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Increasing the reliability of the identification by high-performance liquid chromatography by means of selective and/or sensitive detection

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Abstract

An approach for quantitative assessment of the reliability of identification at high-performance liquid chromatography is proposed. The quantitative assessment of identification is useful for determination of selectivity at validation of the analytical methods. Chromatograms and spectra of the analytes are presented as maps in which characteristics as retention times, detector's signals, maxima and minima and another characteristics of spectra are used for identification. A formula for quantitative determination of the contribution of these characteristics on the reliability of identification is given. Using the more selective diode array detector than the convenient UV detector increases the reliability by several orders. A similar result was obtained when the UV detector was replaced with the more sensitive and selective fluorescence detector. Despite of the small contribution of the separation to the reliability its influence is very important for distinguishing of isomers because their spectra are identical.

Keywords: Detection, LC; Selectivity; Tylosin; Closantel; Sulfonamides

1. Introduction

Various data obtained from the chromatographic analysis as retention times, Kovatsh indexes, peak area, UV or MS spectra are used for identification of the analytes. At present MS is the most reliable method for identification. However, there is not yet a quantitative expression for representing the reliability of the identification. It is impossible to determine the increasing of the certainty of identification when we use DAD (diode array detection) instead of UV detection, for example. The quantitation of the reliability of the identification is closely related to the selectivity of the analytical methods. The selectivity is an important criteria of the method validation, which guarantees that the peak of analyte represents only the analyte and no other compounds [1,2]. That is why we propose an approach for the quantitation of the reliability of identification.

2. Theory

Identification in chromatography is realized by comparing of retention times and peak areas of the analyte with those of standard substances. If we represent the chromatograms as maps with r and s strips along the abscissa (retention time) and the ordinate (signal intensity), the probability, $P_{r,s}$, of the peaks fully overlapping and the compounds which they represent being accepted as identical, is

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$$P_{r,s} = \frac{1}{r,s} \quad (1)$$

where r represents the retention time and s is the signal intensity.

The probability $(1-P_{r,s})$ is a measure for distinguishing these compounds. The probability, $P_{r,s}$, means also that two different compounds whose peaks are fully overlapped will be accepted as identical, although in fact they are not.

It is obviously that the increasing the number of r and s strips will increase the reliability of the identification. Recently, the influence of the efficiency of the column on the reliability of the identification of complex mixtures by their characteristic compounds has been quantitatively evaluated [3]. It was shown that doubling of the number of characteristic peaks as a result of a higher efficiency or/and two-dimensional chromatography increases the reliability of the identification by several orders.

The efficiency has been expressed by the peak capacity n_c

$$n_c = 1 + \frac{\sqrt{N}}{4} \cdot \ln(1 + k_{max}) \quad (2)$$

here N is the number of theoretical plates of the separating system and k_{max} is the maximal practically acceptable value of the retention factor.

The probability P^q that an analyte of the complex mixture with a number of compounds q will be eluted as a single compound peak (SCP) and will not overlap other compounds of the mixture [4] is

$$P^q \approx e^{\frac{-2q}{n_c}} \quad (3)$$

This expression give us an opportunity to calculate the number of strips, r, in Eq. (1). If we accept some value of certainty, for example $P=0.95$, that all compounds q will be separated as SCPs we can predict the necessary peak capacity of the column. In this case, because all compounds are separated as single peaks, the peak capacity n_c, will be equal to the number of strips, r, in Eq. (1). The statistical model of overlap [4] was modified by Davis [5,6]. The significance of these works is the overcoming of the restriction that SCPs must be distinguished with constant density through the separation [2] allowing their number to be determined more exactly. The improved method is more precise but the procedure is difficult and time consuming.

The retention of the analyte represents the contribution of its moieties and structure. These characteristics of the analyte exert an influence on the detector's response, too. For example, because the responses of UV detector for aromatic and aliphatic compounds are quite different the s-values in Eq. (1) will be different. The amplitude of the detector's signals is more impressive when specific fluorescence, electrochemical or mass spectrometric detectors are used. These differences of the detector's response give another opportunity for more reliable identification by increasing the number of strips s.

UV–Vis, DAD acquires spectra on-line through the entire chromatogram. The spectra acquired during the elution of a peak are normalized and overlaid for graphical presentation. If the normalized spectra are different, assessed by the peak purity algorithm, the peak consists of at least two compounds. The level of impurities that can be detected depends on the spectra difference, on the detector's performance and on the software algorithm. However, the impurities in the analyte's peaks can be demonstrated if their retention times are different from that of the analyte. These algorithms do not give an opportunity for determination of the uncertainty at the identification by overlaying of UV–Vis spectra.

There is another possibility for distinguishing of the substances by their characteristic spectra. Let us represent the characteristic absorption maxima as maps with n and m strips along the abscissa (wavelength, λ) and the ordinate (absorbency intensity), respectively (Fig. 1). The characteristic absorption maxima and minima, k, are strictly arranged in the map by their wavelengths on the abscissa. We refer to this specific arrangement as a 'finger print' of the analyte (identified object). The overlapping of the pattern spectrum of a 'standard' object with the pattern of the analyzed object means that they are identical in respect to the characteristic apexes and valleys of the spectrum. The number of strips along the abscissa n, is determined by the capability of the detector for separation of the light's spectrum. The number of strips, m, along the ordinate (signal) is determined by the sensitivity of the detector towards

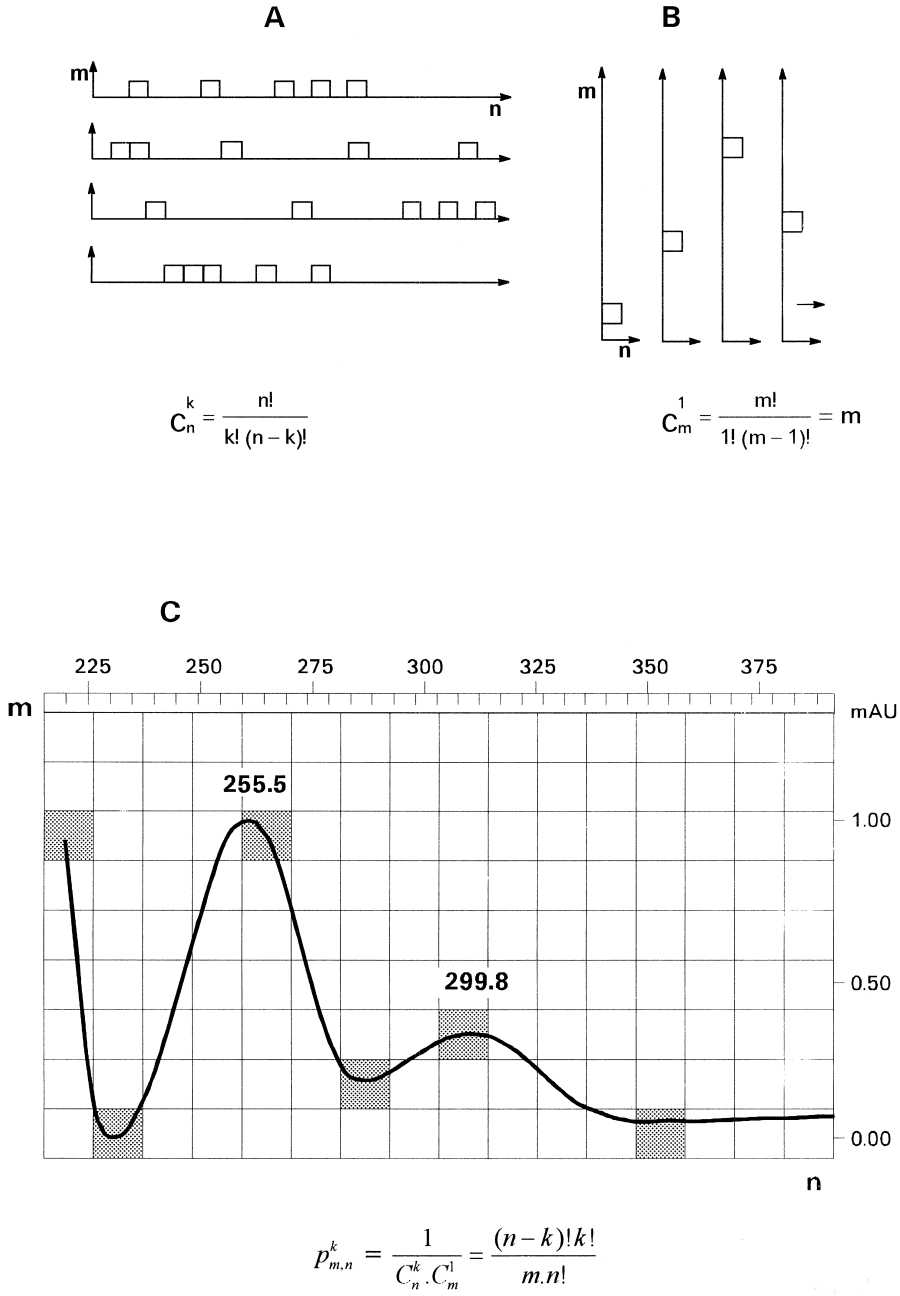

Fig. 1. Combinations of n elements from kth class toward the abscissa (A), combinations of m elements from 1st class toward the ordinate (B) and all arrangements at k number of separated strips (C).

the analytes (absorption at UV detector, excitation and emission at fluorescence detector, etc.).

If we have only one characteristic absorption maximum in the UV spectrum of the analyte the possibility, $P_{n,m}$, the last one to be in any quadrant of the map is

$$P_{n,m} = \frac{1}{n,m} \tag{4}$$

This is a simple mode of detection at the convenient $\lambda = 254$ nm or at any other suitable wavelength at UV–Vis variable wavelength detector.

When the whole spectrum is used all number of segments $n = (\lambda_{max} - \lambda_{min})/\Delta\lambda$ can be used as characteristic parts of the UV spectrum.

The following combinations C_n^k of n elements from kth class exist towards the abscissa for a k number of absorption maxima with progressively increasing wavelength (Fig. 2)

$$C_n^k = \frac{n!}{k!(n-k)!} \tag{5}$$

Towards the ordinate the combinations C_m^l will consist of m elements from 1th class

$$C_m^l = \frac{m!}{l!(m-l)!} = m \tag{6}$$

The number of all arrangements at k number characteristic apexes of the spectrum and n number of separated strips will be

$$C_n^k \cdot C_m^l = \frac{n!}{k!(n-k)!} \cdot m \tag{7}$$

The probability to result in one and the same arrangement in the map of these k points will be

$$P_{m,n}^k = \frac{1}{C_m^l \cdot C_n^k} = \frac{(n-k)!k!}{m \cdot n!} \tag{8}$$

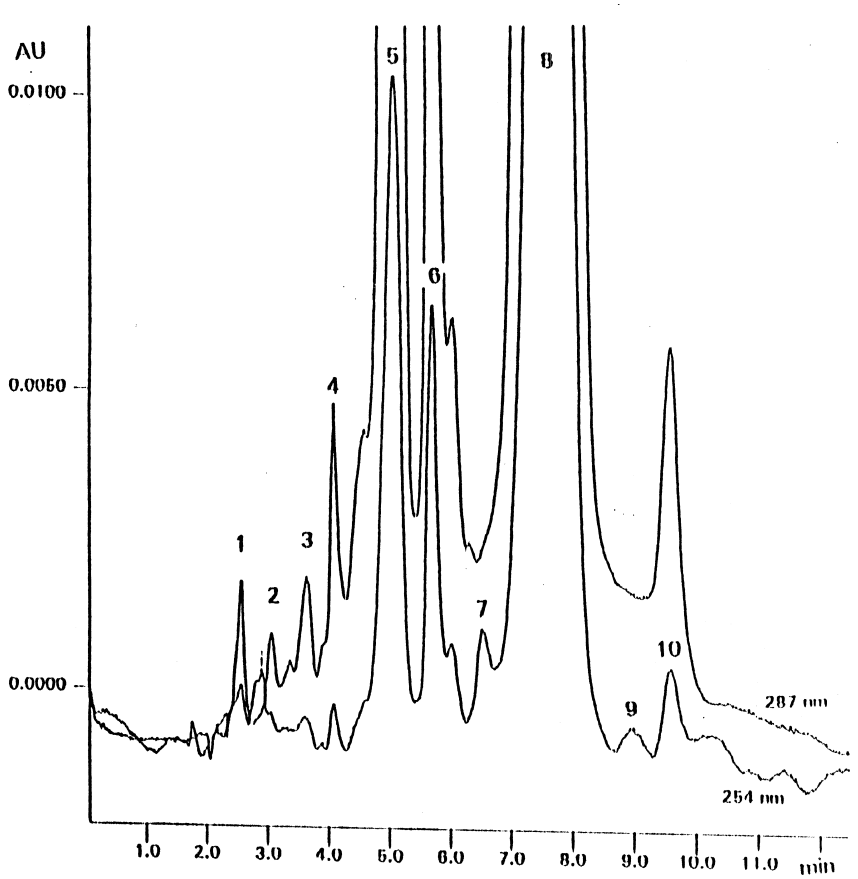

Fig. 2. Chromatograms of tylosin and its homologues, detected at convenient $\lambda = 254$ nm (A) and at the maximal absorption $\lambda = 286$ nm (B), respectively.

This probability is accepted as a threshold value for distinguishing of the analytes by their spectra. Below this value the analytes will be not distinguished, nevertheless some of them are different.

The probabilities $P_{r,s}$ and $P^{k}_{m,n}$ determined by the efficiency of the column and the selectivity and the sensitivity of the detector, respectively, are orthogonal because they are independent [7,8]. That is why the combined probability can be expressed as

$$P_{r,s;m,n} = P_{r,s}P^{k}_{m,n} \quad (9)$$

This value can be used as a quantitative measure at identification of the analytes by a chromatographic system with a DAD.

The aim of this work is a quantitative assessment of the contribution of the sensitivity and selectivity of different detectors for HPLC on the reliability of the identification of the analytes.

In order to test if two numbers (retention times, detector's signals or wave lengths, etc.) are different considering their individual standard deviations, i.e. they can be distinguished, a two-sided *t*-test can be performed. Commonly a 95% confidence level is used and the uncertainty, α, is $(1-95)/100=0.05$. In applying the two-sided *t*-test for the comparison of two means, $\bar{X}_A$ and $\bar{X}_B$, at five runs and looking up in the *t*-test table, the value of the difference, *u*, is then evaluated from:

$$u = 1.03\sqrt{\sigma^2_A + \sigma^2_B} \quad (10)$$

Here σ^2_A and σ^2_B are the variance in each of the values of compounds A and B being compared, respectively. If the absolute value of the difference in the means, $|\bar{X}_A - \bar{X}_B|$, is greater than *u*, then the values are considered different at 95% confidential level, and if $|\bar{X}_A - \bar{X}_B|$ is less than *u*, then X_A and X_B are not distinguishable.

3. Experimental

3.1. Chemicals

Acetonitrile and methanol were HPLC-grade (Merck, Darmstadt, Germany). Dibasic potassium phosphate (Merck), diethylamine, and orthophosphoric acid (Fluka, Buchs, Switzerland) and fluorescamine (EGA, Germany) were analytical-grade quality. The water was purified and deionized by Milli-Q system (Millipore, Milford, MA, USA). The solvents for HPLC were filtered with 0.45-μm filters (Millipore) and degassed by ultrasonic bath. Tylosin, closantel and sulfonamides, used as standard substances, were obtained from Biovet, Pesttera (Bulgaria), Janssen (Belgium) and Sigma–Aldrich (Germany), respectively.

3.2. Apparatus

Two HPLC instruments were used: (1) a Varian LC system consisted of a Star 9012 solvent delivery pump, Polychrom 9065 diode array detector and Varian Star data system; UV–Vis Varian 9050 detector; Rheodyne injector with 100-μl loop and (2) a Waters LC chromatograph consisted of a Waters 600 pump and a injector mod. U6K; MS 991 diode array detector; Waters 470 scanning fluorescence detector and data system with a 991 software.

A Merck analytical column (125×4 mm LiChrospher, 5 μm 100 RP_{18} ODS1) was used in this study. The pH value of the mobile phase was measured with a pH meter Chemcadet (Cole Parmer). The void volume of the chromatographic system was determined by injection an aqueous solution containing sodium nitrate.

4. Results and discussion

Because the aim of this work was the investigation of the influence of the sensitivity and selectivity of different detectors on the identification of the analytes one column with definite efficiency was used for the experiments. In this way the term $P_{r,s}$ in Eq. (1) was constant and the variation in the probability for overlapping, $P_{r,s;m,n}$, was determined only by the detector.

Fig. 2 presents a chromatogram of tylosin and its impurities (nine compounds) detected at $\lambda=254$ nm. Using Eq. (2), an n_c value of 28 was calculated at $k_{max}=30$ and $N=1050$ theoretical plates, i.e., the separation system can separate 28 compounds, statistically arrayed with a resolution $R_S=1.0$. Eq. (3) gave a P^q value of 0.72 at a certainty 0.95. This means the system is able statistically to separate only

one compound as a single compound peak among ten compound of the sample and the number of strips r in Eq. (1) is only one. The probability that all ten compounds will be separated as SCPs at this efficiency is 0.49 and statistically the r value in Eq. (1) will be 13, ($0.49\times28=13.7\rightarrow13$). All peaks, ($q=10$), will be obtained as SCPs at $n_c=390$ which necessitates

$N=2.16\cdot10^5$ theoretical plates (at $k_{max}=30$) or

$k_{max}=2.46\cdot10^{21}$ (at $N=1050$)

These tremendous values of efficiency and capacity factor are unpracticed and another approach for distinguishing of the compounds should be used for their reliable identification.

Because the reproducibility of the peak high ($\bar{X}_{254}=62$ mAU) of Tyson was 4.8%, a u value of 7.0% or 4.33 mAU was calculated by Eq. (10). Using this result the s value of fully separated strips along the ordinate was 14, ($s=62/4.33=14.3\rightarrow14$). The probability that the peak of Tyson will not be distinguished among the other compounds determined at $\lambda=254$ nm by Eq. (1) was $2.6\cdot10^{-2}$ ($P_{13,14}^{\lambda=254}=1/13\times14=5.5\cdot10^{-3}$).

A variable wavelength UV detector gives an opportunity for detection at the apex of the maximum absorption in the analyte's spectrum. This allows a bigger discrimination of peak heights to be obtained than this one at $\lambda=254$ nm and reflects a higher s value and more reliable identification. At $\lambda_{apex}=286$ nm, (Fig. 2), the mean value of the peak height, $\bar{X}$, was 250 mAU and the s value of 57 was calculated, ($s=250/4.33=57.7\rightarrow57$). In addition, at $\lambda=286$ nm peaks nos. 7 and 9 disappear and obviously they are not isomers of tylosin. Because the number of compounds which had to be distinguished is $q=8$ and the probability of each one being separated as a SCP was 0.56 (Eq. (3)) the r value was 15, ($28\times0.56=15.8\rightarrow15$). The probability of overlapping of tyson with the other substances was $P_{15.57}^{\lambda=286}=1/15\times57=1.2\cdot10^{-3}$. This value is 45 times lower than the probability of the detection at $\lambda=254$ nm and means that the distinguishing of tylosin is more reliable because the sensitivity of the detector at $\lambda=286$ nm is approximately four times higher than this one at the universal $\lambda=254$ nm.

Besides the reached higher sensitivity the variable wavelength UV–Vis detector possesses higher selectivity that at $\lambda=254$. When we scrutinize the UV spectrum as a map, (Fig. 1), the probability of the analyte's absorption maximum being in any quadrant is

$$P_{n,m}=1/nm=1/[(\lambda_{max}-\lambda_{min})/u_\lambda\cdot A/u_A] \qquad (11)$$

Here ($\lambda_{max}-\lambda_{min}$) is the bandwidth of the detector and u_λ is the wavelength distinguishing (for modern instrument u_λ is $1.5\div5$ nm); if detector operates in the range 200–365 nm, $n=82$, $(365–200)/2=82.5\rightarrow82$. The absorption A is expressed by the normalized peak height (1.00), usually measured at the wavelength of maximum absorption, and u_A is the value of distinguishing of the signal. In the case of tylosin at $\lambda=286$ nm, the A/u_A value is equal of the s value of 57. The probability that the apex at $\lambda=286$ nm will be in a quadrant $n=43$[(286–200)/2] and $m=57$[1.00/(0,0175)=57.7→57] is: $P_{n,m}=1/82\times57=2.1\cdot10^{-4}$. This value expresses the probability that the absorption apex of another unknown compound being in the same quadrant and will be accepted as tylosin. The combined probability of distinguishing of tylosin by the chromatographic separation and the UV-variable detector, $P_{r,s;m,n}=P_{r,s}\times P_{n,m}$ is $1.2\cdot10^{-3}\times2.1\cdot10^{-4}=2.5\cdot10^{-7}$. The distinguishing is $2.2\cdot10^3$, ($5.5\cdot10^{-3}/2.5\cdot10^{-6}=2.2\cdot10^3$) times more reliable than that of detection with fixed wavelength.

A single chromatographic analysis by HPLC using a fixed-wavelength detector yields data specific to that wavelength but ignores the rest of the dispersed radiation generated throughout the electromagnetic spectrum. DAD gives the unique possibility to acquire information of the absorption of the analyte through the entire UV–Vis spectrum [9]. All parts of the spectrum, represented as k value in Eq. (8), can be used for distinguishing of the analytes. For example, when we use the maxima and minima in the spectrum, represented in Fig. 1, the k value is 6, and if the reproducibility of the chromatographic system determines values of $n=100$, [$(\lambda_{max}-\lambda_{min})/u_\lambda=200/2$)] and $m=50$, ($A/u_A=1.00/0.02$) the probability for coincidental overlaying of this spectrum with another one will be $P_{m,n}^k=P_{50,100}^6=(100-6)!6!/(50\cdot100!)=1.7\cdot10^{-11}$. When we use only one characteristic part, for example the highest

apex of the spectrum, then Eq. (8) is equal to Eq. (4).

Doubling the number of the k value and/or the number of strips n and m as a result of higher resolution, larger bandwidth or higher reproducibility of the signal increases the reliability of distinguishing of spectra by several orders. Values of $n = 41$, $[(366–200)/4 = 41.5]$ and $m = 22$, $(1.00/0.045 = 22)$ were determined with Varian 9065 polychrom DAD. When sulfonamides were analyzed and ten characteristic points of their UV spectra, $(k = 10)$ were used, the probability $P^{10}_{41,22}$ was $4.1 \cdot 10^{-11}$. The values of $n = 400$, $[(800–200)/1.5]$ and $m = 28$, $(1.00/0.036)$, were obtained when the same compounds were analyzed with Waters 991-MS DAD and probability was $P^{10}_{28,400} = 5.4 \cdot 10^{-19}$. Comparing these probability shows that increasing of n and m values does the reliability of identification $7.6 \cdot 10^{-9}$ times more reliable.

The sensitivity of the fluorescence detector is several orders higher than that of the UV detector. This fact can also be used for increasing the reliability of the identification. Let detector excite an analyte, for example, in the wavelength range $\lambda^{min}_{ex} = 300$ nm and $\lambda^{max}_{ex} = 420$ nm, and emits up to $\lambda^{max}_{em} = 600$ nm. Because of the dispersed radiation λ_{em} is always at least with 10 nm longer than λ_{ex}. If λ^{min}_{ex} is 300, λ^{min}_{em} will be 310 and at $\Delta\lambda = 5$ nm the number of distinguished λ_{em} will be 310, 315, . . . , 600 nm or $(600 - 310)/5 = 58$. When λ_{ex} is 305 this number will be $(600 - 315)/5 = 57$ and the detector will distinguishes the analytes at 57 different λ_{em}. The sum of all combinations is represented by the formula:

$$\sum_{i=0}^{j} \frac{\lambda_{max} - [(\lambda_{min} + 10) - i\Delta\lambda]}{\Delta\lambda} \quad (12)$$

where $j = \left[\frac{\lambda_{max} - (\lambda_{min} + 10)}{\Delta\lambda} \right] - 1$

The number of all distinguished λ_{em} when λ_{ex} increases with 5 nm in the interval 300–600 nm will be the sum of these combinations and in this case it is 1508. This value means that the fluorescence detector is able to work at 1508 combination of (λ_{ex}, λ_{em}) and by them it can distinguish analytes with a probability $P^{Fl}_{ex,em} = 1/1508 = 6.6 \cdot 10^{-4}$. On modern fluorescence detectors with $\Delta\lambda = 2$ the number of combinations (10582) is considerably higher.

Fig. 3 presents chromatograms of closantel (500 μg/l), obtained with UV detection at $\lambda_{max} = 270$ nm (A), and with fluorescence detection at $\lambda_{ex} = 335$ nm

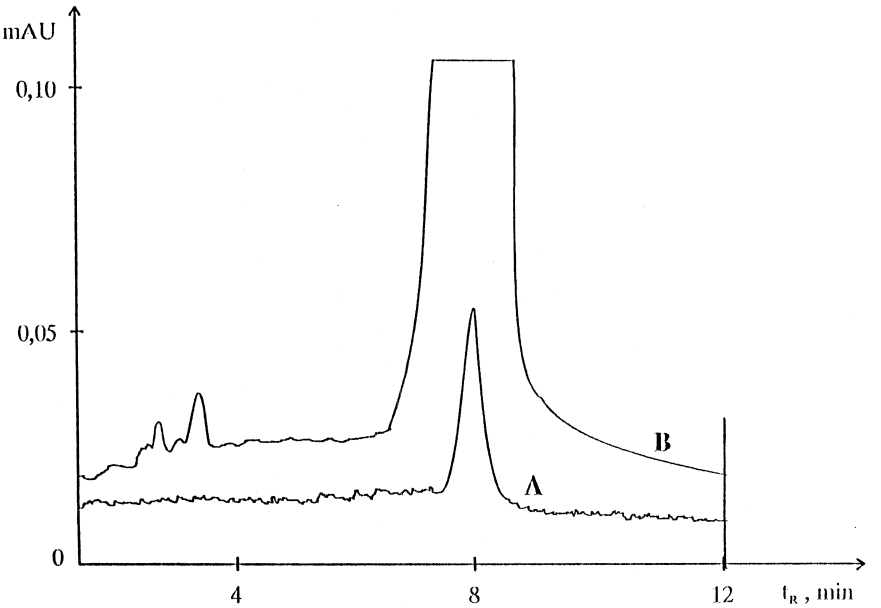

Fig. 3. Chromatogram of closantel (500 μg/kg) obtained by UV detection at $\lambda_{max} = 275$ nm (A) and fluorescence detection/$\lambda_{ex} = 335$ nm; $\lambda_{em} = 510$ nm (B).

and $\lambda_{em}=510$ nm (B). UV and fluorescence detectors were connected in cascade. The peak capacity of the separation system at $N=1350$, $k_{max}=24$, (12 min/0.5 min), was 30. If the number of compounds in the sample q is 10, the probability all of them being separated as SCPs, P^q, is 0.51 and the number of strips r in Eq. (1) is 15, ($30\times0.51=15.4$). The UV detector ($\lambda_{max}=270$ nm) yielded a S/N ratio of 10 and the peak's high was 0.04 mAU with 12% reproducibility. Because it was impossible a lower concentration of closantel to be quantitatively determined the s value was 1 and $P^{UV}_{r,s}$ was $6.2\cdot10^{-2}$. We accepted this signal of the UV detector as a unit for comparison of the signal of the fluorescence detector. The signal of the last one was 0.512 mAU, i.e. the sensitivity was 320 times higher than that of the UV detector. This sensitivity gave an opportunity for a quantity of closantel, which was 320 times smaller than that detected by the UV detector, to be determined and 320 different concentration to be distinguished, i.e. the s value was 320 and $P^{Fl}_{r,s}=2.1\cdot10^{-4}$, $P^{Fl}_{320,15}=(1/320\times15)$. Taking into account of the $P^{Fl}_{ex,em}$ value, the probability of distinguishing of closantel by fluorescence detector was $P^{Fl}_{r,s}\times P^{Fl}_{ex,em}=1.3\cdot10^{-7}$. This value means that the fluorescence detector increases $4.8\cdot10^{5}$ ($6.2\cdot10^{-2}/1.3\cdot10^{-7}$) times the reliability of distinguishing of closantel by its concentration in the sample.

Despite the high reliability of identification gained by using a UV–Vis variable detector, fluorescence

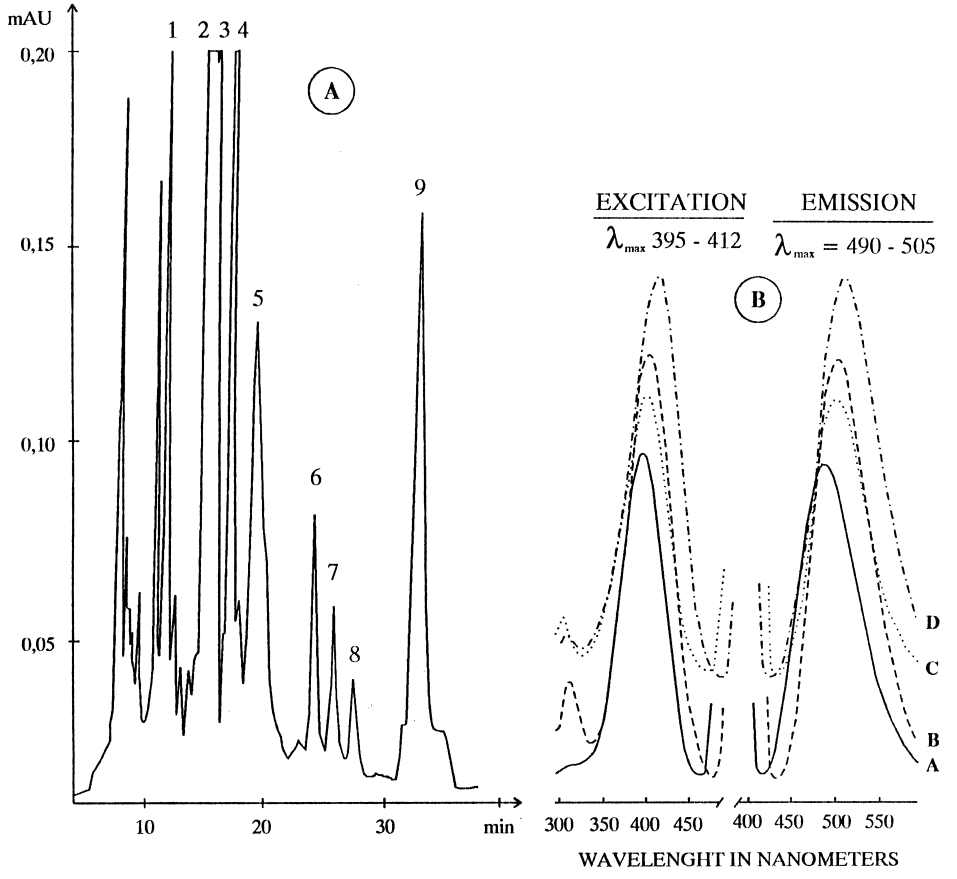

Fig. 4. (A) Chromatogram of meat sample containing residues of sulfonamides: 1=sulfanilamide (11 μg/kg), 2=sulfadiazine (24 μg/kg), 3=sulfadimidine (13 μg/kg), 4=sulfamethylpyridazine (15 μg/kg), 5=sulfachloropyridazine (8 μg/kg), 6=sulfadoxine (4 μg/kg), 7=sulfamethoxazole (3 μg/kg), 8=sulfadimetoxine (1 μg/kg), 9=sulfaquinoxaline (18 μg/kg). (B) Excitation–emission spectra of some fluorescamine derivatives, (20 μg/kg): A=sulfanilamide, B=sulfadimidine, C=sulfadoxine, D=sulfadimetoxine.

Table 1
Values of the uncertainty described in the paper of the identification at the detection with UV, DAD and fluorescence detectors in HPLC analysis

n_c	P^{10} [a]	r [b]	UV fixed $P_{r,s}$ [c] $u=7\%$; $s=14$ [d]	UV (variable-wavelength)				DAD				Fluorescence detection	
				$P_{r,s}$ $u=7\%$; $s=57$ [e]	200–365 nm, $n=82$ [g]		200–900 nm, $n=350$		200–365 nm, $n=41$ [h], $m=22$ [i]		200–800 nm [j], $n=400$ [h], $m=28$ [k]		$\lambda_{min}=300$, $\lambda_{max}=600$	
					$P_{m,n}$ [f]	$P_{r,s}\times P_{m,n}$	$P_{m,n}$	$P_{r,s}\times P_{m,n}$	$P^k_{m,n}$	$P_{r,s}\times P^k_{m,n}$	$P^k_{m,n}$	$P_{r,s}\times P^k_{m,n}$	$\Delta\lambda=5$ [l]	$\Delta\lambda=2$ [m]
$n_c=20$ at: $k=30$; $N=530$ $k=60$; $N=342$	0.37	7.4→7	$7.1\cdot10^{-3}$	$2.5\cdot10^{-3}$	$2.1\cdot10^{-4}$	$5.2\cdot10^{-7}$	$5.0\cdot10^{-5}$	$1.2\cdot10^{-7}$	$k=5$ $6.1\cdot10^{-8}$ $k=10$ $4.1\cdot10^{-11}$	$1.5\cdot10^{-10}$ $1.0\cdot10^{-13}$	$k=5$ $4.3\cdot10^{-13}$ $k=10$ $5.4\cdot10^{-19}$	$1.1\cdot10^{-15}$ $1.3\cdot10^{-21}$	$P^{fl}_{320,7}\times P^{fl}_{ex,em}=$ $3.0\cdot10^{-7}$	$P^{fl}_{320,7}\times P^{fl}_{ex,em}=$ $4.3\cdot10^{-8}$
$n=28$ at: $k=30$; $N=1050$ $k=60$; $N=989$	0.49	13.7→13	$5.5.10^{-3}$	$1.2\cdot10^{-3}$	$2.1\cdot10^{-4}$	$2.5\cdot10^{-7}$	$5.0\cdot10^{-5}$	$6.0\cdot10^{-8}$	$k=5$ $6.1\cdot10^{-8}$ $k=10$ $4.1\cdot10^{-11}$	$7.3\cdot10^{-11}$ $4.9\cdot10^{-14}$	$k=5$ $4.3\cdot10^{-13}$ $k=10$ $5.4\cdot10^{-19}$	$5.2\cdot10^{-16}$ $6.5\cdot10^{-22}$	$P^{fl}_{320,15}\times P^{fl}_{ex,em}=$ $1.3\cdot10^{-7}$	$P^{fl}_{320,24}\times P^{fl}_{ex,em}=$ $2.0\cdot10^{-8}$
$n=40$ at: $k=30$; $N=2070$ $k=60$; $N=1438$	0.61	24.4→24	$3.0\cdot10^{-3}$	$7.3\cdot10^{-4}$	$2.1\cdot10^{-4}$	$1.5\cdot10^{-7}$	$5.0\cdot10^{-5}$	$3.7\cdot10^{-8}$	$k=5$ $6.1\cdot10^{-8}$ $k=10$ $4.1\cdot10^{-11}$	$4.5\cdot10^{-11}$ $3.0\cdot10^{-14}$	$k=5$ $4.3\cdot10^{-13}$ $k=10$ $5.4\cdot10^{-19}$	$3.1\cdot10^{-16}$ $3.9\cdot10^{-22}$	$P^{fl}_{320,15}\times P^{fl}_{ex,em}=$ $8.6\cdot10^{-8}$	$P^{fl}_{320,24}\times P^{fl}_{ex,em}=$ $1.2\cdot10^{-8}$

[a] Necessary probability all ten compounds to be separated.
[b] $r=n_c\times P^{10}$ $(q=10)$.
[c] $P_{r,s}=1/r,s$.
[d] $s=[62\ \text{mAU}/(62\ \text{mAU}\times 0.07)]=14.3\rightarrow 14$.
[e] $s=[250\ \text{mAU}/(62\ \text{mAU}\times 0.07)]=57.7\rightarrow 57$.
[f] m is equal to s at UV-variable detection ($m=57$).
[g] $n=(\lambda_{max}-\lambda_{min})/u_\lambda=(365-200)/2=82.5\rightarrow 82$.
[h] $n=(\lambda_{max}-\lambda_{min})/u_\lambda=(365-200)/4=41.5\rightarrow 41$.
[i] $m=1.00/0.045=22$.
[j] $n=(\lambda_{max}-\lambda_{min})/u_\lambda=(800-200)/1.5=400$.
[k] $m=1.00/0.036=28$.
[l] $P^{fl}_{ex,em}=6.6\cdot10^{-4}$.
[m] $P^{fl}_{ex,em}=9.5\cdot10^{-5}$.

detector or DAD, a probability exists that analytes with one and the same chromophores or fluorophores will not be distinguished. For example, because the compounds in Fig. 2 are isomers of tylosin, they have the same UV spectra, which results in the same m, n, s and k values of the probability $P_{r,s;m,n}$ (Eq. (8)). In this case the only way for identification is chromatographic separation combined with UV spectra. We have already seen that the column gave an probability $P^{10} = 0.49$ for distinguishing of all compounds of mixture, ($q = 10$). This contribution to the probability $P_{r,s;m,n}$ accomplishes the identification of isomers. In the case of determination of sulfonamides's residues as fluorescamine derivatives in biological samples the fluorescence detector, despite its high sensitivity and selectivity, was not able to distinguish the individual sulfonamides because their excitation and emission were very close [10]. In this case the chromatographic separation was indispensable for distinguishing of fluorescamine derivatives, (Fig. 4) and the contribution of the column expressed by the probability sulfonamides to be separated as SCPs was very valuable.

For the practice the uncertainty $P_{r,s} = 5.5 \cdot 10^{-3}$, which we obtained at the chromatographic separation and UV detection at 254 nm, means that tylosine will be distinguished among 5500 combination of compounds with different retention times and/or absorption at 95% confidence level. The number of combinations among which tylosine can be distinguished is $5.4 \cdot 10^{-19}$ when DAD is used (Table 1). It means that this analyte will be recognized among much more substances with different retention times and/or UV adsorption. That is why the probability $P_{r,s;m,n}$ can be used as a measure of the selectivity of the analytical methods. If the number of the substances in the analyzed sample is known it is easy the analyst to take a decision by this probability that the applied method is selective. However in the practice these cases are very rarely. Industrial, environmental and biological sample are very complicated and usually the number of compounds is not determined. More over, every day chemists discover new substances of the nature and their number continually grows. This uncertainty is a barrier at applying of $P_{r,s;m,n}$ and exactly determination of the selectivity is impossible. But, remarking the value of $P_{r,s;m,n}$ as a criteria for the selectivity does the method more reliable and increase its raggedness. Of course, future experiments and discussions are necessary before the application of this parameter for assessment of the selectivity of the chromatographic methods.

5. Conclusion

The contribution of HPLC detectors with different selectivity and sensitivity on identification of the analytes has been quantitatively estimated. Chromatograms and spectra of analytes are presented as maps by their characteristic: retention times, intensity of the detector's signal, maxima and minima of the spectra, etc., and a formula for distinguishing of them is proposed. Reliability of the identification depends on the specificity of the detector's signal: UV absorption, UV or fluorescence spectra, etc. The increased sensitivity renders more reliable identification because the detector distinguishes large interval of analyte's concentrations. Doubling the number of characteristic parts of the spectra, number of strips in the map as a result of higher resolution, larger bandwidth or higher reproducibility of the detector's signal increases the reliability of identification by several orders. Despite of the small contribution of the separation its influence on the reliability of identification is very important at distinguishing of isomers because their spectra are identical.

References

[1] Validation of analytical methods for food control, in: A Report of Joint FAO/IAEA Expert Consultation, 2–4 December 1997, Vienna, Austria, Food and Agriculture Organization of the Nations, Rome, 1998.
[2] L. Huber, LC·GC 4 (1997) 1.
[3] G. Stoev, J. Chromatogr. A 845 (1999) 3.
[4] J.M. Davis, J.C. Giddings, Anal. Chem. 55 (1983) 418.
[5] J.M. Davis, Anal. Chem. 66 (1994) 735.
[6] J.M. Davis, Chromatographia 44 (1997) 81.
[7] Z. Liu, D.G. Patterson Jr., Anal. Chem. 67 (1995) 3840.
[8] F. Cuesta Sanchez, J. Toft, B. van den Bogaert, D.L. Massart, Anal. Chem. 68 (1996) 79.
[9] H.-J. Sievert, A.C. Drouen, in: L. Huber, S. George (Eds.), Diode Array Detection in HPLC, Marcel Dekker, 1993, pp. 51–127.
[10] J. Arthur, F. de Silva, N. Strojny, Anal. Chem. 47 (1975) 714.