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FLUORESCENCE 2D AND 3D SPECTRA ANALYSIS OF TRYPTOPHAN, TYROSINE AND PHENYLALANINE

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The analysis of fluorescence 2D and 3D spectra of tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) measured in physiological solution at 293 *K* are presented. The spectral characteristics of 2D fluorescence spectra of amino acids (AAc) are recorded in the following conditions: for Trp $\lambda_{ex}/\lambda_{em}$=280/357, for Tyr 275/303 and for Phe 257/283 *nm*, and the 3D spectra of AAc recorded in λ_{ex}=200–450 and λ_{em}=200–500 *nm* ranges are given. It has been revealed that 3D spectra of AAc are characterized by two strong excitation/emission peaks located at $\lambda_{ex}/\lambda_{em}$=230/350 and 280/350 *nm*. The high energy emission (absorption) at 230 *nm* is due to $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ transitions of the carboxylic or amide group and low energy emission (absorption) at 280 *nm* is due to $\pi \rightarrow \pi^*$ transition in the indole ring. In 3D spectra Rayleigh scattering peak and second-order scattering peak are identified as well.

Keywords: tryptophan, tyrosine, phenylalanine, fluorescence, 2D, 3D spectra.

Introduction. Fluorescence spectroscopy and its multiple applications to the protein analysis have undergone rapid development during the past twenty years due to new theoretical and technical decisions. Fluorescence spectroscopy methods (steady-state and time-resolved fluorescence, synchronous and excitation emission matrix-EEM, two-dimensional) are widely used for monitoring bioprocesses, protein-ligand interactions, dynamics and kinetics of protein folding reactions [1–7]. The basics of fluorescence spectroscopy and the use of fluorescence spectroscopy methods for chemical and biological systems have been reviewed in many publications [8–11].

This article is not intended as an overview of the main aspects of these publications. It focuses only on the analysis of naturally occurring three amino acids conjugated with aromatic ring: tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) fluorescence 2D and 3D spectra.

Materials and Methods. Chemically pure grade Trp, Tyr and Phe were purchased from "Reanal" (Hungary). Solutions of amino acids (AAc) were prepared using physiological solution at 293 *K*. Concentrations of AAc were: [Trp]= $7.00 \cdot 10^{-6}$, [Tyr] =$1.04 \cdot 10^{-4}$ and [Phe]= $2.3 \cdot 10^{-4}$ $mol \cdot L^{-1}$. Fluorescence spectra were registered on a Varian Cary Eclipse spectrophotometer (Australia). 2D spectra were recorded over the interval λ=260–500 *nm* at an excitation wavelength of 257, 275 and 280 *nm* for Phe, Tyr and Trp respectively. 3D fluorescence spectra were recorded in the following mode: excitation range λ_{ex}=200–450 *nm*; emission range λ_{em}=200–500 *nm*; $\Delta\lambda_{incr}$ =10 *nm*,

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number of scans 26. The inlet and outlet slits were 5 *nm* wide. Quartz cells with *l*=1 *cm* were used for measurements. The reduced diagrams were plotted and analyzed using the ORIGIN 8.0 software.

Results and Discussion.

Analysis of Trp Fluorescence 2D and 3D Spectra. Absorption of Trp is due to $\pi \rightarrow \pi^*$ transitions in the indole ring. The UV/vis spectrum of Trp is characterized by a short wavelength band at 220 *nm* (^1B_b transition) and a long wavelength band at 260–290 *nm*, consisting of the two overlapping transitions (^1L_a and ^1L_b). Sensitivity of Trp to the polarity of environment is due to ^1L_a transition, which is believed to have the main contribution in the emission. The fluorescence 2D spectrum of Trp in aqueous solution is characterized by a wide structureless emission band with a maximum of about 357 *nm* and width 60 *nm* when λ_{ex}=280 *nm*. Shifts in Trp emission are strong as excitation leads to considerable increase in the dipole moment (≈4D), which is the result of orientational relaxation processes of chromophore and solvent dipoles. Such behavior makes Trp fluorescence an important and informative tool for studies of protein structure and dynamics protein and protein–ligand interactions [9]. Fluorescent properties of Trp are more pronounced than both Tyr and Phe. Characteristics of Trp fluorescent spectra depend on the solvent polarity. As the polarity decreases, the spectrum shifts to shorter wavelengths and increases in intensity. For this reason, Trp residues, buried in hydrophobic pockets of folded proteins, exhibit a spectral shift of 10 to 20 *nm*. This phenomenon has been used to study protein denaturation [11]. Alterations in tertiary and secondary structures of proteins can be estimated using fluorescence excitation emission matrix method. Resulting 3D spectra represent fluorescence intensity distribution when the emission spectrum is recorded for all excitation wavelengths of a sample.

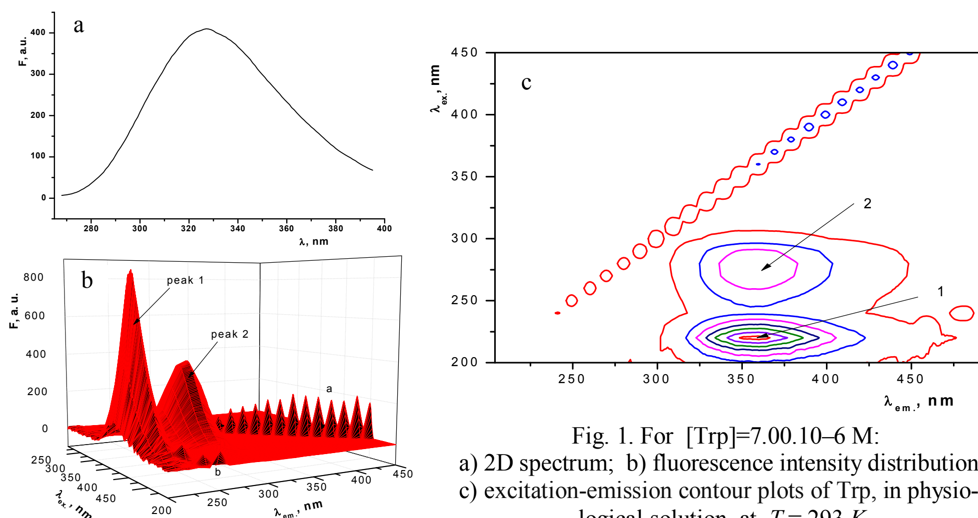

Fig. 1. For [Trp]=7.00.10–6 M:
a) 2D spectrum; b) fluorescence intensity distribution; c) excitation-emission contour plots of Trp, in physiological solution at $T = 293$ *K*.

2D, 3D fluorescence spectra of Trp in aqueous solution and projection of the 3D spectrum in *xy* plane are presented in Fig. 1. 2D spectrum of Trp is characterized by a strong emission at λ_{em}=357 *nm* (λ_{ex}=280 *nm*). Two characteristic excitation/emission peaks for Trp are identified on the fluorescence 3D spectrum, with centers located at $\lambda_{ex}/\lambda_{em}$=220/360 and 280/360 (*nm*). Spectral characteristics of the 3D spectrum in terms of peak position and peak intensities are given in Tab. 1. The fluorescence intensity is much stronger when λ_{ex}=280 *nm*, than λ_{ex}=230 *nm*, and the ratio of fluorescence intensity is

approximately 1 : 2.1. The high energy emission (absorption) at 230 *nm* is due to $n \to \pi^*$ or $\pi \to \pi^*$ transitions of the carboxylic or amide group, and low energy emission (absorption) at 280 *nm* is due to $n \to \pi^*$ transition in the indole ring. Peak "a" ($\lambda_{ex} = \lambda_{em}$) and peak "b" ($2\,\lambda_{ex} = \lambda_{em}$) in the spectrum refer to Rayleigh scattering peak and second-order scattering peak respectively [12]. In KH_2PO_4–NaOH buffer Trp has two strong fluorescence areas: $\lambda_{ex}/\lambda_{em}$ = 220/350 and 230/350 (*nm*) [13].

Table 1

3D fluorescence spectra characteristics of Trp in physiological solution at T = 293K

Peak 1		Peak 2		Rayleigh scattering	
$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.	$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.	$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.
220/360	833.2	280/360	395.8	240/240→450/450	22.7→171.6

Analysis of Tyr Fluorescence 2D and 3D Spectra. Tyr can be excited at wavelengths similar to that of Trp, but emits at a distinctly different wavelength. While Tyr is less fluorescent than Trp, in many proteins it can provide significant signal as it is often present in large numbers. Tyr fluorescence can be quenched by the presence of nearby Trp residues via resonance energy transfer, as well as by ionization of its aromatic hydroxyl group [11].

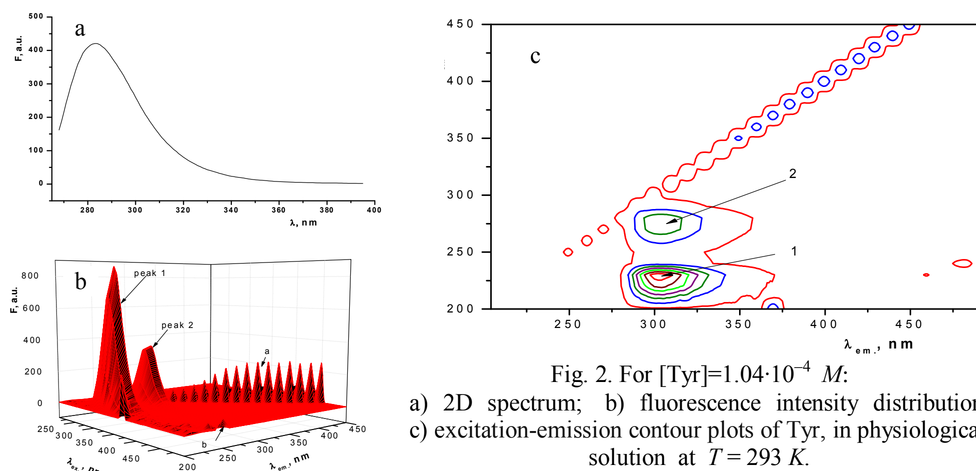

Fig. 2. For [Tyr]=$1.04 \cdot 10^{-4}$ *M*:
a) 2D spectrum; b) fluorescence intensity distribution; c) excitation-emission contour plots of Tyr, in physiological solution at *T* = 293 *K*.

Table 2

3D fluorescence spectra characteristics of Tyr in physiological solution at T=293 K

Peak 1		Peak 2		Rayleigh scattering	
$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.	$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.	$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.
230/305	835.9	280/305	363.2	250/250→450/450	36.3→249.8

2D, 3D spectra and projection of the 3D spectrum in *xy* plane of Tyr are presented in Fig. 2. The 2D spectrum of Tyr is characterized by a strong emission at λ_{em}=303 *nm* (λ_{ex}=275 *nm*). Two characteristic excitation/emission peaks for Tyr are identified on the 3D spectrum: $\lambda_{ex}/\lambda_{em}$=230/305 and 280/305 (*nm*). Spectral characteristics of the 3D spectra in terms of peak position and peak intensities are given in Tab. 2. The fluorescence intensity is much stronger when λ_{ex}=280, than 230 *nm* (1 : 2.3). Rayleigh scattering peak and second-order scattering peak are identified as well. As it

is described in [13], Tyr has three strong fluorescence areas, the center of which are located at $\lambda_{ex}/\lambda_{em}$=202/304 and 220/304 and $\lambda_{ex}/\lambda_{em}$=274/304 (*nm*) in KH_2PO_4–NaOH buffers. Fluorescence intensity at the positions of $\lambda_{ex}/\lambda_{em}$=202/304 and 220/304 (*nm*) restronger, than that of its main peak at $\lambda_{ex}/\lambda_{em}$=274/304 (*nm*).

Analysis of Phe Fluorescence 2D and 3D Spectra. 2D, 3D spectra of Phe and projection of the 3D spectrum in *xy* plane are presented in Fig. 3. The 2D spectrum of Phe is characterized by a strong emission at λ_{em}=283 *nm* (λ_{ex}=257 *nm*). Two characteristic excitation/emission peaks were identified on the fluorescence intensity distribution Figure, with centers located at $\lambda_{ex}/\lambda_{em}$=220/285 and 260/287 (*nm*). The fluorescence intensity of peak 1 ($\lambda_{ex}/\lambda_{em}$=220/285 (*nm*)) is much stronger than that of peak 2 ($\lambda_{ex}/\lambda_{em}$=260/287(*nm*)) on 1:2.5. In KH_2PO_4–NaOH buffer Phe has two excitation wavelengths, the shorter one locates at 210 *nm* and the longer one locates at 260 *nm* [13].

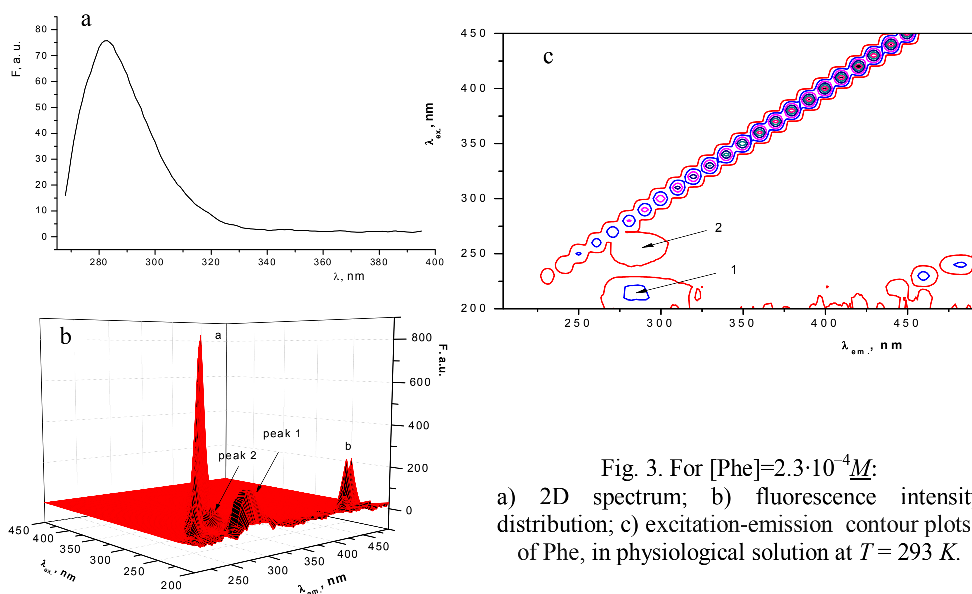

Fig. 3. For [Phe]=$2.3\cdot10^{-4}$*M*:
a) 2D spectrum; b) fluorescence intensity distribution; c) excitation-emission contour plots of Phe, in physiological solution at *T* = 293 *K*.

Table 3

3D fluorescence spectra characteristics of Phe in physiological solution at T=293 K.

Peak 1		Peak 2		Rayleigh scattering	
$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.	$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.	$\lambda_{ex}/\lambda_{em}$, *nm/nm*	F, a.u.
220/285	173.0	260/287	68.4	230/230→450/450	70.7→777.1

The 3D spectra and excitation emission contour plots of Trp–Tyr–Phe mixture (the concentrations of AAc in mixture is the same as in individual AAc solutions) in physiological solution and the intensities of fluorescence peaks are given in Fig. 4 and Tab. 4. From the analysis of fluorescence 3D spectrum of the mixture and 2D spectra of individual AAc it can be concluded that the 4 peaks appearing in 3D spectrum refer to Tyr (peak 1 and peak 2) and to Trp (peak 3 and peak 4). Characteristic peaks of Phe due to low quantum yield and intensity are recovered by the emission of Trp and Tyr. The 3D spectra of proteins, which fluorescence is the result of emitting property of Trp, Tyr and Phe are characterized by two peaks: peak 1 ($\lambda_{ex}/\lambda_{em}$=230/340 (*nm*)) and peak 2 ($\lambda_{ex}/\lambda_{em}$=280/344 (*nm*)) [14].

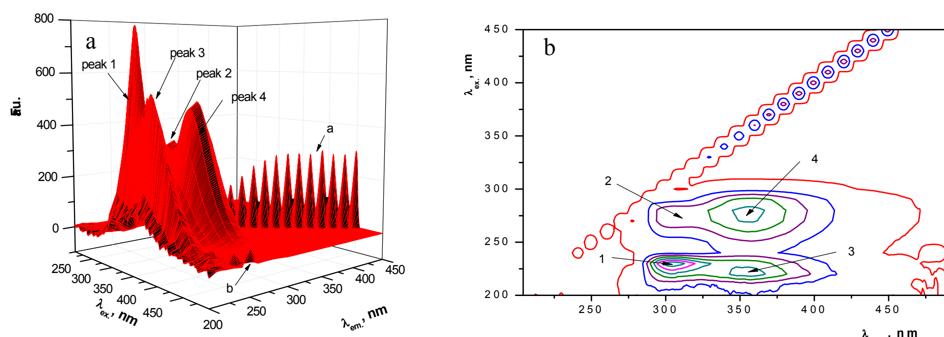

Fig. 4. a) 3D Spectrum (fluorescence intensity distribution); b) excitation-emission contour plots of Trp–Tyr–Phe mixture in physiological solution at $T = 293$ K.

Table 4

3D fluorescence spectra characteristics of Trp–Tyr–Phe mixture in physiological solution at T=293K

Peak 1		Peak 2		Peak 3		Peak 4		Rayleigh scattering	
$\lambda_{ex}/\lambda_{em}$	F, a.u.	$\lambda_{ex}/\lambda_{em}$	F, a.u.	$\lambda_{ex}/\lambda_{em}$	F,a.u.	$\lambda_{ex}/\lambda_{em}$	F, a.u.	$\lambda_{ex}/\lambda_{em}$	F, a.u.
230/305	773.5	280/305	345.2	220/355	528.3	280/357	499.3	240/240, 450/450	23.4→292.8

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REFERENCES

1. **Yuling Y., Gerard M.** Analysis of Protein Interactions Using Fluorescence Technologies. // Current Opinion in Chem. Biol., 2003, v. 7, p. 635–640.
2. **Grigoryan K.R., Ghazaryan A.G.** Characterization of the Platinum(II) Dimetyl Sulfoxide Complex Binding to Bovine Serum Albumin by Fluorescence Spectroscopy Method. // Global J. Anal. Chem., 2012, v. 3, p. 1–9.
3. **Zhang H.** et al. Fluorometric Probing on the Binding of Hematoxylin to Serum Albumin. // Mol. Biol. Rep., 2009, v. 36, p. 2299–2306.
4. **Punith R.** et al. Binding of an Anti-Inflammatory Drug Lornoxicam with Blood Proteins: Insights from Spectroscopic Investigations. // J. Fluoresc., 2011, v. 21, № 2, p. 487–495.
5. **Grigoryan K.R., Markarian S.A., Aznauryan M.G.** Effect of Dimethyl Sulfoxide and Diethyl Sulfoxide on Thermal Denaturation of Human Serum Albumin. // Problems of Cryobiology, 2009, v. 19, № 1, p. 3–9.
6. **Marose S., Lindemann C., Scheper T.** Two Dimensional Fluorescence Spectroscopy: A New Tool for On-Line Bioprocess Monitoring. // Biotechnol. Prog., 1988, v. 14, p. 63–74.
7. **Grigoryan K.R.** Fluorescence Analysis of the Structural Changes of Human Serum Albumin Induced by Low Temperatures in Water–Dimetylsulfoxide Solutions. // Russ. J. Phys. Chem., 2011, v. 85, № 2, p. 372–376.
8. **Permyakov E.A.** Luminescent Spectroscopy of Proteins. Boca Raton: CRC Press, 1993, 164 p.
9. **Ladokin A.S.** Fluorescence Spectroscopy in Peptide and Protein Analysis. // Encyc. Anal. Chem., Chichester: John Wiley & Sons Ltd., 2000, p. 5762–5779.
10. **Brand L., Johnson M.L.** Fluorescence Spectroscopy. San Diego: Academic Press, 1997, 628 p.
11. **Lakowicz J.R.** Principles of Fluorescence Spectroscopy. NY: Kluwer Acad.. Publ., 1999, 698 p.
12. **Zhang Y.Z., Zhou B., Liu Y.X., Zhou C.X., Ding X.L., Liu Y.** Fluorescence Study on the Interaction of Bovine Serum Albumin with *p*-Aminoazobenzene. // J. Fluoresc., 2008, v. 18, p. 109–118.
13. **Yang H.** et. al. Intrinsic Fluorescence Spectra of Tryptophan, Tyrosine and Phenylalanine. 5th Intern. Conference on Advanced Design and Manufacturing Engineering, 2015, p. 224–233.
14. **Grigoryan K.R., Shilajyan H.A.** Study Of Thermal Denaturing Of Human Serum Albumin in the Presence of Potassium Chloride by the Excitation/Emission Matrix Method. // J. Appl. Spectroscopy, 2013, v. 80, p. 786–789.