs. 2.7 and 2.8), corresponding to the light absorption of each substance:

$$D = \varepsilon_1 C_1 d_1 + \varepsilon_2 C_2 d_2 + \dots + \varepsilon_n C_n d_n = d \sum_{i=1}^n \varepsilon_i C_i$$

2.2 Correctness and Accuracy of Spectrophotometric Data

The correctness of measurements is conditioned by the presence or absence of systematic errors of experiment, and accuracy of random ones. Therefore, for accuracy assessment it is necessary to perform a number of repeated measurements, and for evaluation of their correctness some analogous measurements of standard reference samples. The lower the average quadratic (standard deviation) S is, the better will be the reproducibility of results, i.e., they are in the narrower interval and are more precise. Correctness is characterized by the difference between the average result of measurements and the true value of measured characterization (by measurement error).

Deviations from Bouguer's law, considered in Sect. 2.1.1, causes systematic errors both of MEC values and of positions of bands absorption maxima (λ_{\max}). An operator should remember that the narrower the absorption maximum, and the shorter the waves in the region in which it is located, the more values of λ_{\max} and ε can be misrepresented because of the heteromonochromaticity of emission (Shabalin and Petrova 1969; Litvin et al. 1973b; Rubin 1974; Shtern and Timmonis 1974; Rubin 1975). Special attention should be paid to systematic errors caused by misadjustments of a wavelength scale. For the real time control of a wavelength scale it is recommended to use solutions or glass filters with rare earth elements having extremely narrow absorption bands (French 1957b; Dodd and West 1961; Fog and Osnes 1962), for example, the filter No. 1 from the complete set of Russian spectrophotometers (SP-10, SP-14, and SP-18), adapted for instruments calibrating during their release or repair. Also, a reader could use neodymium filters supplied with some spectrophotometers (firms "Opton," "Shimadzu," "Varian"), which are international standards. In the same way, derivatives of holmium (firms "Hitachi," "Opton," "Perkin Elmer," "Shimadzu") or benzene vapors ("Beckman," "Jobin Yvon," "Varian," etc.) are used in international practice.

The test of a spectrophotometer transmission scale (of optical densities) is hampered by the absence of generally accepted standards of optical density (Bershtein and Kaminskii 1975; Lebedeva 1977; Fraifelder 1980).

In practice, as D standards, solutions of stable inorganic compounds characterized by comparatively mildly sloping spectra in UV and visible spectral ranges are most frequently used (Burke et al. 1972; Lebedeva 1977). Of these, 0.006006% solution of potassium bichromate in 0.01 N H_2SO_4 took the widest hold and could be recommended (Burke et al. 1972). Optical density of this solution was repeatedly measured in instruments of different types (Bückert and Raffaele 1963; Calder 1969). Values, reported by different authors vary considerably: $D = 0.7464\text{--}0.7670$ for $\lambda = 235\text{nm}$; $D = 0.8660\text{--}0.8793$ for $\lambda = 257\text{nm}$; $D = 0.2865\text{--}0.2955$ for $\lambda = 313\text{nm}$; $D = 0.6408\text{--}0.6475$ for $\lambda = 350\text{nm}$ (Bershtein and Kaminskii 1975). It now seems that the most reliable MEC is one of potassium bichromate in HClO_4 , accepted by the National Bureau of Standards, USA (Burke et al. 1972) (Table 2.1).

Table 2.1 MEC values for solutions of $K_2Cr_2O_7$ in $HClO_4$, $pH = 2.92 \pm 0.02$, temperature $25^\circ C$ (Bungard et al. 1999)

$K_2Cr_2O_7$ (mg/L)	Spectral region (nm)			
	350	313	257	235
	MCE values			
20.22	3,155.2	1,427.0	4,227.2	3,629.4
40.09	3,159.8	1,426.6	4,241.2	3,640.4
60.12	3,161.8	1,427.6	4,254.4	3,654.2
80.17	3,168.0	1,430.0	4,275.2	3,671.6
99.92	3,171.0	1,431.2	4,288.0	3,683.0

To characterize optical densities and to assess various forms of spectra, for example as considered by Talsky (Talsky 1994), sets of inorganic and organic solutions are recommended and successfully used together with potassium bichromate in analytical spectrophotometry.

It is necessary to remember that, for the same objects, results of D measurements are different for different spectrophotometers (including instruments of one type and when they are previously reliably adjusted), and usually registered figures are distinguished by 0.02–0.05 units of optical density (Brode et al. 1953; Shabalin and Petrova 1969; Shtern and Timmonis 1974; Fraifelder 1980). At the same time, λ_{max} values published by different authors vary by not more than 2 nm in the majority of cases (Phyllips 1962; McWilliam 1969). As ready standards for determination of optical density values it is possible to use neutral light filters from standard sets for SP-16 and SP-26, produced by the Optical-Mechanical Association of Saint-Petersburg (Russia).

2.2.1 Main Factors Influencing the Accuracy of Spectrophotometric Measurements

The possible totality of measurement errors should be considered when performing spectrophotometric analysis. This totality is composed of smaller errors from separate operations:

1. Random errors of preparation of analyzed solution, influence of turbidity, and fluorescence of the analyzed substance or admixtures contained in the solution.
2. Tray error due to different light absorption of cuvettes uncompensated due to different thickness of cuvettes with absorbing solvent and multiple internal light reflections (0.05–0.1% of transmission) (Burnett 1973). Nonreproducibility of the state of optical faces of cuvettes and their position in a cuvette holder is especially important. This error limits the general accuracy of analysis at low density values (Tereshin 1959a, b; Challise and Williams 1964; Ishii and Satoh 1982).
3. Error of blank experiment as in 1 above.

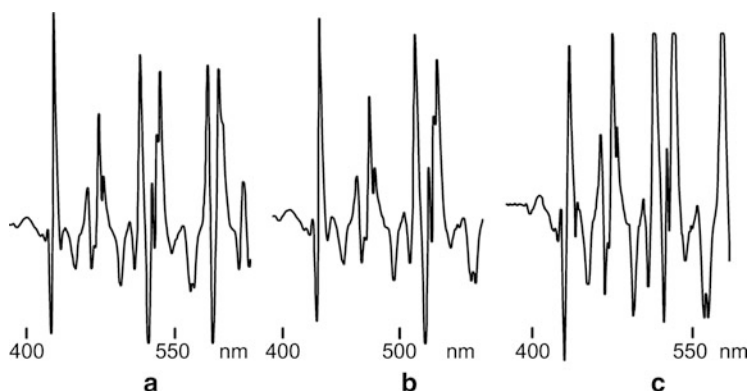


Fig. 2.2 Stability of record reproducibility of the second derivative of the absorption spectrum of the control filter of didymium light filter (absorber) at different μ -factors: (a) the initial spectrum; (b) amplification 1.3; (c) amplification 1.0 (Marenko and Saakov 1973; Marenko et al. 1972; Rutman et al. 1976a, b; Saakov and Shpotakovskii 1973; Rutman et al. 1978)

4. Error in the setting of an analytical wavelength, including a reading error of a wavelength scale and discrepancy between the position of the dispersive element (prism, lattice) and the indicator on a wavelength scale. When working in the region of a gently sloping absorption maximum of analyzed substance or biological structure, inaccurate installation of wavelength does not affect measurement accuracy practically. At the same time, it is necessary to remember that for sharp regions of spectral curves this error grows to 0.7–1.5% of measured value (Ismail and Glenn 1964; Komar' and Samoilov 1969; Bershtein and Kaminskii 1975; Ishii and Satoh 1982) or substantially more.
5. Direct error of the spectrophotometric measurement including errors of instrument tuning at 0 and 100% of transmission (Korablev 1973; Burnett 1973; Litvin et al. 1973b; Konev and Volotovskii 1974; Lebedeva 1977).

The reader should not forget that the relative contribution of listed factors depends on the nature of an absorption spectrum of analyzed substance, special features of an instrument, and analysis conditions. The fulfilling of a number of requirements should be checked by an operator before starting the experimental work with repeated control measurements of the light filter sent with the instrument and then by comparison of this result with a firm result for this standard (Figs. 2.2 and 2.3).

If necessary, namely in a case of absence of control light filters and records, it is possible to use light filters from the catalog of colored glass (for example, ZS-7) published by the St. Petersburg Optical-Mechanical Association. It is necessary to remember that the problem of accuracy of spectrophotometric measurements is closely related to a question on optimum optical density (D_{opt}), and also on operating range of densities permissible in the analysis. This interval should be

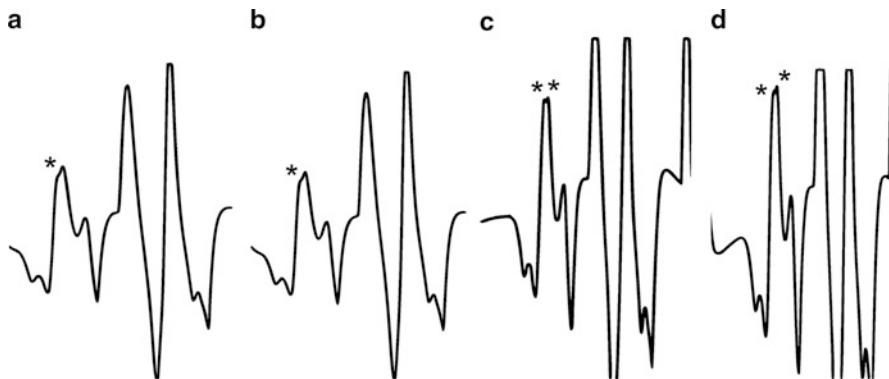


Fig. 2.3 Reproducibility stability of the second derivative of the absorption spectrum of the control filter ZS-7 from a filter set produced by the Leningrad optical-mechanical association (LOMO): (a) the spectrum of initial filter; mu-factor (MU) equals to: (b) 1.2; (c) 1.0; (d) 1.5 (Rutman et al. 1976a, b, 1978; Saakov and Spotakovskii 1973))

selected in such a way that over the entire range the standard deviation would not exceed the doubled standard deviation at D_{opt} :

$$S_D/D \leq 2(S_D/D)_{\min}$$

In the paper of Twymann and Lothian (Twyman and Lothian 1933) relative standard deviation of D takes the form

$$S_D/D = 0.4343 \cdot S_T/D \cdot 10^{-D}. \quad (2.3)$$

Equation 2.3 is deduced by authors supposing that the analyzed substance complies with Bouguer's law, and standard deviation of transmission S_T does not depend on the T value. Differentiating Eq. 2.3 and setting the derivative to zero, it is possible to obtain the value $D_{\text{opt}} = 0.4343$ ($T_{\text{opt}} = 36.8\%$).

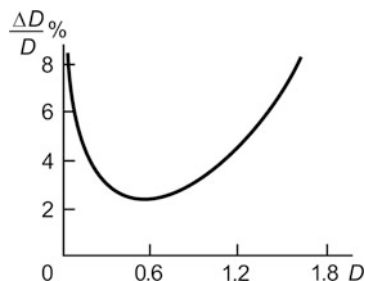
From Eq. 2.3 it follows that the value of relative error substantially depends on density of the investigated substance:

$$\Delta D/D = \Delta C/C = 0.434 \Delta T/D \cdot 10^{-D}$$

In Fig. 2.4, the graph of $\Delta D/D$. error dependence is shown as a function of measured optical density D , at accuracy of a spectrophotometer scale $\Delta(I/I_0)$ equal to 1%. As is seen from the graph data, the transmission band 20–65% should be chosen as the optimum region for absolute method measurements. In this case, the minimum relative error corresponds to $0.434D$.

Consequently, the operating range of optical densities satisfying Eq. 2.3 will be 0.2–0.7, which corresponds to the transmission interval 0.2–0.65. However, Komar' and Samoilov (Komar' and Samoilov 1963, 1967), experimentally found some discrepancy of dependence of S_D on D . This discrepancy is maximal in the

Fig. 2.4 The graph of $\Delta D/D$ error dependence as function of D in direct spectroscopy method (Rubin 1974)



region of large D values. For a large number of spectrophotometric measurements in off-the-shelf devices, performed with the usual methods, a reader should choose the value of D_{opt} within the range $(0.4\text{--}0.8)D$ (Fraifelder 1980).

It is necessary to make sure that for a particular instrument its real measurement error, D_{opt} and the operating density range should be determined only on the basis of an experimental study performed by an operator before the routine analysis and taking into account the recommendations made above (Nagibina and Prokof'ev 1961; Shabalina and Petrova 1969; Sverdlova 1973; Shtern and Timmonis 1974; Morton 1975; Lebedeva 1977).

The influence of many listed factors on an error of spectrophotometric analysis can be compensated if analyses of unknown solutions or homogenates are carried out under same conditions, i.e., same spectrophotometer, cuvettes, volumetric glassware, and weights, and with the same error characteristic of the work of a particular operator (Tarasov 1968; Rubin 1974).

When analyzing complex mixtures or homogenates of biological tissues, the reader should use a combination of spectral approaches and methods of analysis of registered spectra. The purpose of such combinations is to determine not only one main substance in a mixture but several components or changes in their states (Brice and Swain 1945; Nagibina and Prokof'ev 1961; Clayton and Thiers 1966; Calder 1969; Shlyk 1971; Moskvina et al. 1973; Spitsyn and L'vov 1985; Perfilov et al. 1985).

Accuracy and correctness of the quantitative and qualitative spectrophotometric analysis of any particular mixture of main compound and admixtures, and also analysis of heterogeneous biological structure, depend on the combination of many factors. They include, first, strictness of fulfilling conditions and limitations basic for the used method. Furthermore, accuracy and correctness of an analysis depend on the impurity content and trend of spectral curve, on correct choice of analytical wavelengths, errors of spectrophotometric measurements, and accuracy of preparation of biological and parallel samples. Therefore accuracy and correctness of data analysis of a particular system or structure with some spectral method can and must be found in the special preliminary study (Gonopolskii 1969; Litvin et al. 1973b; Bershtein and Kaminskii 1975). Because of high sensitivity of the method, the above-mentioned conditions become especially important in the registration of derived spectra.

“Sensitivity” is tightly bound with “accuracy” and “correctness” of spectral analysis. For example, the sensitivity of color reaction is expressed as a number of micrograms of compound having the optical density equal to 0.001 in the layer of

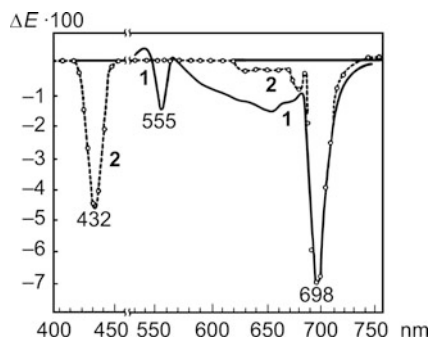


Fig. 2.5 Influence of the exciting light and darkness, and also of redox agents on the pigment P700 absorption in the suspension of chloroplasts fragments processed by acetone (Kok 1961; Khit 1972): 1 the difference spectrum “oxidized form *minus* restored form”; 2 the difference spectrum “light variant *minus* dark variant”

solution with cross section of 1 cm. Further, let us describe one of the methods of the derivative spectrophotometry for increasing sensitivity of spectrophotometers.

2.2.2 Difference (Differential) Spectrophotometry

In described methods of analysis, optical density of an analyzed solution is measured relative to a comparison solution (according to an accompanying manual), which is a pure solvent (a so-called blank solution). Therefore it is obvious that direct spectrophotometry is a borderline case of difference (differential) method when C_0 and $D_0 = 0$. If instead of the cuvette with the solvent we use the solution of any second substance, then registering the absorption spectrum with a direct method (“usual”) the difference in optical densities of the two objects will be directly measured, i.e., the difference absorption spectrum.

We would like to emphasize that the term “differential” is frequently met in the scientific literature, especially in the English language, and it expresses the essence of the Russian term “difference,” but not “differential” or, more precisely, “differentiated.”

If I_0 is the intensity of light incident to the cuvette (solution) and I_1 and I_2 are the intensities of light passed through two solutions (object), then $\lg I_0 - \lg I_1 = D_1$; $\lg I_0 - \lg I_2 = D_2$, and so $\log(I_1/I_2) = D_2 - D_1 = \Delta D$.

After direct measurement of intensities of light passed through compared solutions, it is possible to find the difference between optical densities of these solutions (Figs. 2.5 and 2.6).

The registration of changes of difference spectra substantially increases the accuracy of measurements and sometimes it allows one to register such absorption spectra changes reliably even when the difference is not revealed with the usual method.

In studies of biological samples, the registration of the difference of absorption between two samples as function of wavelength is frequently required when initial

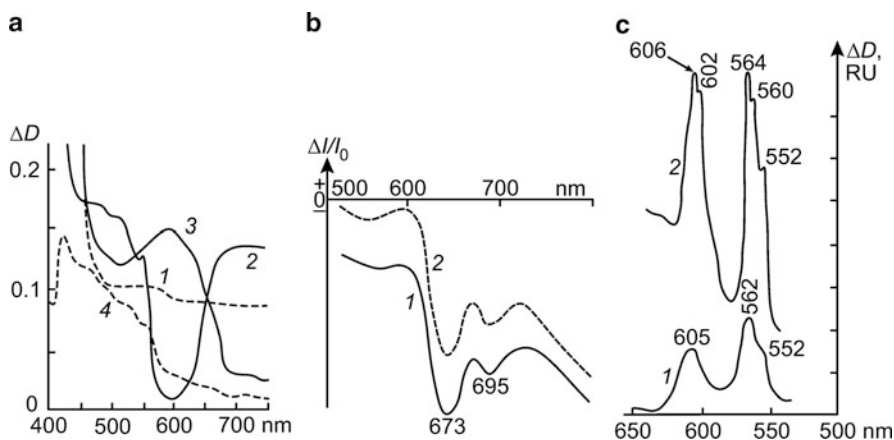


Fig. 2.6 Variants of differential (difference) spectra application: (a) the difference absorption spectra for the unpurified solution of the mixture of water-soluble proteins containing ferredoxin, flavoprotein, plastocyanine and some others 1 the zero line; 2 addition of ferricyanide to the sample; 3 addition of ascorbate excess to the sample; 4 additional introduction of hydrosulfite (Einor 1970); (b) differential spectra “oxidized variant *minus* restored one” of pigment-protein complexes from intergranular thylakoids fragments of pea chloroplasts containing the photo system 1 (1) and from the corn parenchyma (2) (Ostrovskaja (ed) (1975), p. 162); (c) the differential spectrum “restored sample *minus* oxidized one” of the suspension of mitochondria isolated from a rat liver, recorded at room temperature (1) and at temperature -120°C (2) (Rubin 1974, p. 47)

states of samples are very similar. A reader should remember that in this case the higher value of optical density of each solution (sample) can be used than one applied in usual spectroscopy. In this case the optical density of one of solutions is conditionally agreed equal to zero. In the case of identical samples the zero line passes through the ordinate of their density. Transmission of the second solution (or the sample undergoing test) may be or may become either less or more than the transmission of the first solution; with respect to this, the optical density at different wavelengths can have both positive and negative values in the diagram of the difference spectrum, i.e., be bipolar (Figs. 2.5 and 2.6).

In practice, such a difference spectrum is recorded if one places one substance (for example, ubiquinol, cytochrome, oxidized by NAD^+ or NADP^+) into the cuvette of comparison, and places ubiquinone, cytochrome restored by NADPH_2 into the cuvette for samples. As with the example of the difference in spectra of ubiquinol and ubiquinone it is possible to illustrate the basic properties of difference spectra:

1. Extinction values can be negative and positive – bipolarity of signal.
2. Absorption maxima and minima are shifted, and extinction values are different from their values in absolute spectra.
3. Zero absorption points – isosbestic points – correspond to wavelengths, where oxidized (initial) and restored (final) forms of substance absorb equally.

Measurements of difference spectra have advantages in the case of small differences in optical densities of two compared samples, and this is taken into account during the performance of this form of analysis. Thus, the accuracy of measurements in this case is proportional to ΔD . So the possibility to compare two substances or two states of the same substance (sample) arises; therefore the important point of analysis is the fact that in both cuvettes the initial concentrations of components are identical. During the work with native tissues or solutions of pigments it is necessary to perform equalization of densities in one absorption maxima. For plant tissues or their homogenates and for algae the densities are usually equalized in the red maximum of chlorophyll absorption ($\lambda = 683\text{--}685\text{ nm}$).

The difference absorption spectrum of native structures in photobiological studies corresponds to changes occurring in the object under influence of light, temperature, or chemical agents. A decrease of optical density (negative maximum) indicates a decrease of substance concentration in the final state compared with the initial, i.e., a decrease (increase) of product concentration at considered λ in the experimental cuvette compared with the control one, and an optical density increase takes place because of increase of the substance absorbing in the considered region of the spectrum.

To make clear the importance of this method it is sufficient to stress that a number of great achievements, for example in the fields of biochemistry of photosynthesis, study of redox reactions of cytochromes, and energetics of photobiological processes were achieved with its help (articles by B. Chance (Chance 1951), L.N.M. Duysens (Duysens 1954, 1956), B. Kok (Kok 1959, 1961, 1969), H.T. Witt (Witt 1971, 1979; Witt et al. 1961), and L.O. Einor (Einor 1973)). These papers were also described in the book of L.O. Einor (Einor 1973), detailed and covering different problems of chloroplast energetics. It was unfairly forgotten, but remains until now (it was published in 1973) one of the best books in world literature on problems of reconstruction of photosynthesis energetics and on analysis of progress made with application of differential spectrophotometry.

2.2.3 Measurement Errors of Difference Spectrophotometry

If D_0 is the density of comparison solution and D_X the density of studied solution, the density of a second solution measured comparatively to the first one (D_0) will be equal to the difference of absolute optical densities:

$$\begin{aligned} D_{\text{diff}} &= D_X - D_0 = \varepsilon d(C_X - C_0) = \varepsilon d \Delta C; \\ D_{\text{diff}} &= -I_g T_{\text{diff}} = -\varepsilon d(C_X - C_0) \end{aligned} \quad (2.4)$$

To estimate measurement accuracy of this method it is necessary to compute the value of $\Delta C_X/C_X$, which in the case of Bouguer's law compliance is equal to $\Delta D_X/D_X$. After elementary calculations (they are presented in Rubin's books

(Rubin 1974, 1975) the optimal optical density for D_{diff} corresponding to a minimal measurement error is found:

$$D_{\text{diff}} = 0.434 - D_0 \quad (2.5)$$

With D_0 increase a measurement error decreases from 0.434 (at $D_0 = 0$) to 0 (at $D_0 = 0.434$) and then remains equal to zero with the further D_0 increase. At $D_0 = 0$ an operating range of optimum densities should be chosen equal to 0.2–0.7 of D , and with the increase of D this interval will increase. We would like to emphasize that to increase measurement accuracy of difference spectra, optical densities of investigated samples should be high enough and with maximally close values chosen by the experimenter for the selected absorption maximum. In practice this is realized by very careful equalization of densities in one of maxima of the investigated absorption spectrum or of native biological material. For example, depending on study purposes homogenate density of leaves or algae suspensions should be set at 480, 515, 650, and 683.5 nm (in absorption maxima of chlorophylls a and b), for erythrocytes – at 560 nm, for cytochromes – at 550–559, 560–563 nm (Einor 1970, 1973).

In the case $D_0 = 0$, $D_X = D_{\text{diff}}$, Eq. 2.5 is automatically turned into the formula for measurement errors from the method of direct spectrophotometry. This stresses once more that the method of direct spectrophotometry is a particular case of the different one. After setting equal to zero the derivative of the right part of Eq. 2.5 we find the optimum optical density D_{diff} corresponding to a minimum measurement error Eq. 2.5.

Formal analysis of Eqs. 2.4 and 2.5 can result in the conclusion that the highest accuracy can be obtained after comparison of two practically opaque solutions. This paradox appears because only a measurement error of the analyzed solution D_X was taken into account in the equations. If we add into the reasoning a measurement error of a comparison solution D_0 and a tray error, then dependence of a measurement error D_{diff} does not become equal to zero with increase of D_0 , but it has minima (Korablev 1967; Lebedeva 1977). Thus, it is possible to calculate the optimum D_0 value for given D_X or the optimum D_X value for given D_0 .

Research experience shows that, as a result of accumulation of errors characteristic of both direct and difference spectrophotometry, the determination accuracy gain can be only in 5–7 times, instead of the theoretically found one in 10–12 times (Barkovskii and Ganopol'skii 1969). Nevertheless, it is necessary to stress that the great achievements of the last 30 years in the field of biophysics and biochemistry of photosynthesis and of chloroplast functions occurred due to the application of this analysis technique (Einor 1973; Witt 1971; Saakov 1987).

In each specific case an assessment of reasonability of application of direct and difference methods, and also determination of optimum values D_0 and D_{diff} , can be performed only after a preliminary detailed experimental study of properties of an analyzed substance or a biological system and of an instrument used for analysis (Willis and Miller 1959; Gonopolskii 1969; Platonova et al. 1970; Blyum et al. 1972; Blank 1973; Golovachev 1976).

Using difference spectrophotometry – one of the special methods of absorption spectrophotometry – it is possible to study small changes of optical density of an investigated system, and also when extinction value is great. A difference spectrum is found by the subtraction of one absolute absorption spectrum from another. Thus, it helps to obtain an answer about changes in the system under influence of external factors (of a chemical or physiological nature). This is especially important in studies of pathologic changes of blood state, energetics of mitochondria and chloroplasts, and also in comparison of algae mutants having only small visually detected difference.

In Barkovskii's and Ganopolskii monograph (Barkovskii and Ganopol'skii 1969) existing methods and approaches of difference spectrophotometry are described fully enough and critically. So we will only briefly describe basic questions of this analysis technique useful for practical work and also necessary for understanding the material in Chaps. 3 and 4.

Unfortunately, high-performance difference spectrophotometers are not produced by any Russian companies. We do not suppose that it is necessary to discuss details of the modification of Russian commercial recording spectrophotometers for high sensitivity and high speed. Interested researchers could search through the book edited by A.B. Rubin (Rubin 1974), the substantial part of which is dedicated to questions of practical construction of high-performance difference spectrophotometers. Also Yu. Borisov and colleagues effectively worked on modification of Russian spectrophotometers for the purpose of difference spectrophotometry (Borisov and Mokhova 1964; Borisov et al. 1970).

2.3 Derivative Spectrophotometry

In spite of the successful development of spectrophotometric analysis techniques, study of biological objects in their native state, and of multicomponent bio-organic and chemical systems, encounter a number of difficulties.

On the one hand this is caused, as a rule, by high optical density, photosensitivity and strong light diffusion of a biological system, and – on the other by its complexity and heterogeneity. Difficulties of the first kind can be excluded by application of more advanced spectrophotometers with high sensitivity and with compensation for light diffusion. Difficulties of the second kind are caused by the fact that the absorption spectrum of a biological object is the superposition of several rather wide and closely placed absorption bands of different substances, each of which can have several modifications characterized by their own physical-chemical properties (chlorophyll-protein and hemoglobin complexes, chelate compounds and other). Because of this, the total spectral curve has rather a complex form and some components either do not manifest at all or are displayed the form of only weakly expressed bends, inflexions, shoulders, or protrusions.

Thus, a researcher has to struggle with the difficulties of ascertaining the number and positions of extrema, precise identification of substances and their quantitative ratio.

It is significant that for a biological object a resolution limit is mainly dictated not by the technical capabilities of spectroscopic instrumentations, because there are spectrophotometers with high resolution of Angstroms and fractions of Angstroms, but by an object's structure revealed as a mixture of wide and closely placed absorption bands. Since some maxima of a total spectral curve can be manifested as weakly expressed protrusions, bends, and band asymmetry, and since a spectrum curvature is visually assessed, this visual assessment introduces additional errors and uncertainty in answer to the question of existence or position of various absorption bands composing the spectral outline.

If we suppose that an absorption spectrum consists only of Gaussian type bands differing from each other only in width, intensity, and position relative to the wavelength axis, then even under such ideal condition the precise interpretation of the spectrum is a very difficult problem. Absorption spectra of many samples have not just one but several maxima, and their forms substantially vary from the Gaussian and from any another curve described with known mathematical formula (curves of Lorenz, Rayleigh, etc.) (Martin 1957; Rutman et al. 1976b; Dubrovkin and Belikov 1988).

These described difficulties require considerable skill from researchers to solve the necessary task of the most precise possible identification of separate absorption bands in the total spectrum (Fig. 2.7). This part of the problem is extensively described in the review of Giese and French (1955). Using a large quantity of model figures obtained with machine analysis, the authors of the paper illustrated many variants of overlapping bands and their possible separation in the case of differentiation of models.

The criteria of separation of two symmetrical, closely placed absorption bands were also discussed by Meister (Meister 1966a) and Litvin and co-authors (Litvin and Gulyaev 1969; Gulyaev and Litvin 1970). In the work of Meister the resolution limit of two identical absorption bands having a Lorenz curve form (dispersion outline) is described. The Sparrow criterion for separation of two symmetrical band is enough to reveal a notch between them. The borderline case is a total curve with a flat top; in other cases two initial curves are not resolvable (see Figs. 2.10b and 2.13).

In the paper of Litvin and co-authors, in addition to the Sparrow criterion, Rayleigh's criterion is considered, but in contrast to Meister, the authors apply both criteria to the Gaussian curve. According to the Rayleigh criterion two symmetrical bands are resolved if the value of the notch between them is not less than half of the maximum value of ω -band. From the Sparrow criterion the resolution limit of two Gaussian bands is equal to 0.849ω , and Lorenz's bands – to 0.557ω . From the Rayleigh criterion the resolution limit reaches the value $(1.5-1.0) \omega$ for Gaussian curves (Lester 1970; Dubrovkin and Belikov 1988).

Thus, the Rayleigh's criterion is stricter than the Sparrow criterion, and is more reliable. However, due to the high level of quality of spectroscopic instrumentation, in practice the Sparrow criterion is more frequently used. The latter could be written as the ratio of the minimum distance between the tops of two symmetrical bands $\Delta\lambda_0$ to the width of these bands at an ordinate equal to 0.5 from the maximum

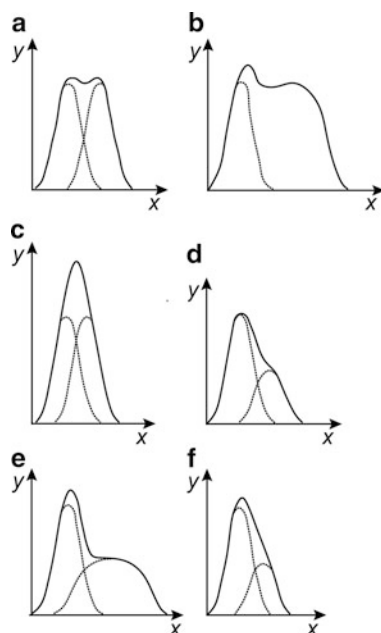


Fig. 2.7 Influence of hidden absorption bands on change of the total absorption curve outline

ω -value, i.e., $\Delta\lambda_0/\omega$. If it is necessary to resolve two asymmetrical bands varying in width and in amplitude, then resolution limit changes. In particular, if the amplitudes of two Gaussian bands are in the ratio $A_1/A_2 = 1/5$, and their half-widths are equal, then the resolution limit will be equal to 1.0ω (Dubrovkin and Sobolev 1976). If with the same ratio of amplitudes the ratio of half-widths is $1/2$, then the resolution limit is equal to 0.835ω . This question is described in detail in several papers (Lester 1970; Skujins 1986b; Dubrovkin and Belikov 1988) and interested readers could investigate this problem from the original sources.

To overcome previously mentioned difficulties, in recent decades some methods increasing the analytic-informational capabilities of spectral methods have appeared. One of them is the method of low-temperature spectrophotometry (Frei 1960; Litvin and Gulyaev 1969; Rubin 1974, 1975). It is based on the fact that, with decrease of object temperature to the temperature of liquid nitrogen, absorption bands become narrower by 20–30%. This narrowing is mainly due to the decrease of widths of molecular energy levels, change of degrees of freedom of molecules, and the forces of intermolecular interaction.

However, this technique has a number of disadvantages and limitations, causing complication of the experimental procedure. Furthermore, this method could not be applied to direct studies of processes taking place in a living cell under physiological conditions.

An experimenter reading graphs of spectral curves knows that some maxima of the total absorption curve and of transmission or luminescence spectra are

manifested as ill-defined protrusions, inflexions, or band asymmetry. Incomparably better results for identification and interpretation of the bands in a total spectrum of a biological subject are obtained by a method which has been given the name of derivative spectrophotometry and it has begun slowly, but persistently, to win places in analytical laboratories from the middle of the twentieth century (Hammond and Price 1953; Pemsler 1957; French et al. 1954; French 1957a; Martin 1957, 1959; Habermann 1960a, b; Aramu and Rucci 1966; Meister 1966a, b; Stauffer and Sakai 1968; Gulyaev and Litvin 1970; Gaudillere 1974; Matsushima et al. 1975; Dubrovkin and Sobolev 1976; Spitsyn and Korepanov 1980; Abdel-Hamid et al. 1984; Skujins 1986b; Dubrovkin and Belikov 1988; Bosch Ojeda et al. 1995; Hagris et al. 1996).

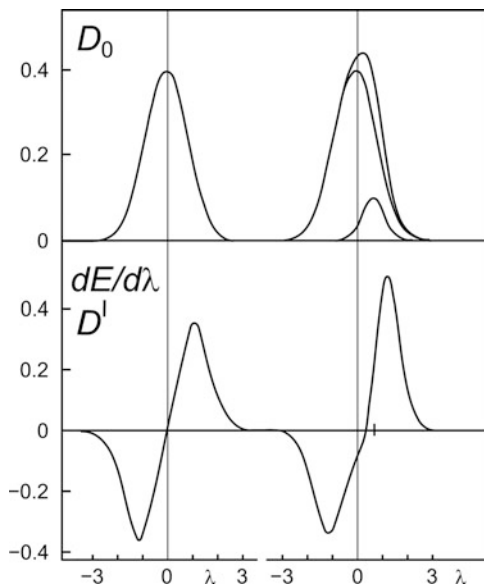
In due course the discussed approach was proposed by Sir Prof. E. Rutherford as the method of identification of ill-resolved peaks of mass-spectra (Dymond 1924; Dubrovkin and Belikov 1988). The author of the first publication (Dymond 1924) illustrates usage of the differentiation method for first-order derivative and expresses appreciation to Prof. E. Rutherford for the proposed study method and for permanent interest in the work. Revival of this approach and a new impulse for its development took place at the beginning of the 1950s; this was covered in papers of both chemists and biologists (Hammond and Price 1953; French and Church 1955; Giese and French 1955; Butler and Hopkins, 1970; Singleton and Collier 1956; French 1957a; Martin 1957, 1959). It should be immediately specified that differentiation of spectral curves substantially increases contrast of detailed spectrum presentation due to the revealing of high Fourier-frequencies (Dubrovkin and Belikov 1988). This means that changes induced in the spectrum become more visible after differentiation of spectral curves.

The method won acknowledgement in laboratories of different scientific specialization in Europe and America, and some of achievements are summarized in *reviews* (Williams and Hager 1970; Hager 1971; Rutman et al. 1976a,b; Fell 1980; Miller et al. 1982; O'Haver 1982; Dubrovkin 1983a; Perfilov et al. 1985; Spitsyn and L'vov 1985; Skujins Sigurds 1986a; Saakov et al. 1987) and in the monograph of G. Talsky (Talsky 1994). The considered approach does not have limitations characteristic of the method of low-temperature spectrophotometry, and allows one to increase substantially selectivity and informativity of the spectral method of investigation of complex biological structures (French et al. 1954; French 1957a, b; Frei 1960; Meister 1966b; Kaler et al. 1967; Litvin and Gulyaev 1969; Navarro et al. 1972; Litvin et al. 1973a, b). It virtually increases the resolution of normal spectrophotometers and excludes errors and uncertainties during visual resolution of a question about presence of various absorption or luminescence bands.

The method of derivative spectrophotometry allows one to realize the ability of detection of small differences in spectral outlines, because small abnormalities of the regularity of initial curve are clearly found in harmonics of derived spectra.

The theoretical mathematical validation of method is extensively presented in a number of articles and reviews (Martin 1957; Bonfiglioli and Brovetto 1964b; Hager and Anderson 1970; Williams and Hager 1970; Dubrovkin 1983a; Perfilov et al. 1985; Spitsyn and L'vov 1985; Skujins Sigurds 1986b; Dubrovkin and

Fig. 2.8 Two curves as the model of the absorption spectrum and their calculated conversions to derived spectra, respectively. The *left upper curve* has the symmetrical D^I spectrum. The *right plot* of the same curve shows addition of another small absorption band resulting in hardly noticeable asymmetry of the total outline. This is more clearly revealed on the derived curve (French 1957b)



Belikov 1988; Talsky 1994) and additional reproduction of its bases quoted from known articles will not be done in this book.

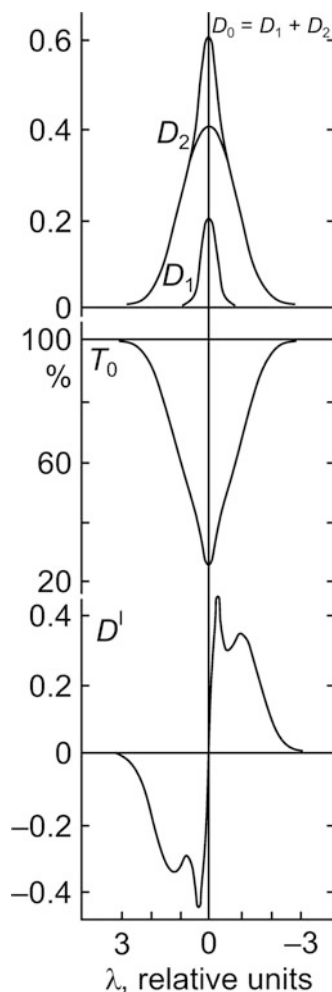
Without particular consideration of mathematical description of derivative spectrophotometry, for the convenience of a reader, to familiarizes himself with the method and its capabilities and to clarify and explain the process, we illustrate in Figs. 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, and 2.14 the special features of this method, whose numerous corresponding graphics were presented earlier (Litvin and Gulyaev 1969; Butler and Hopkins 1970; Epel and Butler 1972; Marenko and Saakov 1973; Hager 1973; Bershtein and Kaminskii 1975; Leclerc et al. 1975; Rutman et al. 1976b; Talsky et al. 1978a; O'Haver 1978; Ioffe et al. 1984; Perfilov et al. 1985; Skujins Sigurds 1986b; Dubrovkin and Belikov 1988; Talsky 1994).

The derivative of the absorption spectrum of a substance or solution is the result of differentiation of optical density of the spectral curve D with respect to wavelength, to wave number λ , or to time t of spectrum scanning, when the latter is strictly synchronized with wavelengths scanning.

It can be seen from Figs. 2.8 and 2.9 that in the region of maximum of the initial curve the first-order derivative has two peaks – positive, corresponding to *maximum speed of optical density increase*, and negative, corresponding to *maximum velocity of optical density fall* for the initial curve. The spectral curve becomes bipolar. At λ_{\max} of initial spectrum its first (D^I), and also all odd derivatives (D^{III} , D^V , D^{VII} , etc.), are equal to zero (see Fig. 2.11a–c). Thus, the precise position of the maximum of the initial spectral curve corresponds to zero point on the X-axis. Because of this, the form of these functions is dramatically different from the form of the initial spectrum (Fig. 2.11). Thus, the first-order derivative spectrum characterizes a curve slope to the X-axis or, which is the same, the graph of function

Fig. 2.9 The model of the spectral outline consisting of two bands with different width, but with the identical position of maxima.

The central curve (T_0) is the calculated transmission curve, and lower one – the calculated D^I curve of transmission, on which the peak corresponding to minor component is visible. This means that the derived spectrum can make seen two peaks identical on absorption, if their band widths are different enough (French 1957b; Giese and French 1955)



change rate (i.e., of the initial curve of spectrum). Possible protrusions, bends, or shoulders presenting on the initial curve will be more protuberantly manifested as outline changes of the derivative graph (Figs. 2.8, 2.9, 2.10, and 2.11c).

A second derivative (D^{II}) of the symmetrical absorption curve has one intensive negative peak and two positive satellites of approximately four times smaller intensity. Half-width of a main peak is approximately one third of a half-width of an initial absorption band. A number of even derivatives (D^{II} , D^{VI} , D^X , D^{XIV}) have negative peaks at λ_{\max} of the initial spectrum and therefore with reversal of sign (of direction of Y-axis) are similar to it in some ways (Martin 1957; Skujins 1986b; Dubrovkin and Belikov 1988; Talsky 1994). After conversion into D^{IV} a half-width of the main peak decreases by approximately five times, theoretically, in comparison with the initial spectrum. However, an operator should clearly know that in this case the ratio of intensities of the main peak and positive satellites also

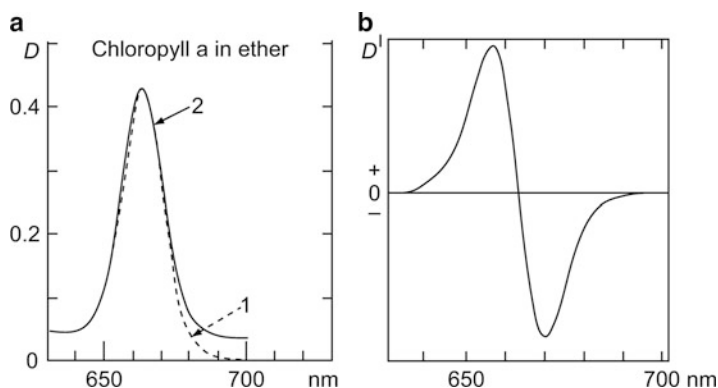


Fig. 2.10 The absorption spectrum of the purified solution of chlorophyll *a* in the sulfur ether measured with the instrument “Beckman DK2” (a) the curve 2, and its D' (b). Integration of the curve *b* is shown with the curve 1 (a) (French 1957b)

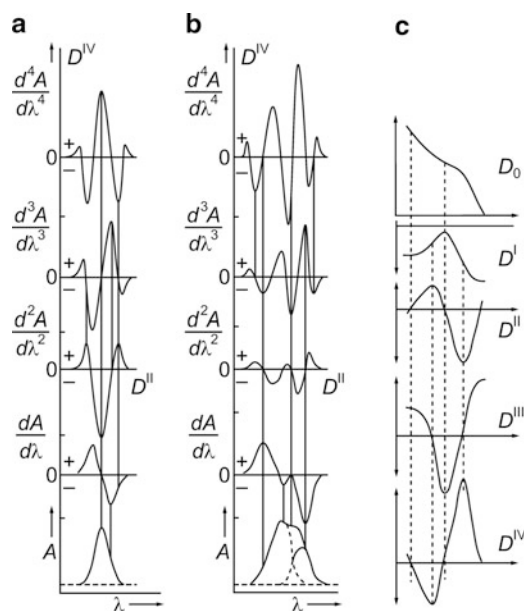


Fig. 2.11 Differentiation of model analytical Gaussian curves: (a) the initial curve and its $D^I - D^{IV}$; (b) the initial curve of overlapped Gaussian curves and their $D^I - D^{IV}$; (c) manifestation and intensification of signal after differentiation of bend shoulders (inflections); the initial curve (D_0) and its $D^I - D^{IV}$ (Talsky 1994; Talsky et al. 1978a)

reduces (Figs. 2.11 and 2.12) (Dymond 1924; Giese and French 1955; Butler and Hopkins 1970; Bershtein and Kaminskii 1975; Talsky et al. 1978a; Skujins 1986a). Points of inflection manifest as extrema, and even for derivatives, as an intersection of zero line. At the same time, the center of the shoulder bend is revealed in even

Fig. 2.12 Change character of ratio of main peaks and satellites intensities; compare D^{II} (* and ^) and D^{VI} , D^{X} (*), and also D^{IV} (* and ^) and D^{VIII} , D^{X} (* and ^)

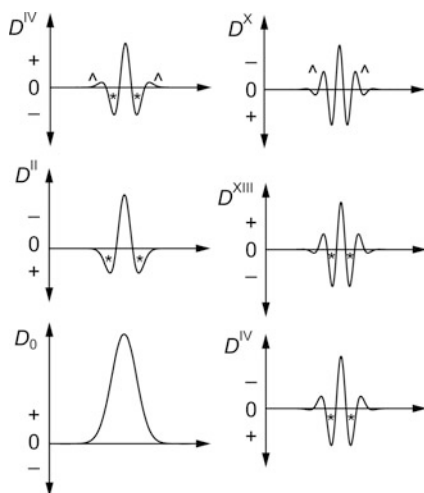
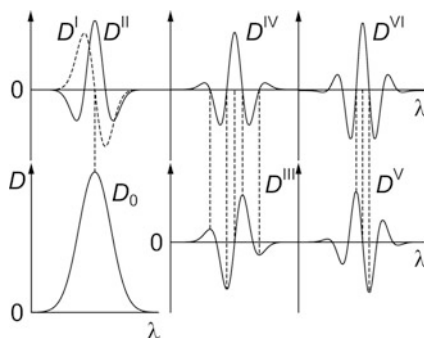


Fig. 2.13 The Gaussian curve D_0 and its analytical derivatives of the first to the sixth (Ioffe et al. 1984)



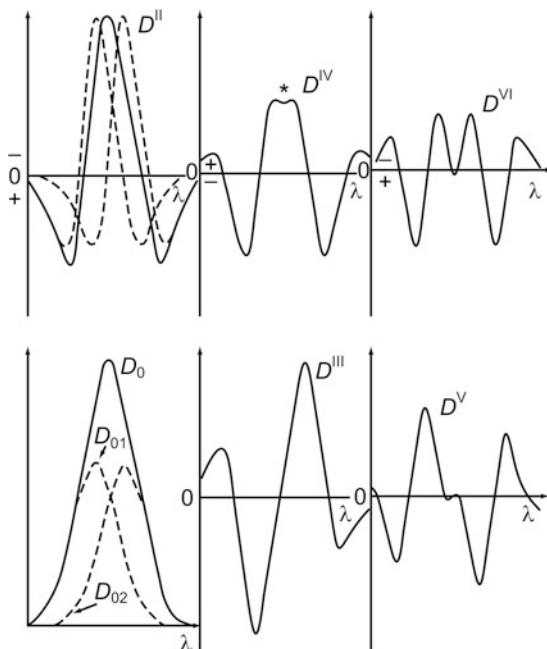
derivatives in the form of acute maxima (D^{II} , D^{IV}) (Talsky et al. 1978a; Talsky 1994).

However, registration of the first-order derivative (from now on we will use introduced designations instead of words) has a number of disadvantages. First, the form of curve is complicated, because the derivative possesses both positive and negative values, i.e., the signal is bipolar; moreover, not one but both maxima (positive and negative) on the graph of derivative (Fig. 2.11) correspond to each maximum of the initial curve.

We would like to emphasize again that at λ_{max} of the initial spectrum its D^{I} and also all odd derivatives of higher orders are equal to zero, i.e., the position of the absorption maximum corresponds to the zero point on the X-axis. Because of this the graphic representation of this function is sharply different from the form of the initial spectrum. Half-width of the D^{I} absorption band remains practically the same as that of the initial absorption or transmission spectrum.

From all the above evidence we can conclude that the application of D^{I} does not increase resolution of the method, but only specifies positions of absorption bands.

Fig. 2.14 Two symmetrical curves (spectral Gaussian curves) and analytical derivatives of the total curve. Asterisk shows the separation of two peaks located from each other at a distance of half width of each of them in the D^{IV} spectrum (Ioffe et al. 1984)



Thus it is more promising to use D^{II} for the analysis of spectra, and with improvement in electronics – D^{IV} (Dymond 1924; Martin 1957, 1959; Butler and Hopkins 1970; Kvaratskheli and Demin 1983).

Conversion of D^I into D^{II} is similar to the conversion of the original function D into D^I . The second derivative physically corresponds to acceleration of change of the initial curve D . Finding extreme points for any function with D^{II} allows one to determine exactly the quantity and position of maxima and minima of the initial curve. Inflections, protrusions, and shoulders in the initial spectrum manifest themselves on the D^{II} curve as separate maxima. The form of the D^{II} curve as curves D^{VI} , D^X , D^{XIV} , etc., is close to the form of the original function, but with the *opposite* sign. Therefore, some even derivatives (D^{II} , D^{VI} , D^X , D^{XIV}) have *negative* peaks at λ_{\max} of the initial spectrum and consequently with change of sign (change of the Y-axis direction) they have some similarity to the initial spectrum, whereas *the form and the polarity* of curve outlines D^{IV} , D^{VIII} , D^{XII} , D^{XVI} , etc., correspond to the original function (Fig. 2.11). This allows one to compare the initial and derived spectral outlines quickly, and simplifies the solution of problem of experimental results interpretation. Theoretical assessment of preferential usage of optical density derivatives of one order or another for the increase of determination of selectivity of elements with strongly overlapped bands has been described (Kvaratskheli and Demin 1983; Dubrovkin 1983a). However, conclusions from these papers are ambiguous and there is the possibility of further discussion. Briefly, easily accessible to the reader, theoretical bases and special features of the method are presented in Part 4.1 of the monograph by Ioffe and colleagues (Ioffe et al. 1984).

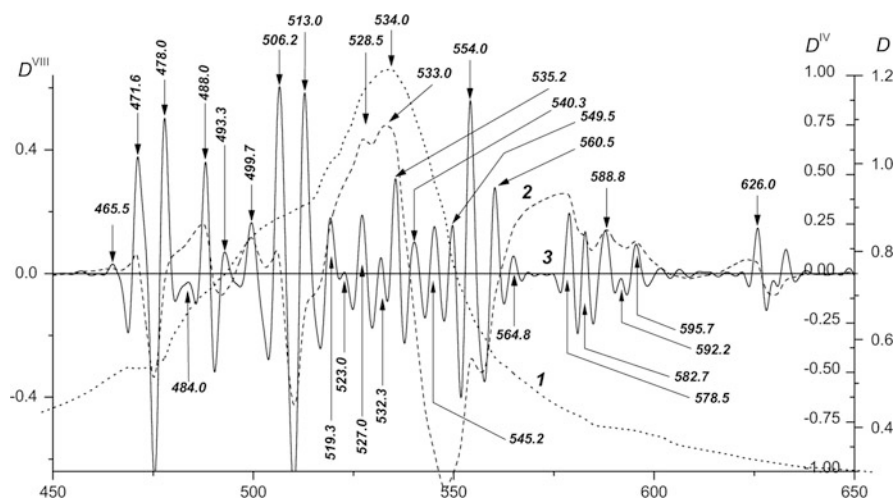
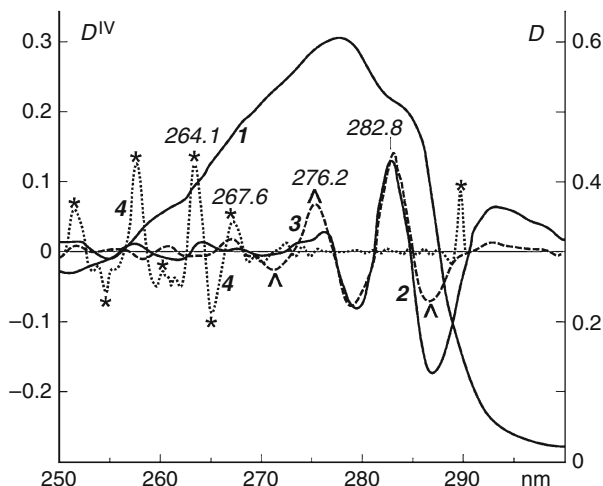


Fig. 2.15 The absorption spectrum of prodigiosin *1* and its fourth *2* and eighth *3* derivatives (Ryasantzeva et al. 2011)

Registration of higher derivatives requires a significant increase of sensitivity, selectivity, and complication of equipment (Dymond 1924; Butler and Hopkins 1970; Leclerc et al. 1975; Rutman et al. 1976a; Cuellar et al. 1978) but, on the other hand, values of main and parasitic maxima become commensurable and it is very difficult to identify them in the process of spectra interpretation. It is quite understandable that with the first usages of derivative spectrophotometry the methods of first derivative registration were widely used (Hammond and Price 1953; French et al. 1954; Habermann 1960a, b; Kaler et al. 1967; Inoue et al. 1975; Skujins Sigurds 1986a), then of the second one (Meister 1966b; Drews 1967; McWilliam 1969; Gulyaev et al. 1971; Saakov 1971a, b, c; Hager 1971; Marenko et al. 1972; Navarro et al. 1972; Marenko and Saakov 1973; Hager 1973; Saakov et al. 1973; Saakov and Shpotakovskii 1973; Baranov et al. 1974; Udovenko and Baranov 1974; Saakov et al. 1976; Ishii and Satoh 1982), and only later did methods of the higher derivative registration arise and find their application in scientific studies, first by Dymond (1924) and then by Butler and Hopkins (1970) and Cuellar et al. (1978). Development of the computer technology and corresponding software allows one to solve problems on the calculation of derived spectra of high orders with only standard spectrophotometric equipment available in the laboratory (Challise and Williams 1964; Savitzky and Golay 1964; Goldstein 1970; Cuellar et al. 1978; Skujins Sigurds 1986b; Talsky 1994; Eliseev et al. 2000).

In Fig. 2.15, an example of derived spectra application for the analysis of prodigiosin (pyrrolylpyrrolmethylene) is shown. This substance is from the group of natural red pigments, isolated from strains of *Streptomyces* and *Serratia marcescens* (*Bacillus prodigiosus*) (Hubbard and Rimington 1950; Rapoport and Holden 1962; Hearn et al. 1968; Boger and Patel 1988) and used for the fight against leukemia and melanoma cancerous cells (Yamamoto et al. 1999; 2000; Diaz-Riuz et al. 2001; Montaner and Perez-Tomas 2001).

Fig. 2.16 The absorption spectrum of insulin *I* synthesized by E. Lilly, and its fourth 2 derivative, fourth derivatives of tyrosine 3 and phenylalanine 4 spectra

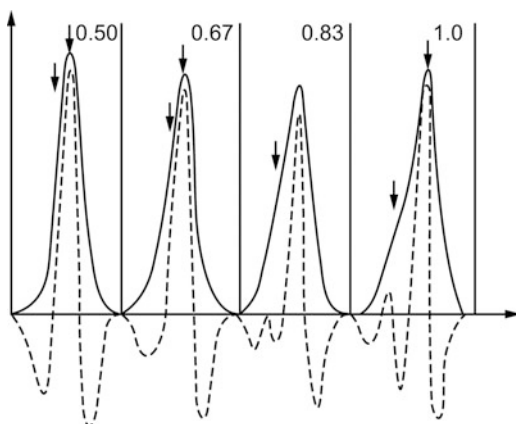


Prodigiosins are a new group of H^+/Cl^- co-transporters breaking proton transfer (Hearn et al. 1968; Kataoka et al. 1995; Sato et al. 1998; Melvin et al. 2002). As shown in Fig. 2.15, in acidic ethanol the main maximum of pigment absorption is decomposed into five or six absorption bands not detected earlier and unknown in the literature (Saakov and Petrova 1996; Saakov 1996b; Saakov et al. 1998), and the shoulder bend at 500–510 nm is revealed on the D^{VIII} spectrum as three bands at 499.7, 506.2, and 513.0 nm (Hubbard and Rimington 1950; Rapoport and Holden 1962). The known region of the spectrum, 470 nm, is decomposed into five bands: 471.6, 478.0, 484.0, 488.0, and 493.3 nm. Furthermore, special features of the D^{VIII} spectrum allow one to assess the purification efficiency of isolated prodigiosin preparations or its protein complexes with the necessary certainty in accordance with manifestation of the above-mentioned spectrum bands, and also according to change of extrema in the region 578.5–595.7 nm (not considered earlier in the literature) under the influence of stress or chemical factors (Saakov and Petrova 1996; Saakov 1996b; Saakov et al. 1998; Saakov and Moshkov 2003; Rysantseva et al. 2012).

In contrast to the low-temperature method, the method of derived spectra registration is actively used for studies of processes taking place in living (native) cells under physiological conditions and unimpaired biochemical processes, including investigations of different mutants (Frei 1960; Navarro et al. 1972; Inoue et al. 1973; Saakov and Hoffmann 1974; Baranov et al. 1975; Kvitko et al. 1976, 1977; Saakov et al. 1978a, b).

The example of D^{II} analysis of insulin preparation important for diabetics is shown in Fig. 2.16. Molecules of gelatin and polymyxin do not contain tyrosine and tryptophan, but have a phenylalanine. Insulin includes both phenylalanine and tyrosine. This is confirmed by Fig. 2.16 showing the smooth outline of the insulin absorption spectrum (*I*) and its (non-smooth) fourth derivative (2). Positive extremum of the derivative is at 281.8 nm, that is equal to the main extremum of D^{IV} of tyrosine (3) (Saakov et al. 1998).

Fig. 2.17 Resolution of two overlapped bands from second derivatives of the absorption spectrum (Bershtein and Kaminskii 1975; Gulyaev et al. 1971). Numbers at curves correspond to distances between peaks in units of band half-width



An additional advantage of the derived spectrophotometry method is that D^I registration substantially decreases the constant component of side absorptions, such as light diffusion, shift, and, in plant studies, change of a leaf, excessive moistening, or drying (Meister 1966b; Saakov et al. 1973; 1976; Udovenko and Saakov 1976; Skujins Sigurds 1986a). Usage of D^{II} allows one to eliminate an effect of the mentioned side absorptions linearly depending on λ ; only if listed components have quadratic dependence on λ does this influence manifest on the D^{II} graph as constant component (Rutman et al. 1976b). The presented considerations allow one to limit a number of a priori statements or objections about spurious signals of light scattering in boundary regions of a spectrum.

Derivative spectrophotometry is often used for the resolution of almost or completely overlapped spectral lines (French 1957b; Gulyaev et al. 1971; Udovenko and Saakov 1976; Skujins Sigurds 1986a). Using a computer Litvin and co-authors (Gulyaev et al. 1971) calculated the model curves (Fig. 2.17) illustrating the appearance of two overlapped spectral bands in the second derivative, with different intensity and half-width, and located at different distances from each other. Analysis of the curves in Figs. 2.11, 2.12, 2.13, and 2.14 shows that, even if the overlapping of two bands is not revealed in the initial spectrum, for the D^{II} curve the two bands are found using the appearance of two negative maxima or the asymmetry of positive satellites. This exact special feature of the method should be always recalled by an experimenter during spectra analysis (Fig. 2.11b).

The method of derivative spectrophotometry, as one of the special approaches (techniques) of absorption spectrophotometry, allows one to find and to sharpen details of spectral curves hidden within wide spectrum bands or obscured by medium turbidity in “usual” direct spectrophotometry, to find the correct number and improved positions of maxima of blurred or overlapped bands (Fig. 2.18).

In Figs. 2.19 and 2.20 derived spectra in comparison with initial absorption spectra of some known chemical compounds are presented for illustration of the special features of fourth order derivatives. Nickel chloride (hexahydrate) is used in galvanotechniques as the chlorine carrier for nikelizing.

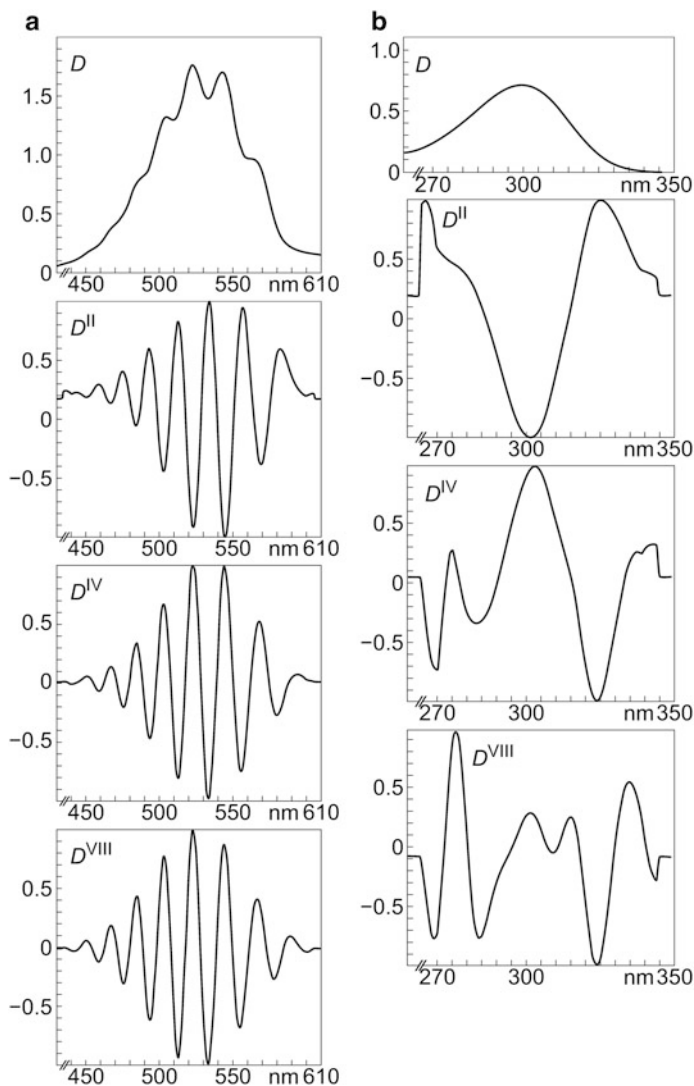


Fig. 2.18 Dynamics of absorption spectra change: (a) KMnO_4 and (b) KNO_3 in dependence on the order of derivative

Methylene blue (*N,N,N',N'*-tetramethyl thionine chloride hydrate, 3,7-*bis*-(dimethylamino)-phenothiazin-5-ium chloride) is relatively poorly soluble in water and ethanol, is soluble in the hot water, and is easily reduced ($E_0 = +0.53$ V). It has oxidation–reduction properties and can play the role of a hydrogen ion acceptor. In aqueous solutions the monomer form absorbs light with $\lambda_{\text{max}} = 668$ nm, and dimeric – with $\lambda_{\text{max}} = 613$ nm. It is an important organic thiazine dye. In analytical chemistry it is used for determination of chlorates, perchlorates, cobalt, cadmium, calcium, and

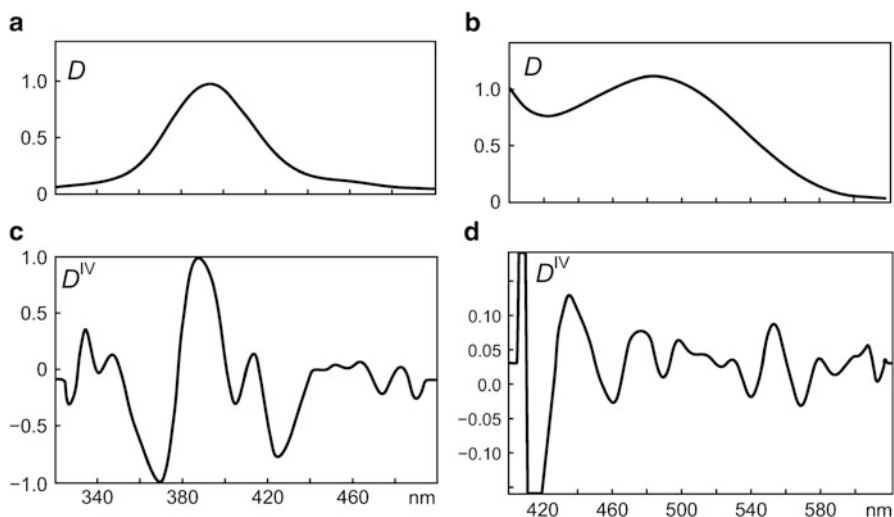


Fig. 2.19 Spectra of water solutions of NiCl_2 (a, b) conc. 40 g/L, and of Congo red (c, d) conc. 20 mg/L

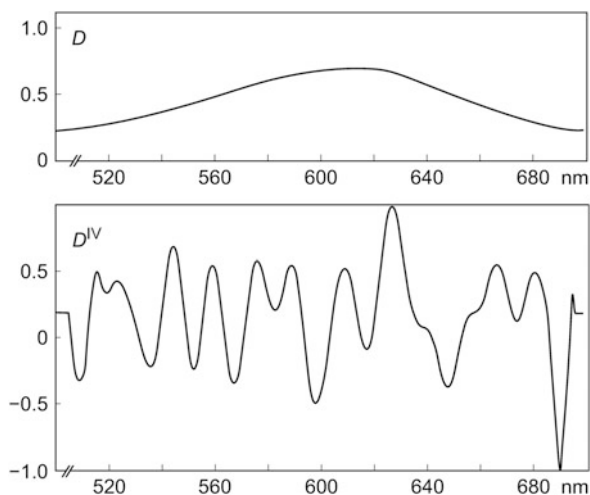


Fig. 2.20 The absorption spectrum of methylene blue (D) and its fourth derivative (D^{IV})

magnesium. In medicine it is applied as an antiseptic and the antidote against poisoning with cyanides, carbon monoxide, hydrogen sulfide, aniline, and derivatives. In microbiology it is used for the lifetime staining of preparations and also of histological preparations. Dyes of this class are able to intercalate into nucleic acid structure and become tightly bound to guanosine residues of DNA/RNA. After irradiation by light of wavelength 590 nm the photo-activated dye oxidizes oxygen

to singlet state. Singlet oxygen damages the genetic material of viruses, and thus prevents infection of blood plasma during its use in blood transfusion.

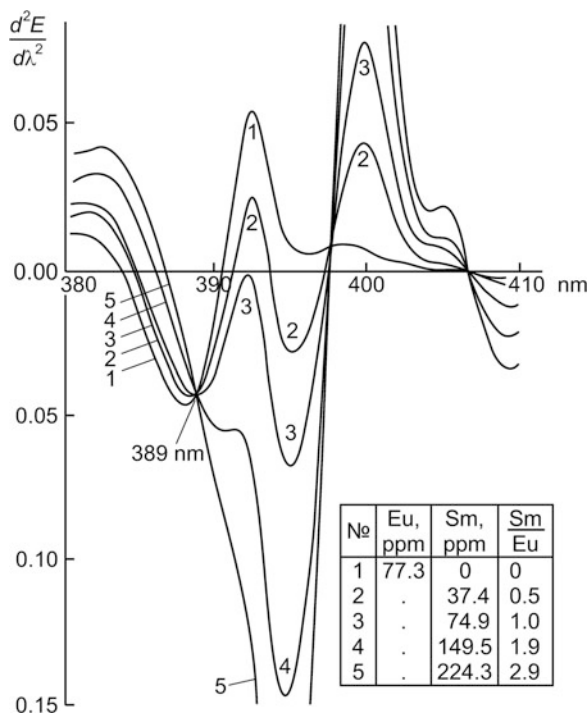
Congo red – disodium salt of 4,4'-bis(1-amino-4-sulfo-2-naphthylazo) biphenyl – is an azo dye and acid–base indicator. The absorption spectrum has $\lambda_{\text{max}} = 505$ nm. In weak-acid medium with pH 5.2 this dye has a red color; in acid medium with pH 3.0 it is blue. The indicator is used for photochemical detection of nitrates.

Therefore the method can be recommended for the identification of components of complex reaction mixtures (Singleton and Collier 1956; Challise and Williams 1964; Samsonova and Gak 1971; Shibata et al. 1976; Kitamura and Majima 1983; Bosch and Sanchez 1995). The considered method helps in the detection and quantitative determination of pollutions, impurities, and undesirable minor components in multicomponent media because the detection of low-intensity peaks of admixtures, overlapped by intensive absorption or transmission bands of base material, is possible (see Fig. 2.11b) (Singleton and Collier 1956; Morton 1975; Inoue et al. 1975; Baranov et al. 1976; Such et al. 1980; Spitsyn and L'vov 1985; Skujins Sigurds 1986a). This method of analysis is also used for the assessment of the structure of polymeric compounds (Dodd and West 1961; Calder 1969), sterols (Olson and Alway 1960), the analysis of electron transitions in organic and inorganic compounds (Ismail and Glenn 1964; Lewis et al. 1970; Hager 1971; Aleksandrova et al. 1982; Perfil'ev et al. 1983b, 1985; Nazarenko et al. 1982; Spitsyn and L'vov 1985), steric (conformational) changes in chelate complexes (Rozengart and Saakov 2002, 2003; Saakov and Rozengart 2005), to characterize a native state of biological structures and special features revealed due to mutations of the plastid apparatus or changes of hemoglobin and plasma of the blood (Fog and Osnes 1962; Navarro et al. 1972; Litvin et al. 1973a, b; Saakov and Hoffmann 1974; Baranov et al. 1974; Kvitko et al. 1976, 1977; Saakov et al. 1978a; Siek and Rieders 1984; Saakov 2005a, b).

Materials considered in the article by Skujins Sigurds (1986a) led us to the investigation of the determination of detection limits of uranyl nitrate in the region 330–505 nm: during uranium extraction from worked out fuel elements or other objects, the well water-soluble uranyl nitrate $\text{UO}_2(\text{NO}_3)_2$ is produced after treatment with nitric acid. In the flow injection cuvettes (optical cells) of the spectrophotometer, continuous qualitative and quantitative analysis of uranyl nitrate and also the uninterrupted automated sampling for checking of completeness of extraction from the initial products can be performed using characteristics of derived spectra of the second (Fig. 2.21) or fourth derivatives, and taking, as the basis, for example, spectral bands in the region 397–433 nm. Substance concentrations can vary over a wide range. In the case of darkening of glass of cuvette walls caused by radiation – they should be replaced. Darkened glass does not distort the picture of spectrum configuration, because it works as a neutral light filter. The above is also valid for the control of primary information on trace content and for continuous sampling of heptyl and dimethylhydrazone, for example, in air.

With the use of derived spectra, positive results are obtained for determination of nano-quantities of alkyl- and alicyclic ketones (Meal 1983) and nano-quantities of nickel and other elements and compounds while performing registration of

Fig. 2.23 Change character of the D^{II} spectrum of aqueous Eu solution with increase of Sm concentration in it



absorption spectrum (Ren et al. 1985) and also after registration of D^{I} and D^{II} spectra for combined analysis of elements of 8 group – ruthenium and palladium – in complexes with thiobarbituric acid (Morelli 1983), the analysis of lanthanides in microquantities, 4f-elements samarium, europium after D^{II} and D^{IV} registration (Figs. 2.22 and 2.23) (Ishii and Satoh 1982) and determination of elements of the fourth group – zirconium in the presence of hafnium with picrin amine E using the D^{I} method. Also the problem of the analysis of 5f-elements of the actinoid group – uranium at 20-fold in presence of thorium and zirconium – was successfully solved (Kvaratskheli et al. 1983; Semenov, Perfilova (2000)).

To obtain the qualitative picture of derived spectra it is necessary to find conditions in accordance with the stated study problem and the outline of the absorption spectrum (Spitsyn and L'vov 1985). Intensification or weakening of the manifestation of recorded bands of derived spectrum depends on values of band half-widths in the initial spectrum, on gradient and symmetry of its band. The outline of the derived spectrum is more intensively revealed proportional to the narrowing of the band of the initial spectrum. Manifestation of the derived spectrum is less for a more mildly sloping peak. Spectrum scanning rate and selection of time constant (τ , see Sect. 3.9.1, Fig. 3.46) of spectra record regimes play the significant roles. *This means that it is necessary to individually look at methods of derived signal measurement for every substance or mixture of substances.*

We would point out that the most complete reviews concerned with the description of an application range of derivative spectrophotometry methods are covered in several articles on the physicochemical field of studies (Komar' and Samoilov 1967; Bershtein and Kaminskii 1975; Dubrovkin and Belikov 1981; Miller et al. 1982; Gans 1982; O'Haver 1982; Sneddon et al. 1982; Perfilov et al. 1985; Howell and Hargis 1986; Belikov 2002). In a number of other papers different approaches and techniques of derivative application in the chemistry of tranquilizers (Abdel-Hamid et al. 1984), alkaloids (Hassan and Davidson 1984), and phenols (Shibata et al. 1976), for determination of indomethacin, ibuprofen, salicylic acid (Such et al. 1980; Kitamura and Majima 1983; Mahrous et al. 1985), caffeine and amidopyrine, ephedrine and pseudo-ephedrine (with the instrument "Perkin-Elmer 552") (Davidson and Elsheikh 1982; Korany et al. 1984), heroin, morphine (Davidson and Elsheikh 1982; Lawrence and Kovar 1984; Taulier et al. 1986) and in toxicology (Melvin et al. 2002) are described. This method was successfully used for the analysis of fungicides of the tetramethyldithiocarbamate type (thiram) (Sharma et al. 2003), used for fruit protection from causal fungus (with the instrument "Shymadzu UV-VIS 160"), and also for analysis of different hemoglobin derivatives (Saakov et al. 1973, 1978a; Saakov and Shpotakovskii 1973; Siek and Rieders 1984; Parks and Worth 1985; Melvin et al. 2002), of chlorophyll forms from plants of diverse ecological groups and bacteria (Magomedov and Saakov 1973; Magomedov et al. 1974; Magomedov and Saakov 1978; Whitten et al. 1978) and mixtures of vitamins, for example, of B group. Registration of UV spectra for B group vitamins was performed with the instrument "UV-VIS Perkin Elmer-200," and derived spectra – with the instrument "Perkin Elmer-200-0628" (Such et al. 1980; Kitamura and Majima 1983) (Fig. 2.24).

On the possibility of quantitative determinations from derived spectra. For purposes of quantitative analysis derivative spectrophotometry is not at present applied very intensively although potential capabilities of the method are very promising (McWilliam 1959; Savitzky and Golay 1964; McKay and Scargill 1968; Porro 1972; Ishii and Satoh 1982; Dubrovkin 1983; Perfilov et al. 1985; Skujins 1986a; Saakov et al. 1987; Dubrovkin and Belikov 1988; Talsky 1994). This is seen in Figs. 2.25, 2.26, 2.27, 2.28, and 2.29, for example, for inorganic ions, and also for the quantitative analysis of amino acids (Fig. 2.29).

For such determinations a value of difference in amplitudes of two vicinal extrema (P_1 , P_2) or a minimum amplitude value relatively to the tangent line t drawn to two vicinal maxima (Fig. 2.25) are used more frequently. When analyzing D^I or D^{II} spectra and in turbid media the use of absolute peak value from the zero line z is recommended (Talsky et al. 1978a; Ioffe et al. 1984; Skujins 1986a, b; Talsky 1994).

The technique of quantitative assessment with derived spectra and analysis of possible measurement errors are described in the review O'Haver and Green (1976) and in later papers (Perfilov et al. 1985; Dubrovkin and Belikov 1988). Moreover, the possibility of the quantitative determination of substances in turbid media is accented. For this it is recommended to apply the method based on finding of the distance (t) between the tangent drawn through the two nearest maxima or minima,

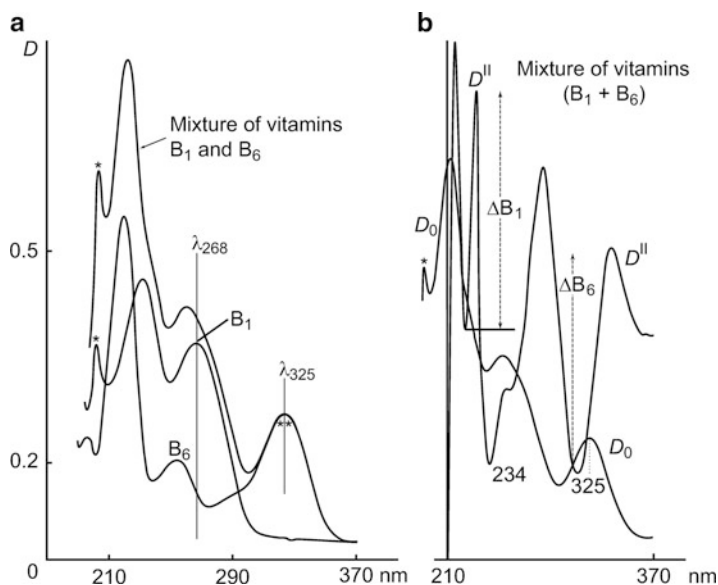


Fig. 2.24 UV absorption spectra of B_1 and B_6 vitamins and of their mixture for concentrations 20 and 10 $\mu\text{g/mL}$, respectively, in phosphate buffer ($\text{pH} = 7$) (a); (b) the absorption spectrum of the $B_1 + B_6$ mixture and D_{II} of the spectrum of this mixture

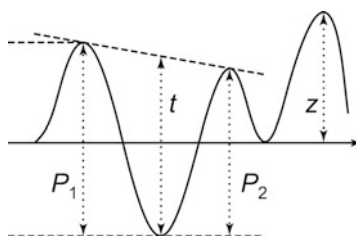


Fig. 2.25 A number of equal variants of calculation of quantitative characteristics of derived spectra, when it is possible to expect linear concentration dependence with retention of values of the band half-width of the substance spectrum. More frequently the value of amplitudes P_1 or of peak amplitude relative to the base line t are used. The possibility of usage of the absolute value of peak from the zero line (z) is not excluded

and the locus of the extremum amplitude (Fig. 2.25) (O'Haver and Green 1976; Schmitt 1977).

If half-width λ_{max} is constant, a peak height from a derived spectrum is proportional to substance concentration, so high accuracy is ensured, together with informativeness and selectivity of the analysis, realization of which by other means is difficult or even impossible.

The above determines the fields of rational application of the method, including cases of the *avia- or satellite monitoring of terrestrial and aqueous surfaces*.

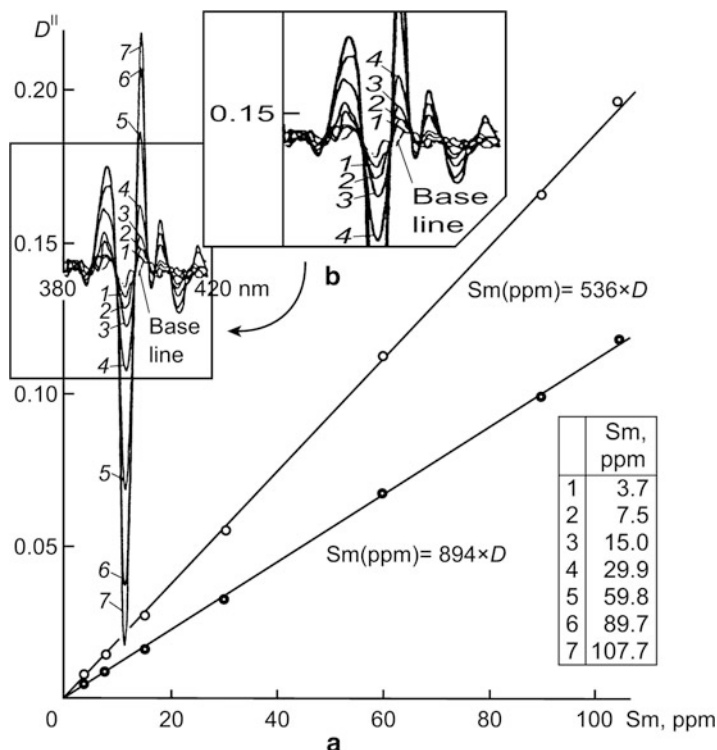


Fig. 2.26 Calibration curves of the determination of samarium concentration using D'' spectra with help of two calculation ways (Ishii and Satoh 1982): (a) original record; (b) expanded scale

Thus, in the figures presented in this part, various approaches to the usage of derived spectra for quantitative and qualitative determinations in the analytical and practical work are shown.

2.3.1 Methods of Derivative Signal Registration and Diagrams of Differential Analyzers

Because derivative spectroscopy has found wide application in qualitative and quantitative analysis (Hammond and Price 1953; Giese and French 1955; French 1957a; Hager 1971; Skujins 1986a; Dubrovkin and Belikov 1988; Talsky 1994), interest in this method constantly grows. The quantity of literature on the theoretical aspects of the method continuously increases (Giese and French 1955; Bonfiglioli and Brovetto 1964b; O'Haver and Green 1976; Hager and Anderson 1970; Ioffe et al. 1984; Skujins 1986a; Dubrovkin 1983), although separate communications are too general and far from practical recommendations.

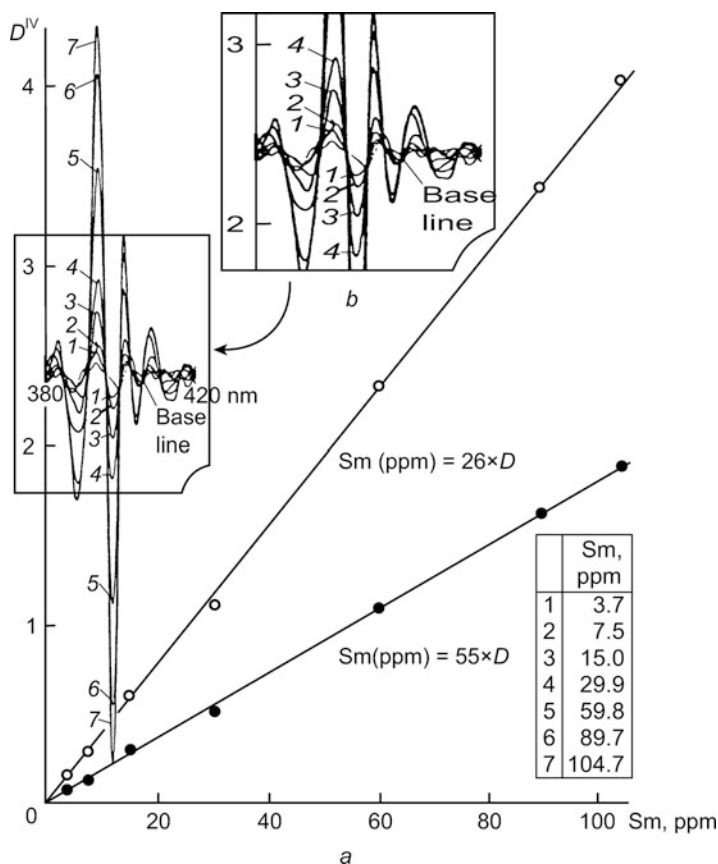


Fig. 2.27 Calibration curves of the determination of Sm concentration from D^{IV} spectra with help of two calculation ways (Ishii and Satoh 1982): (a) original record; (b) expanded scale. Graphs in Figs. 3.25 and 3.26 are drawn according to points of changes of peak amplitudes of D^{II} spectra or according to measurement of the peak amplitude from the base line

Different approaches to practical application of the method are developed simultaneously with analysis of the theory of a problem. In general, there are three main directions: optical-mechanical, numerical, and electronic-analog (Perfilev et al. 1985; Spitsyn and L'vov 1985; Gunders and Kaplan 1965; Dubrovkin and Belikov 1988).

The first group of methods for derived spectra registration includes two-wave spectrophotometry (French 1957a; Pemsler 1957; Bonfiglioli, Brovetto 1964 a,b; Bonfiglioli et al. 1967; Shibata et al. 1969; Porro 1972; Shibata et al. 1973; Wahbi and Ebel 1974; O'Haver and Green 1976; Skujins 1986a). This method is based on the direct differentiation of optical density with respect to wavelength. It is characterized by the high cost of instruments, and also by the capability to register only a first derivative of a studied spectrum without additional expensive alterations of equipment. The method shows advantages over derivative spectrophotometry and with development of computer technology offers new possibilities for researchers of different

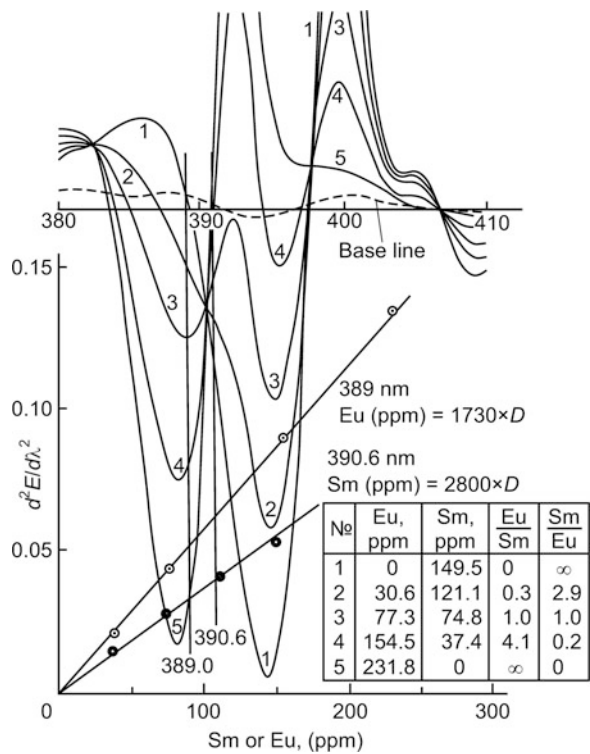


Fig. 2.28 Calibration curves of simultaneous detection of Sm and Eu in aqueous solutions from absorption D^{II} spectra (Ishii and Satoh 1982)

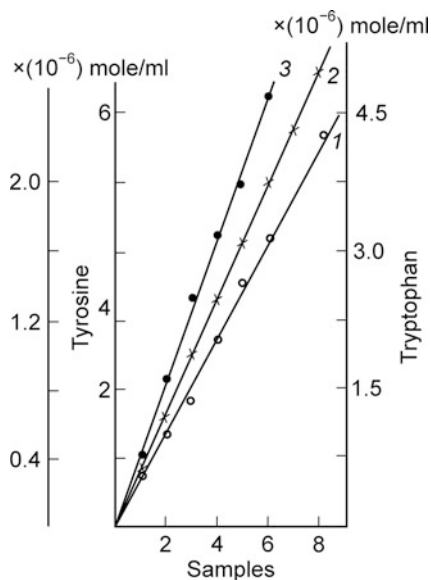


Fig. 2.29 Quantitative determination of aromatic amino acids

specializations (Pemsler 1957; Bonfiglioli, Broveto (1964 b); Talsky et al. 1978a; Dubrovkin and Belikov 1988). The same group includes methods of derived spectra registration with wave modulation (Shibata et al. 1969; Hager and Anderson 1970; Hager 1971, 1973; Wahbi and Ebel 1974; O'Haver and Green 1976; Talsky et al. 1978b; O'Haver 1979). Elements modulating wavelength could be oscillating or rotating mirrors (Brode et al. 1953; Hammond and Price 1953; Collier and Panting 1959; Snellman 1968; Snellman et al. 1970; Shaklee and Rowe 1970; Burke et al. 1972), quartz plates or interference filters (Vierordt 1873; Drews 1967; McWilliam 1959; Williams 1959; Gilgore et al. 1967; Zucca and Shen 1973), the oscillatory entrance either output slit of monochromator or sector shutter (Vierordt 1873; French et al. 1954; Baslev 1966; Williams and Hager 1970; Brandts and Kaplan 1973; Fowler et al. 1974; Mukhtarov and Nikolaev 1979), and light source modulation (Fowler et al. 1974). The positive aspect of derived spectra registration with the method of wave modulation is the decrease of signal-to-noise ratio; however, simultaneously, the intensity of light incident to the object is substantially weakened. Realization of this approach requires complex equipment hardware for optical-mechanical and electronic systems (Giese and French 1955; French 1957a, b; French et al. 1954; Bonfiglioli et al. (1967); Goldstein 1970; O'Haver 1976; 1979).

Using emission modulation in laser gas analyzers, the authors of the paper Mukhtarov and Nikolaev (1979) found that application of the first derivative allows one to decrease the lower limit of concentration determination of SO and NO by three to five orders in comparison with the method of differential absorption. Thus, the determination of nanogram quantities of these gases becomes possible, and this is a direct junction with nanotechnology.

The design complexity of equipment is a common feature of listed methods of derivative signal realization. A detailed example of the instrument diagram is presented in Goldstein (1970).

The second group of methods of derived spectra registration is based on the method of numerical (digital) differentiation (Challise and Williams 1964; Lewis et al. 1970; Lester 1970; Grum et al. 1972; Brandts and Kaplan 1973; O'Haver and Green 1976; Cuellar et al. 1978; Gans 1982), easily realized because of the appearance of spectrophotometers with built-in microprocessors or operating with the computer. In recent years this group of studies has attracted increasing attention (Mikhailyuk 2003).

In Russia (former USSR) the third – electronic-analog – method of differentiation of spectral curves is more widely applied (Kaler et al. 1967; Litvin and Gulyaev 1969; Saakov 1971b; Marenko 1972; Marenko and Saakov 1973; Saakov and Shpotakovskii 1973; Spitsyn and Korepanov 1980; Dubrovkin 1983a), etc. Combination of this method with further digitization of data and further data processing with programs is described, for example, in Magomedov, Stepanova et al. (1974), Magomedov and Saakov (1978), and Saakov (2000a). This method allows one to achieve the desired result with simpler hardware (Talsky et al. 1978).

Let us add that Russian recording spectrophotometers of SP-2 to SP-18 series are such that the recorded electrical signal is proportional to a rate of absorption change, i.e., to the first derivative of an absorption or transmission spectrum. According to this principle, different devices and attachments to spectrophotometers were designed for generation and registration of signals of the first and second

derivatives of absorption and fluorescence spectra (Kaler et al. 1967; Litvin and Gulyaev 1969; Marenko et al. 1972; Marenko and Saakov 1973; Saakov et al. 1973, 1987; Saakov and Shpotakovskii 1973; Zeinalov 1974; Rutman et al. 1976a, b; Dubrovkin and Sobolev 1976; Kucher et al. 1983; Dubrovkin et al. 1978).

We stress that the third method is based on the measurement of the rate of spectrum amplitude change with maintenance of its scanning speed, continuous and uniform (Kaler et al. 1967; Litvin and Gulyaev 1969; Marenko et al. 1972; Marenko and Saakov 1973; Zeinalov 1974). In recording spectrophotometers the spectrum scanning speed is strictly dependent on the scanning time of the wavelength range. If the scanning speed of the spectrum is constant, i.e., $d\lambda/dt = \text{const}$, then for the first derivative $dD/dt = dD/d\lambda(d\lambda/dt)$ (I), and for the second one $d^2D/dt^2 = d^2D/d\lambda^2(d\lambda/dt)^2$ (II), but $d\lambda/dt = \text{const}$ and consequently $d^2\lambda/dt^2 = 0$ and $d^2D/dt^2 = d^2D/d\lambda^2(d\lambda/dt)^2$ (III).

Thus, the first and second derivatives with respect to wavelength and to time are equal to each other within an accuracy of constant coefficients, determined by spectrum scanning speeds.

Material described above is not only concerned with derived absorption spectra registration, but also relates to modern requirements and development trends of derived luminescence spectra registration (Navarro et al. 1972; Green 1974; O'Haver 1976; Almela et al. 1983).

One of the first Russian articles in which authors described (in general) the registration method for the first derivative of an absorption spectrum was Kaler et al. (1967). A similar approach to the technical realization of creation of signal proportional to the first and second derivatives of an absorption spectrum is described in several papers (Litvin and Gulyaev 1969; Gulyaev et al. 1971; Shabalin and Petrova 1969; Litvin et al. 1973a, b; Saakov 1971a; Ishii and Satoh 1982; Matsushima et al. 1975; Meister 1966a; Dubrovkin et al. 1978; Demchenko et al. 1978). However, this technical realization has a number of disadvantages, the main one being an increased noise level distorting the formed signal and, therefore, complicating the correct interpretation of a derived spectrum graph.

We connected a differentiator directly to the entrance of the final power amplifier stage of a spectrophotometer (Marenko et al. 1972; Marenko and Saakov 1973; Rutman et al. 1976a, b; Rutman and Saakov 1978) that allowed us to exclude significant outside signals as mentioned in earlier articles.

The authors of this book decided not to wait for detailed descriptions of development stages and circuit improvements related to new hardware and from accumulation of research experience. Features of improvements performed by authors of this monograph are described in available publications (Marenko et al. 1972; Marenko and Saakov 1973; Saakov and Shpotakovskii 1973; Rutman et al. 1976a, b; Saakov et al. 1976, 1977, 1987; Baranov et al. 1976; Rutman and Saakov 1978) and in papers of other authors (Zeinalov 1974; Dubrovkin and Sobolev 1976; Dubrovkin et al. 1978; Kucher et al. 1983). Practically all our developments have passed many years of testing in laboratory practice and the results based on them were published in Russia and abroad. Developed together with the department of physics of SPbETU "LETI" ways of D^I and D^{II} signal registration were studied

by chemists and biochemists from different laboratories (Kvitko et al. 1976, 1977; Aleksandrova et al. 1982; Kucher et al. 1983; Perfilev et al. 1983a, b, 1985; Spitsyn and L'vov 1985; Skujins 1986a; Talsky 1994), and the results were positively characterized at different scientific forums.

As electronic engineering and locating features develop continuously, the electrical circuits of differentiators previously published by us are interesting at present only as history. For readers interested in familiarization with our improvements and with technical solutions of other authors we recommend several publications (Marenko et al. 1972; Marenko and Saakov 1973; Dubrovkin and Sobolev 1976; Saakov and Shpotakovskii 1973; Udovenko et al. 1974; Saakov et al. 1976, 1977).

With modernization of components of electric network, circuits of different devices for shaping and registration of D^I and D^{II} absorption spectra with Russian serial equipment were published (Dubrovkin and Sobolev 1976; Rutman et al. 1976a, b; Udovenko et al. 1974; Saakov et al. 1976, 1987; Rutman and Saakov 1978; Kucher et al. 1983). However, before its description we will consider the problem of optimization of differentiating circuit parameters.

West European authors referred to a number of the above-mentioned developments made together with the department of physics of SPbETU "LETI" (Skujins 1986a, b; Talsky 1994; Dubrovkin and Belikov 1988). It is regrettable that articles by Russian authors are cited by Western scientists only 15–20 years after of their publication, whereas Russian researchers, even not biochemical specialists, use and cite described developments promptly (Aleksandrova et al. 1982; Kucher et al. 1983; Perfilev et al. 1983a, b).

2.3.2 *Parameter Optimization of the Differentiating Circuit*

We are interested in the calculation of optimum parameters of the differentiating circuit and specific methodological solutions leading to an increase of differentiation accuracy and reliability of circuit operation during the analysis of plant and animal tissues or suspensions and solutions of bio-organic substances.

We think it is relevant that the work of the above-mentioned differentiators is based on the principle of spectrum differentiation not with respect to wavelength, but to time. This principle is realized when spectrum scanning on wavelengths is strictly synchronized with the time of the whole wavelength range transit and, consequently, derivatives with respect to wavelength and to time are equal to each other within an accuracy of constant coefficients depending on the spectrum scanning speed.

Practical realization of this method, as can be seen from above-mentioned diagrams, is simple; however, it requires the correct calculation of the differentiating circuit taking into account the nature of the spectral outline.

An example of this approach is described in Martin (1957, 1959). On the basis of these recommendations we calculated parameters of the differentiating circuit used in studies with Russian and some European recording spectrophotometers.

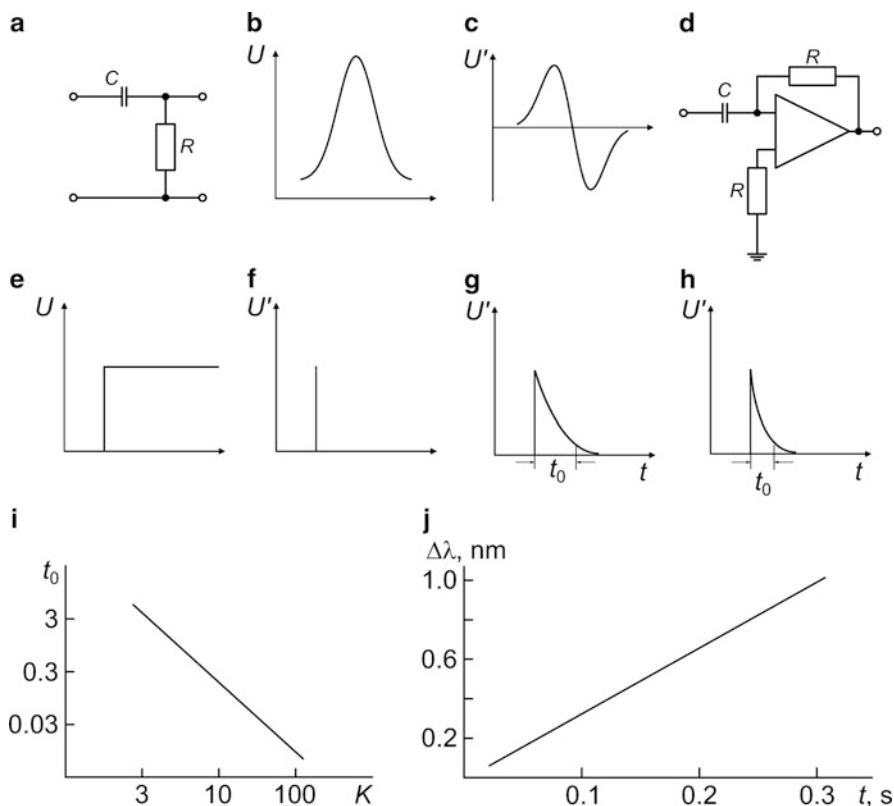


Fig. 2.30 Diagrams and performance records of differentiating circuit and of differentiating amplifier: (a) differentiating RC-circuit; (b) initial signal at the entrance of RC-circuit; (c) first-order derivative of signal b; (d) differentiating amplifier; (e) signal at the entrance of differentiator; (f) signal at the output of ideal differentiator; (g) differentiation of signal (d) with help of RC-circuit; (h) differentiation of signal d with help of the differentiating amplifier; (i) dependence of t_0 on mu-factor of the differentiating amplifier (t_0 – time, during which signal at the output of differentiator decreases to the value of $1/e$); (j) dependence of the average length $\Delta\lambda$ of the differentiation region on the time constant τ of the RC-circuit

Not specifically adapting the mathematical formulae for description of signal at the entrance and output of the differentiating circuit, we will give an instance of basic formulae.

If the signal enters the entrance of the differentiating circuit (Fig. 2.30a), and this signal has the Lorenz curve form (Fig. 2.30b), the maximum value of derivative of this curve at the output of differentiating circuit will be equal to

$$U'_{\max} = \frac{\pm 3\sqrt{3}U_0r\tau}{4\omega} = \frac{10}{3,000},$$

where U'_{\max} is the maximum value of derivative at the output of the differentiating circuit, U_0 the maximum value of signal amplitude at the entrance of the differentiating circuit, r the scanning speed of spectrum, nm/s, $\tau = RC$ the time constant of circuit differentiation, and ω the half-width of Lorenz band (the width of spectral band at height of 1/2 of the maximum value of spectrum amplitude).

In transistor circuits (Rutman et al. 1976a, b; Saakov et al. 1976, 1987) to the entrance of the differentiating circuit the maximum signal U_0 – about 3 V enters; at the circuit output it is necessary to have a signal not less than 20 mV. Since the first derivative of Lorenz curve has two maxima (it is bipolar) of equal amplitude (Fig. 2.30c), then $U'_{\max} = 10\text{mV}$, and consequently

$$\frac{U'_{\max}}{U_0} = \frac{3\sqrt{3}}{4\omega} r\tau = \frac{10}{3,000}; \quad (2.6)$$

$$\frac{r\tau}{\omega} = \frac{1}{225\sqrt{3}}. \quad (2.7)$$

If we use a spectrophotometer with fixed spectral scanning speeds (for example, SP-18 or SP-14) to record the spectra, then for the most convenient speed (for example, № 4) the value of r is equal to $r = 2.9$ nm/s. Because of the half-width of the red absorption band, for example, of photosynthesizing models (tissue homogenate, suspension of algae or chloroplasts, etc.) is usually in the range 30–40 nm, from Eqs. 2.6 to 2.7 it is possible to find the optimum value of the differentiation time constant:

$$\tau = \frac{\omega}{r \cdot 225\sqrt{3}} = \frac{35}{2.9 \cdot 225\sqrt{3}} = 0.03$$

At $r = 2.9$ nm/s all wavelength range of the visible spectral region (400–750) is scanned in 2 min, i.e., in this time the spectrum is divided into 4,000 parts of differentiation. The average length of part will be equal to $\Delta\lambda = 0.0875$ nm ($\Delta\lambda = 350\text{nm}/4000 = 0.0875\text{nm}$). Thus, the average length of the differentiation part linearly depends on the τ -value. Dependence $\Delta\lambda = f(\tau)$ is shown in Fig. 2.30j. However, taking into account that accuracy of wavelength setting in the spectrophotometer is ± 1 nm, the time constant of differentiation can be increased practically to 0.1–0.2 s. In this case $\Delta\lambda = 0.3$ –0.4 nm, and at $\tau = 0.3\text{s}$ we have $\Delta\lambda = 0.87$ nm, i.e., its value is close to the spectrophotometer precision.

Depending on parameters of the concrete spectral band (amplitude and half-width), selection of optimum conditions for differentiation is performed by choice of the scanning speed and of nominal values R and C of the differentiating circuit (DC). During the search for DC ratings the reader should proceed from the condition that capacity resistance must be much more than R (Saakov 2000b). For $\tau = 0.1$ –0.2s rated capacity must be $C = 4$ –10 μF , and therefore R -values can be from the range 10–25 kilohms (kOhm). Having the

package with the above-mentioned R and C, we can change τ from 0.04 to 0.25 s depending on parameters of the concrete spectral band (its amplitude and half-width). *It is also necessary to change the τ -value with change of the spectral scanning speed.* During choice of R and C nominals a reader should proceed from conditions described in the article Saakov (2000b). Optimization of τ -value choice has a great influence on manifestation of the fine structure of derived spectra (see Sect. 3.9.1, Fig. 3.46).

Experience from the use of serial spectrophotometers shows that optimum differentiation conditions can be found when the differentiator ensures a change of the time constant in the range 0.05–0.5 s and nominal values of R are in the range 10–5 kOhm, values of C from 2 to 10 μF (Saakov et al. 1987).

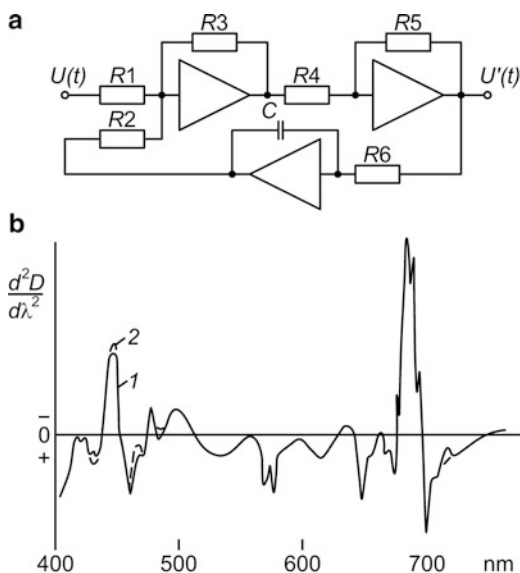
The application of any method always raises the question of its accuracy – in this case the question of accuracy of differentiation. As revealed earlier, the differentiating amplifier (DA) (Fig. 2.30d) has a number of advantages compared with the usual RC-circuit (Maiorov 1956; Rutman et al. 1976a; Taulier et al. 1986). This, first of all, represents a significant increase in the level of output signal; second, the exclusion of influence of the mu-factor instability on the output signal and, third, the significant decrease of the differentiation error, i.e., $t_0 \rightarrow 0$ (Fig. 2.30g).

In Fig. 2.30e–h work diagrams of the differentiating circuit (Fig. 2.30g) and of the differentiator amplifier (Fig. 2.30h) are presented for conditions when the single voltage jump enters (Fig. 2.30e). In Fig. 2.30f the diagram of the output voltage of the ideal differentiator is shown. Dependence of t_0 on the mu-factor (K) of the amplifier with constant R and C is presented in Fig. 2.30i; it is of linear character in logarithmic coordinates. Even if the transfer constant of the differentiating amplifier is equal to 1.0, then t_0 decreases from 3.0 to 0.3 s in comparison with the RC-circuit. Thus, advantages of the differentiating amplifier against the RC-circuit are obvious.

Specialists tried to assess the differentiation errors (Smirnov and Badu 1967). Meister (1966b) showed the presence of such errors, but did not give recommendations on how to decrease them. In Korobkov (1975) the author attempts not very successfully to compensate for these errors. Previously, in articles on derivative spectrophotometry for biological native structures, the accuracy level of differentiation was not determined.

The question about differentiation errors and the value of using differentiating amplifiers instead of usual RC-circuits is examined in Rutman et al. (1976b). The analysis of the literature data allows one to conclude that usage of an RC-circuit results in that, after 1–3 s, the differentiation error depending on the value $\tau = RC$ becomes insignificant (it is 2–5%), and the usage of a differentiating amplifier after only 0.1–0.3 s allows one to reduce error practically to zero at the mu-factor $K = 10$ (Fig. 2.30). With the growth of K ($K = 100$) after only 0.01–0.03 s, $\Delta \cong 0$. Thus, correctly selected parameters R, C, and K reduce the differentiation error to so low a value that it will be inessential. Details of calculation and graphs of the differentiation error change when using the differentiating amplifier are presented in Rutman et al. (1976b).

Fig. 2.31 Diagram of the real differentiator (RD) (a) and the absorption spectrum of homogenate of spiderwort leaves (b)



Concerning the shift value for maximum the second derivative discussed in Korobkov (1975), because the D^{II} spectrum is formed and recorded in parallel with the initial absorption spectrum, and the time constant of spectrophotometer is more than the time constant of the differentiating attachment (otherwise there would be no way for differentiation), it is possible to consider all these shifts as existing only relative to the true absorption band, but not to the band recorded by the spectrophotometer, i.e., comparison of the initial spectrum with its D^{II} registered with differentiating amplifiers does not reveal shifts of bands maxima.

Already at initial steps of usage of the considered method the considered method we performed checks of the differentiation accuracy, applying the procedure proposed in the article Smirnov and Badu (1967). The checking technique is the comparison of transfer functions of ideal differentiator (ID) and of real differentiator (RD), whose block diagram is shown in Fig. 2.31, and in Rutman and Saakov (1978). In this case RD parameters and absolute and relative errors are calculated, and a method to decrease these errors due to change of nominals of separate circuit elements is found.

RD consists of an adder, an inverter, and an integrator connected to a feedback loop. Without rewriting mathematical calculations described in the paper Smirnov and Badu (1967), we would like to note that the accuracy of differentiation of the arbitrary time function (on the supposition of equality of $R1$ and $R2$ and transfer constant of circuit $K = 1.0$) depends on integrator parameters, i.e., on the integration time constant τ_0 . Having a constant accuracy level, when differentiating the arbitrary time function, it is necessary to find τ_0 from the prevailing frequency (Rutman et al. 1976a, b; Saakov et al. 1976, 1987; Rutman and Saakov 1978).

For example, if the half-width of the red absorption band of spectrum is equal to 30 nm, and the scanning speed of the spectrum is 2.9 nm/s, then sinusoid frequency

approximating this band will be approximately 0.1 Hz. Using this value, the time constant of integration is chosen.

We performed comparison of RD (Fig. 2.31) and of other differentiators (Rutman et al. 1976a, b; Rutman and Saakov 1978; Saakov et al. 1987) and concluded that the differentiation accuracy of these devices is satisfactory enough, the relative error not exceeding 10%. In Fig. 2.31b the graph of D^{II} absorption spectrum for homogenate of spiderwort (*Tradescantia sp.*) leaves is shown. The spectrum was registered with RD and a differentiator, manufactured according to the diagram of the differentiating amplifier. Data in Figs. 2.2 and 2.3 allow one to draw conclusions about stability of reproduction of spectra recorded in equipment developed by us. Similar checking of the results stability of derived spectra registration is described in Talsky (1994). These materials prove the correct registration of higher order-derived spectra at temperate devices.

Assessment techniques for the deformation degree of form and position of the spectrophotometer absorption band are sufficiently well presented in the literature (Babushkin et al. 1962; Tereshin 1959a, b; Tarasov 1968; Rubin 1974, 1975; Bershtein and Kaminskii 1975; Talsky 1994), so we will not describe them. If spectroscopic instrumentation does not change the form of the absorption band by itself and does not shift the band maximum, then a differentiator with correctly selected parameters of differentiation will not add error to the position of absorption maximum at the wavelength scale.

The analysis performed in Rutman et al. (1976b) was one of the first attempts at the study and compensation of differentiation errors of the first equipment realizing shaping and registration of D^{I} and D^{II} signals, and probably this analysis is not ideal and has imperfections.

Examples of the usage of differentiating amplifiers in apparatus recording D^{I} and D^{II} signals are described in several articles (Rutman et al. 1976a, b; Saakov et al. 1976, 1987; Rutman and Saakov 1978).

2.3.2.1 Differentiators with New Locating Features

As differentiation accuracy and reproducibility of results depend on stability of parameters of the elements composing a differentiating unit, the differentiator was developed (Rutman et al. 1976a, b; Udovenko et al. 1974) based on integrated microcircuits and according to the diagram of differentiating amplifier (Saakov et al. 1987). We pay attention to the fact that DA substantially amplifies the level of output signal, the noticeable decrease of the error of differentiation, and excludes influence of mu-factor instability on the value of the output signal.

In Fig. 2.32 the schematic diagram of the differentiator based on microcircuits is shown (Rutman and Saakov 1978). The signal is registered from the entrance of the final stage of the power amplifier of the spectrophotometer and comes into the left half of MS1(A1) (assembly of field-effect transistors KPS 104B), which is the matching cascade. The right half of MS1 is the phase-inverting cascade, to the entrance of which the voltage of the reference signal will be applied. The reference

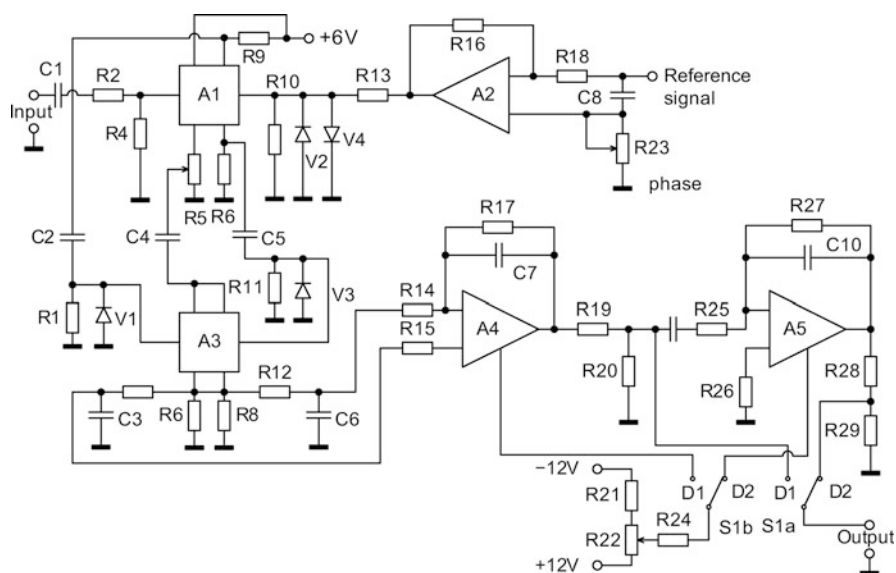


Fig. 2.32 Principal electric diagram of the differentiating attachment on microschemes. A1, A3 – KPS104B; A2, A4, A5 – K1UT531A; V1 – V4 – D219; C1, C2, C4, C5 – 1.0; C3, C6 – 10.0; C7, C9 – 6.0; C8 – 0.5; C10 – 2.0; R1, R4, R10, R11 – 1.0; R2 – 510 kOhm; R3, R12, R16, R17, R18, R22, R26, R27 – 100 kOhm; R5 – R9, R20, R29 – 1 kOhm; R14, R15 – 5 kOhm; R13, R19, R28 – 51 kOhm; R21 – 160 kOhm; R23, R25 – 20k; R24 – 240 kOhm

signal preliminarily passed through a phase inverter based on MS2(A2) (the operational amplifier K1UT531A or MS40 ensuring the precise matching of phases of fundamental and reference signals). In the considered differentiator the principle of synchronous signal detection is used. As the signal is modulated by the frequency of 50 Hz and its envelope is proportional to D^I , after the synchronous detector based on MS3(A3) we register the signal of the first-order derivative of the absorption spectrum. After the active filter based on microcircuit MS4(A4) performing the transformation of symmetrical signal into the asymmetrical one, and also realizing additional filtration of variable component of the signal (cutoff frequency not more than 20 Hz), the signal enters either at the entrance of the graph plotter or the entrance of the differentiating amplifier based on the microcircuit MS5 (A5). From the output of MS5 the signal proportional to D^{II} is recorded. Supply of the differentiating unit is maintained with the stabilized voltage source described earlier (Rutman et al. 1976b). The registration of signal can be performed both with recording instruments of types KSP-4, EPP-09 (with the scale up to 2.5 s), and with the cathode-ray indicator (of type I-10) or with the oscillograph face (S1-18). The electron-beam record is inertia-free, leading to more registration rapid and smaller changes of the spectrum structure. The electron-beam record ensures the selective large-scale record of individual regions of spectrum and can be useful for an operator performing a number of special experiments (Zeinalov 1974; Rutman et al. 1976b; Rutman and Saakov 1978; Saakov et al. 1987).

The considered DU is characterized by high accuracy of differentiation, reliability in operation, and small overall dimensions. The accuracy of DU differentiation is 1–3% (depending on phase ratios in the synchronous detector) and was checked with an analog computer MN-10 M using the procedure described by Smirnov and Badu (1967).

As a result of the above-mentioned positions in SPbETU “LETT” the dedicated analog processor (DAP) was created for processing of electronic data recorded by spectroscopic instrumentation (spectrometers of optical range, spectrofluorometers, microwave spectrometers) (Saakov et al. 1987). DAP in the general case performs the following: search and isolation of informative region of the spectrum, its processing (one- or double differentiation, measurement of extremum value, the integration of spectral curve, etc.), storage of the processing result and its comparison with the given value corresponding to the normal value of the measured parameter of spectrum (amplitude, slope, area, linewidth). From DAP, information can be sent to a digital instrument or a computer. DAP was successfully used in combination with modifications of the instrument “Specord UV–VIS” (“Carl Zeiss,” Jena) (Saakov et al. 1987). One of the DAP variants is used for the optimum coupling of the EPR sensor with the computer ASU TP for preparation of the metallo-organic catalyst for polymerization of isoprene caoutchouc. This ASU TP including EPR sensor and DAP is successfully operated in production by “Nizhnekamskneftekhim” (petrochemical industry in Nizhnekamsk) and allows one to solve the task of continuous automated monitoring and control of the preparation process of the optimum composition catalyst. The usage of integrated microcircuits and semiconductor devices in DAP guarantees high stability, reliability of operation, and operational simplicity.

DAP was successfully used in combination with modifications of the instrument “Specord UV–VIS, Carl Zeiss, Jena” in 1975–1995. The graph plotter of this spectrophotometer has two interconnected slide wires for vertical pen deflection of the instrument’s automatic recorder. One of slide wires is destined for the remote registration of spectra. Connections for this potentiometer are at the slot located on the back side of the spectrophotometer panel (contacts 22, 23, and 24). Applying a voltage of 9 V at contacts 22 and 24 from the stabilized source of voltage (SSV) based on transistors V1 and V3 and the stabilatron V2, we will obtain at connections of the slide wire the voltage proportional to the optical density of sample (see Fig. 2.33).

The load of SSV is the slide wire R1 of “Specord” and the compensation potentiometer R2. The signal, proportional to the position of the carriage of the spectrophotometer, goes through integrating circuit R4 and C1 decreasing high-frequency noise and interferences, and comes in at the entrance of the compensating amplifier based on the microcircuit A1, at another entrance of which there is a compensation voltage from the R2 resistor slider. With resistors R3, R5, R6, and R7 the μ -factor is assigned; switch “S” changes the μ -factor stepwise. With the closed switch S the input amplifier A1 works in the regime of voltage repeater and expands the dynamic range of the recorded voltage signal. Potentiometer R2 is set in such a way so as to compensate possible voltage shifts of the signal source, and

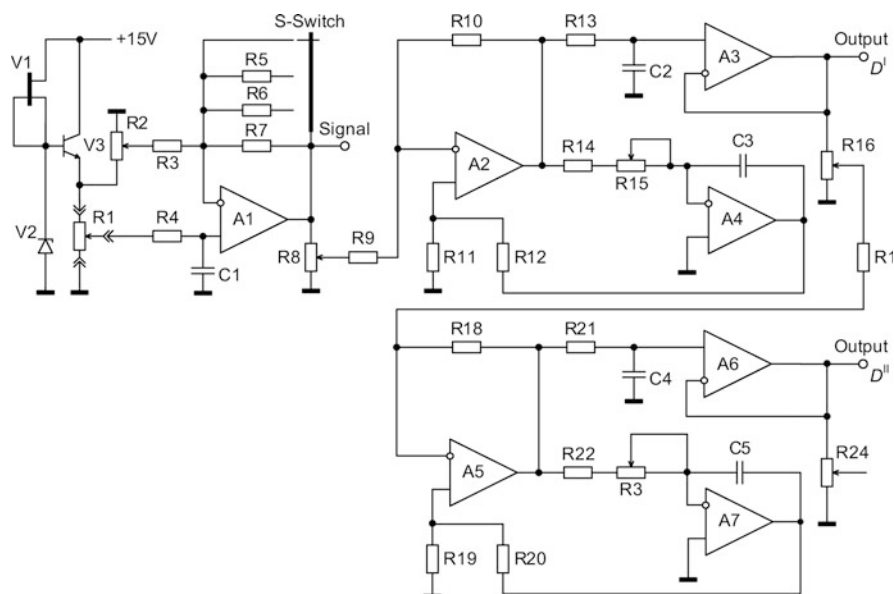


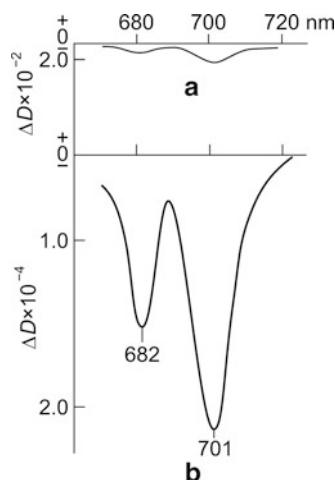
Fig. 2.33 Electric diagram of the dedicated analog processor. *V1* – KP303D; *V2* – D818E; *V3* – KT801; *A1*, *A3*, *A4*, *A6*, *A7* – K140UD8; *A2*, *A5* – K153UD2; *C1*, *C2*, *C4* – 0.1; *C3*, *C5* – 1.0; *R1* – реохорд спектрофотометра; *R2* – 3KOhm; *R8*, *R16*, *R24* – 6.8 kOhm; *R4*, *R9*, *R10*, *R12*, *R14*, *R18*, *R20*, *R22* – 24 kOhm; *R5* – 30 kOhm; *R3*, *R13*, *R21* – 68 kOhm; *R6* – 100 kOhm; *R11*, *R19* – 240 kOhm; *R7* – 300 kOhm *R15*, *R23* – 1.0

also to select differentiated regions of the spectrum. If amplification is large then exceeding the operating range of the compensating amplifier is possible, but with the corresponding position of potentiometer *R2* the more detailed representation of the necessary spectral region will be recorded.

From the output of the compensating amplifier the signal can be recorded by a recording instrument or can go to the input of the differentiator deriving the first-order derivative. The differentiator consists of amplifiers *A2* (*A5*) and *A4* (*A*) and elements *R8*, *R9*, *R10*, *R14*, *R15*, *C3*, *R12*, *R11* (*R16*, *R17*, *R18*, *R22*, *C5*, *R23*, *R19*, *R20*). In brackets the elements of the second differentiator are listed. Through the differentiating circuit the amplifier *A2* is connected with elements *R9* and *R10* of the circuit of negative feedback; moreover the integrator is connected to the output. The integrator is based on the microcircuit *A4* and elements *R14*, *R15*, and *C3*. With the divider $R11 \div R12$ the output of the integrator is connected to the non-inverting input of the amplifier *A2*. This allows one to have at the output of the amplifier *A2* the voltage proportional to the derivative of input signal.

Through the integrating circuit *R13* and *C2* serving for noise decrease, the D^1 signal reaches the input of the second differentiator. Resistors *R8* and *R16* regulate the time constant (τ) of differentiation. In microcircuits *A3* and (*A6*) the amplifiers matching integrating RC- circuits with the next loads are assembled.

Fig. 2.34 Change of the dynamic range of registration of the difference absorption spectrum ("light minus darkness") for spinach chloroplasts: (a) record of the serial spectrophotometer SP-18; (b) the same spectrum registered when using a differentiator (Saakov et al. 1987a) with the turned-off programmed motion



The reader should remember that the operation of differentiation is combined with some worsening of the signal-to-noise ratio. Therefore the optimization of this ratio after differentiation can be performed if instead of integrating chains R13, C2 and R21, R4 shown in Fig. 2.33 we use filters of higher orders.

For better processing of registered information, the functional capabilities of the described device could be enhanced by introduction into its circuit of logarithmic amplifiers or additional differentiating cascades.

Several years later the diagram of the differentiating attachment on the base of two differentiating amplifiers for the spectrophotometer "Specord" from the laboratory of the academician N.S. Poluektov (Odessa) was published and used for the analysis of mixtures of samarium and europium (Kucher et al. 1983). Its disadvantages include the obsolete locating features and impossibility of optimizing the differentiation time constant that decreases sensitivity and real functional capabilities of the device.

One additional possibility, important for the experimenter and realized in DA usage, should be remembered. If we register with the spectrophotometer the signal when playback of the programmed motion is turned-off and a differentiator is used in the D^I regime, then, when registering an absorption spectrum, the possibility of dynamic range increasing approximately by two orders appears (Fig. 2.34). This suggestion can be found in the work of Kaler and co-authors (Kaler et al. 1967), but it was not clearly formulated. Later this concept was mentioned in the paper Golovachev (1976) and in a more final form we reported it at symposia in "Molecular and applied biophysics" (Krasnodar 1974); "Plastid apparatus and the resistance of plants" (Leningrad, N.I. Vavilov Research Institute of Plant Industry 1975); at the second All-USSR conference on spectroscopy in Moscow 1977 (Saakov et al. 1977). It is described in the collection "Spectroscopic methods in physiology and biochemistry" (Leningrad 1987), and other publications (Udovenko et al. 1974; Saakov et al. 1976, 1987; Rutman and Saakov 1978).

From variants of circuits described in the literature it follows that the absence of Russian industrial devices allowing one to record derived spectra forces scientists

to use their own equipment designs, improved with developments from research practice and with advances in electronic engineering.

In this case each experimenter invents a system for his own studies because he does not know of all the available discoveries in this field and he can also explore only his own financial possibilities. The wide separation of researchers does not make it easy for one to coordinate development of optimal technical diagrams and to direct money to device manufacturing at the appropriate specialized enterprises in Russia.

2.3.3 Derivative Spectrophotometry of Difference Spectra

The considered methods of derived spectra registration using Russian and analogous European instruments have extended the analytical abilities of absorption spectrophotometry and opened up new systematic techniques (Klein and Dratz 1968; Williams and Hager 1970; Whitten et al. 1978; Fell and Smith 1982; Chadburn 1982; Cottrell 1982; Melvin et al. 2002).

The combination of methods of difference and derivative spectrophotometry therein proved to be very effective. We proposed the method using Russian recording spectrophotometers and also foreign double-beam instruments with automatic spectrum recording for registration of ΔD^{II} and if necessary of higher difference spectra derivatives (Saakov et al. 1973, 1976, 1987; Mikhailyuk 2003). This method of analysis successfully combines advantages of difference (see Sect. 2.2.2) and derivative spectrophotometry, and it allows one to record the difference spectrum on spectrophotometers with sensitivity below 10^{-3} – 10^{-4} optical density units and thus to solve analytical problems, undoable with other devices (Saakov 1971a; Saakov and Hoffmann 1974; Saakov et al. 1976; Saakov 1987, 2000b, c; Rozengart and Saakov 2002, 2003; Saakov 2003a).

Using Russian recording spectrophotometers, for the optimum shift of zero line a reader can use a technique of unbalance of the ray flux incident to the photo cell of the instrument. For this purpose an optical wedge or a set of neutral light filters (for example, for instruments SP-16, SP-26) or a vertical diaphragm allowing one to change a light flux area are used. Then at an input of an amplifier system of the spectrophotometer a fixed signal of light fluxes imbalance will be present and will specify the position of zero line on the paper of the spectrophotometer curve-tracer.

As the differentiating unit one of diagrams presented in Rutman and Saakov (1978) or Saakov et al. (1987) can be used. The technology of derived difference spectra registration does not differ from the record of derived absorption spectra; however, it requires the strict observance of conditions for difference spectra recording (see Sect. 2.1.2).

The described technique of registration of the second derivative of difference spectra was successfully applied by Japanese researchers for registration of the first derivative of difference spectra of some proteins and aromatic amino acids (Matsushima et al. 1975; Inoue et al. 1975). Quite often foreign researchers use

the methods of Russian scientists without citing their original sources. In practice the method of derived spectra registration can be used for very diverse spectral studies (Bershtein and Kaminskii 1975; Saakov et al. 1978b; Nazarenko et al. 1982; Perfilev et al. 1983a, b, 1985; Skujins 1986a; Mishchenko et al. 1987; Talsky 1994).

We used this method for investigation of spectral discreteness of chlorophyll and hemoglobin molecules, which are almost chemically identical, and discreteness is produced, probably, by the difference in interaction peculiarity of pigment molecules with the protein or lipoid complex (Saakov et al. 1973, 1978b). Using this method the presence in blood spectrum of a number of components indicating the spectral heterogeneity of hemoglobin unnoticeable with registration of usual difference spectra was discovered.

The registration of the derivative of difference spectra increases the volume of information about the registered process and allows one to detect more reliably insignificant changes of absorption lines unnoticeable with the usual techniques of the absorption spectral analysis (Udoenko and Saakov 1976).

Exemplary usage of the method of derived difference spectra registration is presented in Chaps. 3 and 4.

2.3.4 Method of the Pulse Amplitude-Modulated Fluorescence for the Solution of Ecological-Biochemical Problems

In recent years problems of the ecological monitoring of the environment invites increasing attention. Throughout the world, and in particular in Russia, extensive research on the creation of new techniques and procedures of checking of states of different natural objects is performed. One such method is the determination of plant leaves fluorescence by means of their illumination with light having a given wavelength. The measured intensity of this fluorescence and time dynamics of its change allow one to assess the level of damage of functional biochemical systems of the investigated plant.

Thus, if the method of derivative spectrophotometry is concerned with study and assessment of changes of *the structural state* of a biological object, then the registration method of pulse amplitude-modulated fluorescence (PAM) shows changes of *functional* reactions of an organism. Combination of both methods helps one to get more complete information on a state or damage of the cell.

As damage, in this context, the influence of chemical substances and of radiation or thermal irradiations is meant, i.e., of factors frequently occurring during different technogenic catastrophes or natural cataclysms. Thus, the method of fluorescence measurement of plant foliage allows one to assess rapidly scales of many ecological catastrophes without waiting for their eventual consequences.

The method of pulse amplitude-modulated fluorescence (PAM) has won acceptance in Europe and USA in the last 20 years (Schreiber 1983, 1986; Schreiber and Bilger 1987, 1997), and it successfully came out on top in the international

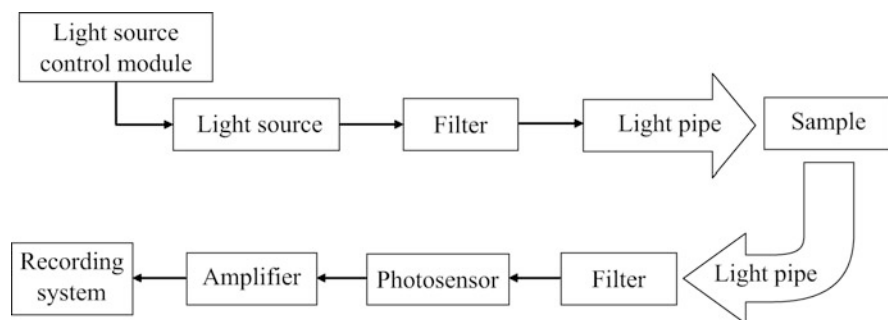


Fig. 2.35 Functional diagram of MF gauge

competition of methods of fluorescence analysis (Application of chlorophyll fluorescence, Lichenthaler (ed) 1988; Bolhar-Nordenkampf et al. 1989; Saakov 2011).

Realization of the PAM method for the solution of ecological-biochemical problems is illustrated with the example of the instrument functional diagram for measurements (Fig. 2.35).

With the development of the instrument the following positions were taken into consideration. The light source must ensure illumination of a model in different regimes. Furthermore, it is necessary to have the capability to modulate light by sine waves at different frequencies up to 100 kHz, and simultaneously the emission wavelength must be more than 560 nm. On the basis of this, the semiconductor radiating diode with increased brightness can be used as the light source. This solution substantially allows one to simplify the construction of the instrument due to the absence of mechanical attenuators for modulation of light flux. As the wavelength of light induced as a result of fluorescence is greater than the wavelength of the exciting light and as these wavelengths are close enough, they must be separated for successful detection of the useful signal of emission. For achievement of satisfactory results, interference filters with bandwidth of 16 nm should be used. For light supply to the model and for transfer of fluorescence to the light detector, flexible glass light guides are used. Radiation induced at a wavelength more than 670 nm is more convenient to record with a semiconductor photodetector. The registration circuit works in the pulsed mode ensuring a good noise immunity and allowing one to perform measurements when external illumination is presents.

The described variant of PAM instrument works as follows. The module of light source control ensures the working of the source in all required regimes and modulates the light flux with the necessary frequency. The semiconductor radiating diode, which is the light source, emits electromagnetic waves with the central frequency about 660 nm. After passing through a short-wave interference filter with transmission in the wavelength region of more than 650 nm and through a glass light guide, the light goes to the sample surface. In another light guide the fluorescence goes through a long-wave filter with the transmission band in the region of 670 nm and falls on a semiconductor photoreceiver. Then the signal is intensified by a bandpass amplifier at the frequency of light modulation for guaranteeing the necessary signal-to-noise ratio, and then the useful component of the signal is detected and goes to the register system.

The microprogram of the master controller is built according to the modular approach and ensures control of light source parameters, of photodetector, amplifier, and register system. The following modules are in the microprogram: the module of initialization, the module of light source control, the module of electronic attenuator control, the module of creation of control commands, the interface module, and the main module.

The program of high level must allow one to change operational modes of MF gauge to record and to show MF spectra with the computer display. For these purposes, facilities for database maintenance are built into the program and it has a graphic interface developed in accordance with modern requirements of ergonomics.

For convenience of working with the instrument, the program should make data export possible as data in a tabular format for the purpose of further processing with specialized packages specifically for work with spectra.

Already the first experiments on the usage of the PAM method showed how promising this approach was (Saakov 1993c, 1996a, 2000c, 2001b, 2002b, 2003d; Saakov et al. 1993; Saakov and Shiryaev 2000) for studies of the influence of gamma-radiation on plants. Further investigations on the influence of extreme factors of environment on the photosynthetic apparatus, namely, of low (Saakov 2001a, 2004c) and high temperatures, and also of drought (Saakov 2002d, 2003b, 2005a), of increased salting with ions Na^+ , SO_4^{2-} , Cl^- (Saakov 2002a) and of herbicides (Saakov 1998b), and also of gas mixtures of different composition (Schreiber 1986; Schreiber and Bilger 1987, 1997; Bolhar-Nordenkamp et al. 1989; Lichtenthaler (ed) 1988) revealed the association between changes of MF coefficients and effectiveness of functioning of the electron transport chain in transformation of light energy into the bonding energy (Fig. 2.36).

Very similar data were obtained in studies of changes of leaf native structure and of chloroplast suspensions with the derivative spectrophotometry method (Saakov et al. 1975; Udovenko and Saakov 1976; Saakov 2003a, d).

Results of studies obtained over a long time with two principally different methods allowed one to formulate a concept on dependence of resistance of a heterotrophic or autotrophic cell to abiotic influences on damage of its energy transformation chains in chloroplasts and mitochondria, i.e., the energy theory of cell resistance. These materials are published about widely enough and have been reported several times at different international conferences (Saakov 1998b, 2001c, 2002f, 2003a, c). Theses of this concept were formulated not immediately, but have been considered over time in publications and reports (Saakov et al. 1975; Udovenko and Saakov 1976; Saakov et al. 1978b; Saakov and Leontjev 1988; Saakov 1990, 1993/1994, 1996b, 1998a, 2000d, 2001b, 2002b; Pronkin and Saakov 1997; Saakov and Shiryaev 2000).

Examples of usage of the derived difference spectra record method are presented in Chaps. 3 and 4. We do not forget the warning of academician V.A. Engelhardt (1955) on the careful attitude to new methods introduced in study practice and promising at first glance, on the possibility of unforeseen dangers connected with

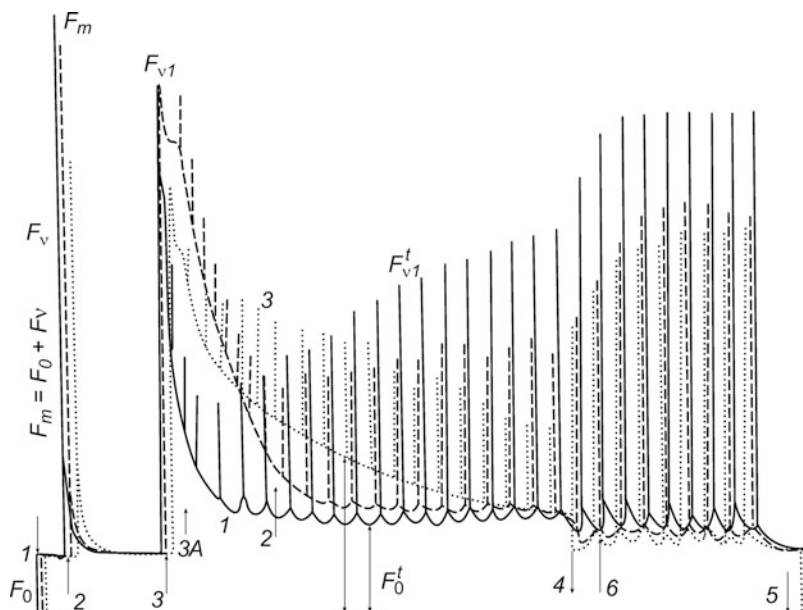


Fig. 2.36 Character of change of the signal harmonic of amplitude-modulated pulse fluorescence of *Nicotiana tabacum* leaf with its dehydration. Curves: 1 control; 2 experiment, dehydration to 45% of the initial one; 3 dehydration to 30% of the initial one. Arrows 1 and 5 switching modulating light on and off, respectively. Light has frequency of 1.6 kHz ($5 \mu\text{E}/\text{m}^2 \cdot \text{s}$), $\lambda < 670 \text{ nm}$; 2 switching one of the saturating 1-s white light pulse ($2,500 \mu\text{E}/\text{m}^2 \cdot \text{s}$) to find F_m and F_v values; 3 and 4 switching actinic light ($1,200 \mu\text{E}/\text{m}^2 \cdot \text{s}$) on and off, respectively; 3A switching 30 s later on of 1-s pulses of the saturating light at background of actinic light to find F_{v1}^t values; 6 switching on of 1-s pulses of the saturating light after switching of the actinic light (Saakov 2001a)

hasty and erroneous conclusions, on requirements for the thought-out and thorough data processing and the critical analysis of results (Engelhardt 1955).

Thus, in Chap. 2 a brief introduction to absorption spectrophotometry bases is presented, systematic approaches for application of the derivative spectrophotometry method are described, and problems solved with the discussed method or its combination with other biophysical methods are considered. The materials in Chap. 2 will help the reader in the understanding of materials of the following chapters of this book.

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