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When they go LOW, we go HIGH! Comparing sensitivity levels for the analysis of fluorescence-labeled proteins

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SUMMARY

The use of a fluorescence detector increases the sensitivity of the chromatographic analysis. In this Tech Note we evaluated the different sensitivity modes of the fluorescence detector with a fluorescein-labeled protein and compared it to the UV signal.

INTRODUCTION

The fluorescence detector is one of the spectroscopic detectors in chromatography. However, it is a lot more sensitive than UV/VIS detectors and responds selectively to compounds with a fluorophore. This detector excites the sample with excitation light and breaks up the emitted fluorescence light with a fluorescence monochromator. It extracts the required fluorescence wavelengths and measures the intensity with a photomultiplier. In bio science fluorescence labelling of proteins is often used to produce versatile tools for a variety of research applications. These labelled proteins are often purified or analysed by FPLC. In this TechNote we used fluorescein (FITC) labeled bovine serum albumin (BSA) as a model protein. The fluorescence detector RF20AX is fully integrated in our FPLC software PurityChrom and can be set to three different sensitivity levels (HIGH, MED, LOW). The aim of this TechNote was to compare different protein concentrations and evaluate which sensitivity levels should be used.

Additional Information

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RESULTS

The FITC-BSA fluorescence signal was analysed for 8 µg and 0.8 µg of sample 1, as well for 13 µg and 1.3 µg of sample 2 with the HIGH, MED and LOW level mode. The corresponding peak areas were compared, and the ratios for the HIGH, MED and LOW level were determined. As the factors were comparable between the two samples with different molar ratios F/P, they are given as mean values over both samples **(Tab. 1)**. Using the sensitivity mode HIGH and MED with the same conditions resulted in much higher fluorescence signal in comparison to LOW sensitivity **(Fig. 1)**. The fluorescence peak area for the HIGH mode is more than 40 times higher than the LOW mode, while the MED mode shows a more than 30 times higher peak than the LOW mode. The peak area for the MED mode is increased by factor 1.33 in comparison to the HIGH mode. Next, the peak area of the fluorescence signal for different sensitivity levels was compared to the corresponding peak area of the UV signal **(Fig. 2 A-C)**. Depending on the molar ratio of the fluorescein and the protein the factors varied. With the HIGH sensitivity level the fluorescence signal peak area was between 24-fold and 29-fold higher than the peak area of the UV signal, while with the MED level the fluorescence peak area was still between 17 and 20 times higher than peak area of the UV signal. The LOW sensitivity resulted in smaller peak areas for the fluorescence signal with a factor in between 0.52 and 0.65 in comparison to the peak area of the UV signal **(Tab. 2)**. Higher functionalization shown by higher molar ratios F/P did not result in higher factors comparing the fluorescence and UV signal (280 nm). Quenching effects of FITC could be an explanation for the phenomena. As this was not the scope of this work, it was not further analyzed.

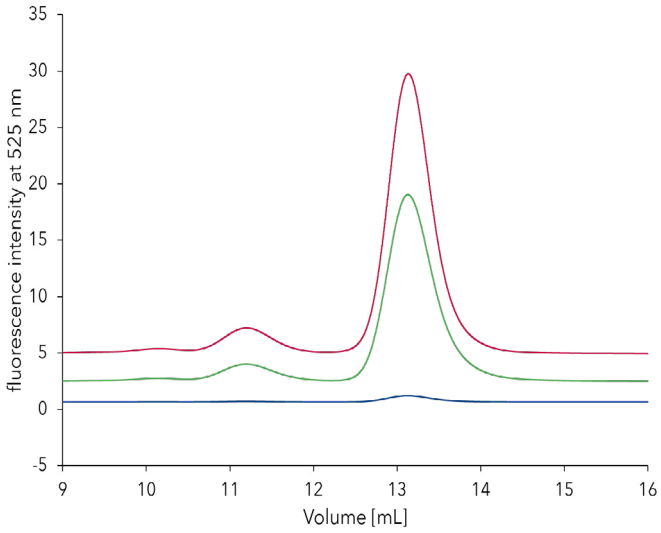

Fig. 1 Overlay of FITC-BSA fluorescence peak measured with different sensitivity modes (red line HIGH, green line MED, blue line LOW). 1.3 µg of sample 2 was injected.

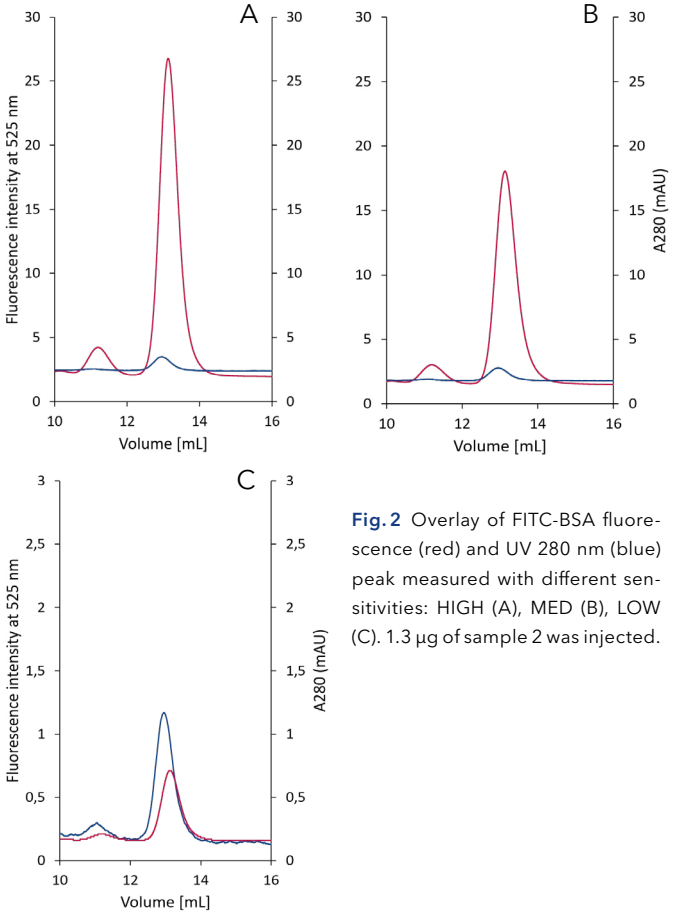

Fig. 2 Overlay of FITC-BSA fluorescence (red) and UV 280 nm (blue) peak measured with different sensitivities: HIGH (A), MED (B), LOW (C). 1.3 µg of sample 2 was injected.

SAMPLE PREPARATIONS

For the labelling of BSA with FITC BSA was dissolved in 0.1 M sodium carbonate (pH 9) at a final concentration of 2 mg/mL. FITC was dissolved in DMSO at a concentration of 1 mg/mL. 1 mL of BSA solution was carefully mixed with 20 µL FITC solution. The labelling reaction took place unter two different conditions. The labelling was carried out at 4° C for 16 hours for sample 1 and at room temperature (RT) for 1.5 hours for sample 2. All solutions and samples were prepared fresh and the solutions containing FITC or BSA were protected from light. After labelling BSA with FITC, the protein-dye solution was purified with a 5 mL Sepapure Desalting column (020X460SPZ) using PBS as buffer. A maximum of 1 mL sample was applied onto the column. The labelled protein was collected and the fluorescein/protein (F/P) molar ratio was determined using the following equation:

$$\text{Molar F/P} = \frac{MW_{BSA}}{MW_{FITC}} \times \frac{(A_{495} / E^{0.1\%\ FITC})}{[A_{280} - (0.35 \times A_{495})] / E^{0.1\%\ BSA}}$$

Molecular weight FITC	389
Molecular weight BSA	66,430
Absorption FITC $E^{0.1\%}$ *	195
Absorption BSA $E^{0.1\%}$ **	44,308.81
Correction factor FITC	0.35 x A_{495}

* Absorption at 490 nm at pH 13.

** Absorption at 280 nm of a protein at 1.0 mg/mL.

The absorbance of the conjugate sample was determined at 280 and 495 nm. For sample 1 a molar ratio of fluorophore per protein 1.13 and for sample 2 a molar ratio of 0.3 was calculated. As protein concentration determination by UV 280 nm is misleading, protein concentration was calculated from the used starting material. For the first labelling (sample 1) 8 mg BSA and for the second labelling (sample 2) 3 mg BSA were used. After purification a total volume of 9.6 mL for sample 1 and 3 mL for sample 2 was collected. From this a concentration reached 0.8 mg/mL for sample 1. 1.3 mg/mL were calculated for sample 2. Using the AZURA Bio Lab system for the first sample 8 µg and 0.8 µg were analysed, for the second sample 13 µg and 1.3 µg.

CONCLUSION

With the fluorescence detector the sensitivity of the chromatographic measurement of fluorescently labelled proteins can be increased. The HIGH sensitivity level results in more than 40 times higher and the MED level results in more than 30 times higher peak areas in comparison to the LOW level mode. The HIGH sensitivity mode can be used for labelled protein amounts in the low µg and high ng range. For higher protein amounts the LOW mode should be used.

MATERIALS AND METHODS

Tab. 1 Factor of peak area increase comparing different FLD sensitivities

Sensitivities	Factor
LOW vs. HIGH	43.2 ± 1
LOW vs. MED	32.5 ± 0.8
MED vs. HIGH	1.33 ± 0.01

Tab. 2 Comparison of fluorescence and UV 280 nm peak area

Sensitivity	BSA FITC Molar F/P	Factor
HIGH	1.13	24 ± 0,2
HIGH	0.3	29.3 ± 0.6
MED	1.13	17.4 ± 0.5
MED	0.3	20.5 ± 1
LOW	1.13	0.52 ± 0.04
LOW	0.3	0.65 ± 0.03

Tab. 3 Method parameters

Buffer A	PBS (phosphate buffered saline, pH 7.4)
Gradient	isocratic
Flow rate	1,8 mL/min
Run temperature	RT
Injection volume	100 µL or 10 µL
Detection wavelength UV	280 nm
Excitation wavelength FLD	495 nm
Emission wavelength FLD	525 nm
System pressure	~ 42 bar
Run volume	min. 1 CV (24 mL)
Data rate	min. 2 Hz

Tab. 4 System configuration

Instrument	Description	Article No.
P6.1L	Metal-free, low pressure gradient FPLC pump with 10 mL ceramic pump head, degasser and 250 µL mixer	APH64EB
ASM 2.2L	**Left**: UVD2.1S variable single wavelength UV detector **Middle**: valve drive VU 4.1 **Right:** valve drive VU 4.1	AY00001
Flow Cell UV	Semi-preparative bio-compatible 3 mm UV Flow Cell, 1/16"	A4045
V4.1	Biocompatible multi-injection valve, 1/16"	AVN94CE
V4.1	Biocompatible two-position valve, 6 port	AVD24CE
CM2.1S	Conductivity monitor with flow cell for up to 100 mL/min flow rate	ADG30GD
RF20A	Fluorescence detector	A59200
Flow Cell FLD	Bioinert Flow Cell for fluorescence detector	A59212
Foxy R1	Fraction collector	A59100
Software	PurityChrom	A2650 A2652
Column	Prepacked SEC (10 x 300 mm) column for small-scale preparative purification, as well as for characterization and analysis of proteins with molecular weights (Mr) from 10,000 to 600,000, column volume (CV) 24 mL