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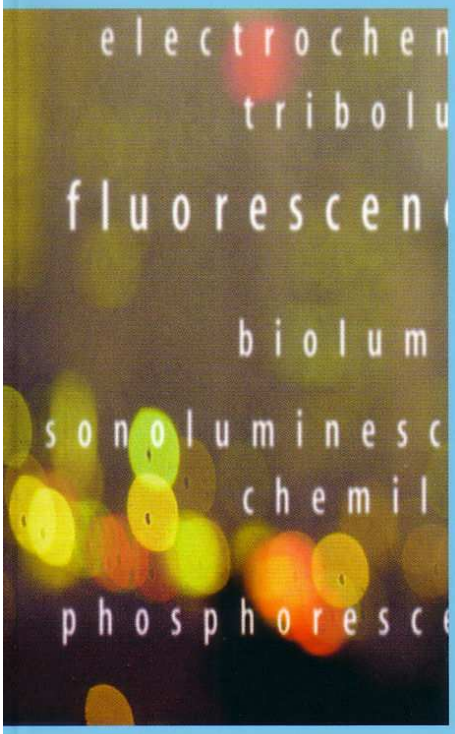

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Direct simultaneous determination of two analgesics in serum

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A direct method for the determination of a mixture of two anti-inflammatory drugs (diflunisal and salicylic acid) in human serum is proposed. A method for the determination of these compounds in serum by the combination of synchronous fluorescence and partial least squares multivariate calibration was developed by Muñoz de la Peña *et al.* (1). In this method, a separation of the compounds from the fluorescence matrix was required. Simultaneous determination in serum can not be carried out by conventional synchronous fluorescence spectrometry due to the high spectral overlap of the analytes and the matrix. This limitation can be avoided by the use of matrix isopotential synchronous fluorescence (MISF); this technique allows the spectral interference of the matrix to be removed. MISF spectra are obtained by joining points of equal intensity on the isopotential trajectory in the three-dimensional fluorescence spectrum from a serum dilution. The trajectory is the portion of the line that passes by the fluorescence maxima of both compounds, ensuring a sensitivity level similar to that of a direct determination in absence of background fluorescence.

The isopotential trajectory and MISF spectra were obtained using a home-made program called FTOTAL, developed by the authors (2). This program allows a total luminescence analysis of any fluorescent compound to be performed by plotting the total luminiscence three-dimensional spectra as an isometric projection or as a contour map. The main advantage of this program is the possibility of generating any bidimensional spectra from any trajectory in the total luminiscence spectrum. These spectra can also be subjected to arithmetic operations, such as derivation or smoothing.

The simultaneous determination was not feasible with the MISF technique only, so its first derivative must be calculated and the results were obtained by applying the zero-crossing technique. Chemical variables were studied and optimized in order to ensure the best possible measurement conditions, maximum fluorescence intensity and adequate selectivity. Analysis was carried out in water using a pH of 7.2 provided by 0.1 mol/L sodium dihydrogen phosphate buffer. Serum samples were diluted 100 times and provided linear calibration plots at diflunisal and salicylic acid concentrations up to 800 ng/mL.

As can be seen in Fig. 1, the typical fluorescence maxima of diflunisal and salicylic acid fall in the UV region, where serum exhibits a broad peak that precludes determination of the analytes without prior separation. In addressing a fluorimetric determination in serum, it is preferable to quantify the analytes at high wavelengths. Based on selectivity,

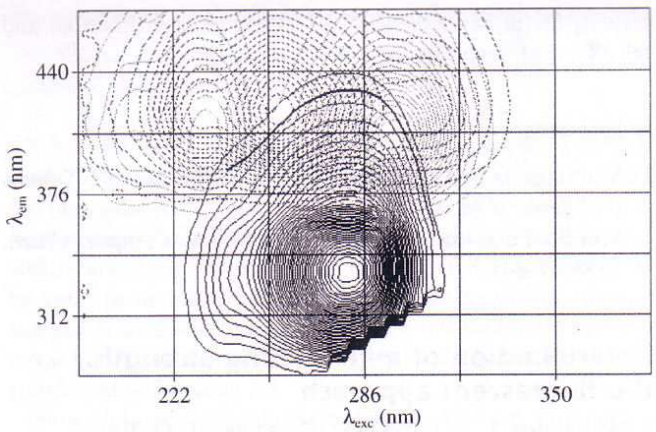

Figure 1. Total fluorescence spectra of the two analytes (diflunisal, broken grey line; and salicylic acid, continuous grey line) in human serum (black line), and the isopotential trajectory.

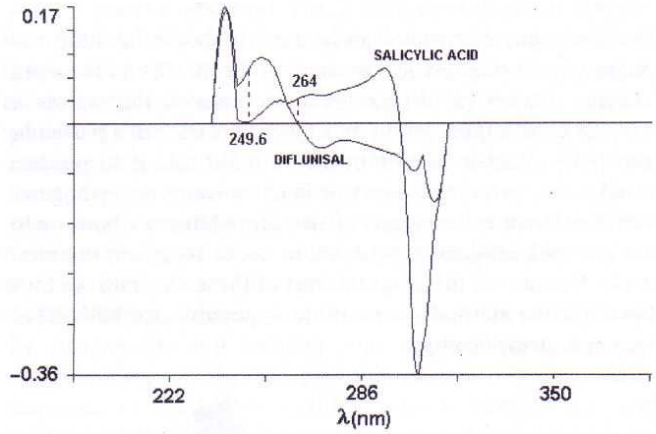

Figure 2. First derivative MISF spectra of salicylic acid (600 ng/mL) and diflunisal (600 ng/mL).

sensitivity and reproducibility criteria, the bands of characteristic wavelengths $\lambda_{exc} = 258$ nm, $\lambda_{em} = 422$ nm and $\lambda_{exc} = 296$ nm, $\lambda_{em} = 408$ nm were selected to determine diflunisal and salicylic acid, respectively, for the following reasons:

- They resulted in an increased fluorescence intensity for salicylic acid.
- They decreased the fluorescence background of serum, thereby diminishing the internal filter of the matrix.
- The contour lines for serum were further apart, which minimizes the differences of the serum samples when the MISF technique is applied.
- Some interferences were lessened.

The goodness of the analytical signal was checked by using variance analysis. Signals recorded throughout the calibration range were subjected to three calibrations for each analyte in both the absence and presence of variable amounts of the other analyte.

Differences between individual calibrations and slopes were compared with those within individual calibrations. Based on the results, diflunisal and salicylic acid can be accurately quantified in the presence of each other. The limits of detection, calculated according to Clayton, who uses error propagation throughout the calibration curve and a non-centralized

security factor, were 36.84 and 37.35 ng/mL for diflunisal and salicylic acid, respectively.

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Determination of milk clotting strength: the fluorescent approach

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Proteases are enzymes widely used in both the food and pharmaceutical industries, accounting for ca. 60% of the world enzyme market (1). Proteases have revealed themselves as very specific in their action on a given substrate in a particular site. This effect is essential, since a main role is to produce other active proteins from their inactive states or 'zymogens'. Another main role consists of the degradation of both endogenous and exogenous proteins in order to generate amino acids. Because of the great interest of these enzymes we must have reliable methods, as accurate as possible, for both detection and quantification.

Several ways have been proposed to detect these activities, mainly based on the disappearance of the substrate or the generation of by products. The substrates may be either the preferential substrate for a given protease or chemical analogues that exhibit similarities to the natural substrates as far their linkage are concerned. One of the most active fields in food science and technology concerns the cheese-making industry. The kinetics of milk-clotting formation has been studied since the 1970s. Because of the changes in the mechanization and robotization in the industry, we must use precise and accurate methods for determining the milk clot strength of a given rennet. Milk-clotting proteases act on the soluble portion of caseins, kappa-casein, thus originating an unstable micellar state that results in clot formation (2).

The first stage of milk clotting consists of a first-order reaction following Michaelis–Menten kinetics (3). This reaction is, in fact, the limiting step in cheese making and is normally conducted by chymosin (E.C. 3.4.23.4), considered to be one of the best aspartic proteases for milk clotting (1). This enzyme hydrolyses the linkage Phe_{105}–Met_{106} in the kappa-casein chain, leading to milk clot formation.

The classic milk-clotting assay consists of determination of the time elapsed until the milk clot appears. Because milk clotting is sensitive to pH and temperature (2), other methods, such as haloes formation in agar milk, colorimetric determination or the determination of the degree of hydrolysis of previously labelled molecules of casein, have been employed. Recently a new method has been proposed (4) (Fig. 1) in order to facilitate this task. The method uses FITC-labelled

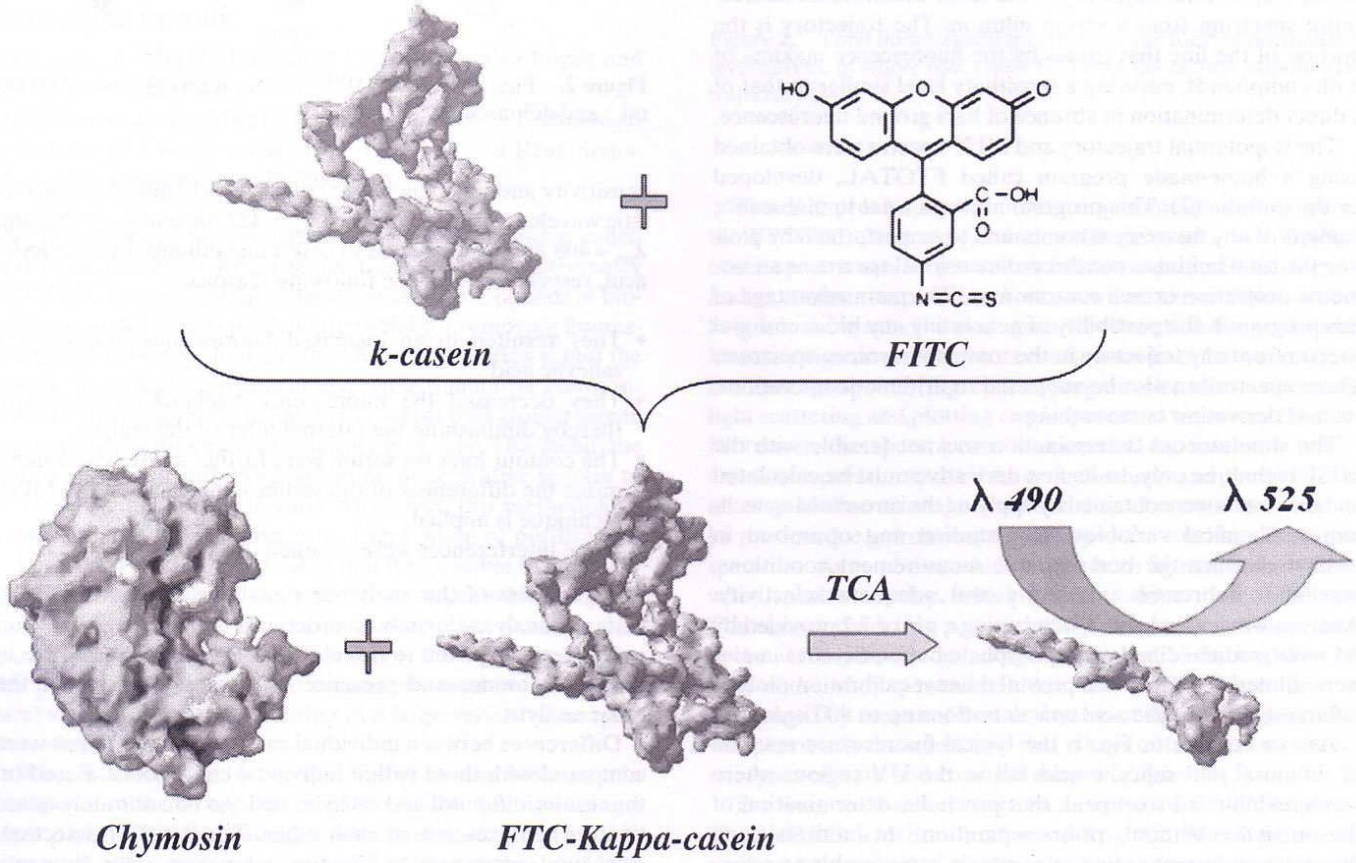

Figure 1. FTC–kappa-casein assay scheme.