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in the late 1980's, wang, mcclements, powers and smith described a fractionation technique in which the actual particle fractionation is carried out off-line by a second chromatographic column (called adsorption disk or sax) loaded with adsorbent particles. afterwards the elution profile is used to correct and scale the data from the on-line detector [43]. the method is robust, simple and rapid, but results in a single line for all sizes, so it becomes difficult to evaluate the distribution of the particle sizes. in addition, it requires the use of a semipermeable column for the adsorbent particles. an alternative fractionation process called by schubert in 1986 was based on the combination of chromatography and electrophoresis, in which a sample is injected and fractionated in a strong eluent by one chromatographic channel, subsequently fractionated in a weak eluent by the opposite channel, and then analysed by laser scattering [100]. the method is simple, fast and quantitative. unfortunately, it has the limitation that it can distinguish between two equally good particles only if they have different volumes, which is not always the case, so it was not considered to be a truly online analysis tool. this method was further improved, for example, by separating a mixture of pvp and polyamides (pvp-am) into three fractions: pvp in the first, am in the second and pvp-am blend in the third [101]. as the mobile phase flows through the channel, solutes are separated according to their size, as opposed to being separated by a stationary phase. this technique has the peculiarity of being capable of fractionating particles with very different hydrodynamic radii, up to an order of magnitude, and has already found interesting applications in biology [128]. the eaf technique allows the simultaneous determination of both size and surface charge of particles, which has also found important applications in nanomedicine [116]. the fff technique was first reported in 1994 [135] and underwent more than 30 years of research, mainly focused on the improvement of its resolution. during these years, the most common variation of the technique has been the modification of the channel. the earliest channels were tapered, with the width decreasing to zero at the center of the channel [135]. then, the channels got larger at the center, but without modifying the tapered shape [136], or the channel started with the center set at a distance from the channel's walls [137]. the first parameter that was modified was the cross-flow rate [138], which is related to the diameter of the channel, and allowed the resolution to be increased by about a factor of ten [139]. then, the eluent velocity, or equivalently the cross-flow rate, was modified [140] and the wall was replaced with a segmented membrane [141]. in this channel, even larger resolutions were achieved, but with a loss of the focus of the channel, due to the multiple segments. instead, a modification of the channel that increases the size of the focusing, without affecting the resolution, is the so-called "slot-outlet" technology. this channel is illustrated in fig. 2. once the focusing process has been completed, the eluent flows continuously. in the center of the channel, there is a serpentine-shaped slot-outlet that allows a variable transverse flow, varying from zero at the side of the channel to a limit determined by the length of the slot-outlet. this setting allows maintaining the focusing process, while reducing the sample dilution in an order of magnitude. the introduction of the flow-diverter, first reported by staudenmayer et al. [143] is another advancement in fff that allows the use of extremely high cross-flow rates, even up to 25 l/h. however, in cases where flow-diverters are not available, other modifications of the channel are worth to be considered. for instance, the parallel flow channels, reported by derencin et al. [129] provide a faster elution with reduced dilution of the solutes. finally, the parallel flow channels shown by ratajczak et al. [129], which are interconnected by a funnel-shaped section that allows the concurrent focusing of different sample materials, also allow the fractionation of multiple samples, at the same time [132]. the motivation behind all the modifications to the channel is the constant need for improving the resolution of the technique. several papers focused on the modeling of the fluid mechanics within the channel; a good example is the work of wright and baker [142] in which they developed a hydrodynamic model to determine the non-linear velocity profiles, valid for a limited range of flow rates.

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a crucial role is played by the flow-rate in the determination of the eluent composition. a systematic study has been carried out to optimize the composition of the eluent for the fractionation of nanoparticles loaded with enrofloxacin. the sorption capacity of silica-based particles was found to be more sensitive to the size of the eluent, whereas the eluent composition was more critical for the swelling capacity of cationic nanoparticles loaded with enrofloxacin [174]. to avoid misleading results, it is important to know that the fractionation process is independent of the particle number concentration of the sample. on the contrary, if the sample concentration is not known, it will affect the fractionation efficiency. the dynamics of the fluorescence intensity associated to the fractionation of nanodiscs loaded with the k1290 probe was evaluated by means of fff. it was found that the detector sensitivity towards the hydrolyzed product was dependent on the particle size of the nanodiscs [174]. in recent years, the number of publications that relate the optimization of fractionation methods using ml methods is increasing. in the particular case of af4, many studies have been published in the last decade. the first effort was published in 2005 with tff-spefa-af4 [33, 35]. the results obtained in this study were comparable to those obtained with the traditional multilevel design of experiments. with the aim of a fast tff-spefa-af4 workflow, a study was carried out on the eluent adjustment using the ama protocol [30]. since then, several articles have been published using different ml methods for the study of the eluent, loading capacity, and kinetics of drug-transfer. a method for the simultaneous fractionation of multiple samples using multiple flow-focusing devices in three separation steps was described [31]. a study on the optimization of af4 fractionation using a neural network, and an in-depth comparison with the classical uma algorithm, was carried out in 2010. in this study, the authors found the same optimal values of the many parameters with the classical uma algorithm and the neural network algorithm [39]. more recently, a study on the optimization of the af4 fractionation of liposomes based on artificial neural network algorithm was carried out, leading to a method with comparable results to the classical ones [41]. there is no doubt that more studies will be needed to achieve a balance between the available options; for the time being, the established protocols remain the gold standard. 5ec8ef588b