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Challenges in HPLC to UHPLC method conversion

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INTRODUCTION AND OBJECTIVES

Regardless of the encapsulation method and procedure, researchers need methods to determine encapsulation efficiency. Usually this is done by quantifying the amount of core material inside the capsules and comparing it to amount used in the encapsulation procedure. One method for quantifying different kinds of compounds is HPLC.

HPLC analyses can be made faster and maybe even more efficient by converting them to UHPLC analyses using shorter columns with smaller particle size. Specifically the use of smaller particle size allows the use of shorter columns without decreasing resolution of the analysis, and shorter columns mean shorter analysis times. This in turn leads to increased analysis efficiency (in terms of number of analyses / time spent) and less eluent waste / analysis. (Nguyen 2006)

In our department, we have an HPLC method for analyzing different carotenoids, which we are using for quantifying lutein in emulsions and spray-dried emulsions. In this study, we converted that HPLC method to UHPLC method trying to maintain at least the same separation efficiency but with significantly reduced analysis time.

MATERIALS AND METHODS

The HPLC/UHPLC instrument used was combined from Nexera and Prominence series modules (Shimadzu, Japan). Nexera series modules were two LC-30AD pumps (one for each eluent) and an SIL-30AC autosampler, whereas Prominence series modules were a DGU-20A₅ degasser, a CTO-20AC column oven, an SPD-M20A diode array detector and a CBM-20A_{lite} communications bus module (a circuit board fitted inside one of the pumps). Both analyses used a gradient solvent system with methanol:water (3:1) as eluent A and ethyl acetate as eluent B. The HPLC column was a Luna C-18 column, length 150 mm, i.d. 3.00 mm and particle size 5 μm (Phenomenex, USA) and the UHPLC column was a Kinetex C-18 column, length 100 mm, i.d. 2.10 mm and particle size 1.7 μm (Phenomenex, USA). The original HPLC method had a flow rate of 0.5 ml/min and the gradient was as follows: 0-10 min 0-70 % eluent B, 10-14 min 70-100 % eluent B, 14-20 min 100-0 % eluent B, 20-35 min 0 % eluent B. The system was computer-controlled with LabSolutions software (version 5.42, Shimadzu corporation, Japan).

Carotenoid samples were lutein esters extracted from marigold flowers, commercial standard free lutein or mixed carotenoids extracted from paprika. All carotenoid samples were dissolved in acetone. All solvents were of analytical purity and suitable for HPLC analysis.

Method conversion was begun with a linear conversion to scale down the flow rate and gradient. In linear conversion, each step of the gradient is calculated to have equal amounts of solvent as column volumes and the linear velocity is kept the same. After this linear conversion we increased flow rate within the pressure limits of the column and the whole system. Finally we modified the gradient in different ways to find the optimum conditions.

RESULTS AND DISCUSSION

After the linear conversion the method length was 23.3 minutes with a flow rate of 0.245 ml/min. After also maximizing the flow rate, the method length was 8.5 minutes with a flow rate of 0.5 ml/min, but the resolution of the lutein ester peaks was poor. We started to add angles to the gradient curve, and this seemed to increase peak resolution. As more angles resulted in better resolution, we decided to try "pump B curve" function from the software, which makes an exponential gradient change between two given time points instead of a linear change. Positive values of curve function give a change with increasing slope and negative values give a change with decreasing slope. After several test gradients, we came up with the following: 0-0.3 min 0-30 % eluent B (linear), 0.3-2.1 min 30-70 % eluent B (curve -4), 2.1-4 min 70-100 % eluent B (curve 2), 4-5 min 100-0 % eluent B (linear), 5-8.5 min 0 % eluent B. This gradient is graphically shown in figure 1. With this gradient the resolution of lutein ester peaks was superior both to the linear conversion (with maximized flow rate) and the original HPLC program, as can be seen in figure 2. In figure 2, the time axis of the HPLC chromatogram has been scaled to show the lutein ester peaks in the same position for easier comparison.

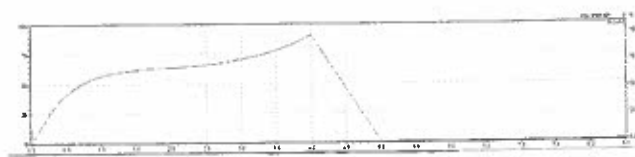


Figure 1. The modified UHPLC gradient showing percentage of eluent B

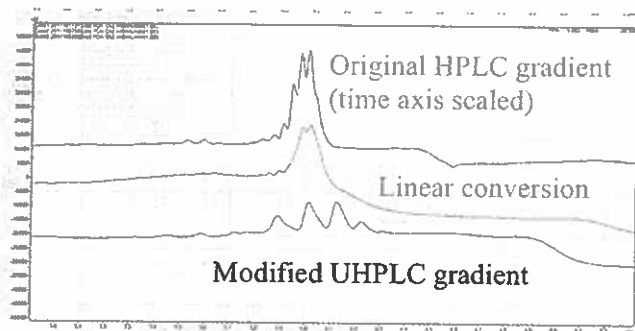


Figure 2. Comparison of peak resolution of lutein esters between HPLC, linear conversion and modified UHPLC gradient analyses

This gradient seemed to work very well, but until that point we had only used the extracted lutein esters as the sample. When we tried to analyze mixed carotenoids extracted from paprika, majority of the peaks eluted earlier than lutein esters into a part of the chromatogram, where the baseline was not level, as can be seen in figure 3.

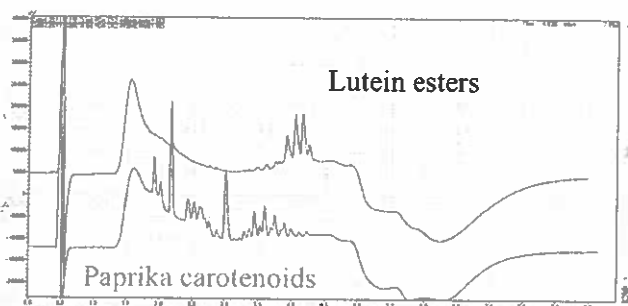


Figure 3. Lutein esters and paprika carotenoids analyzed with modified gradient UHPLC analysis

To make the gradient work for mixed carotenoid samples, we went a little backwards, and used a linear gradient in the beginning of the program. This delayed the retention of early peaks and leveled the baseline a bit, but also decreased the resolution slightly. The resolution was still better than in the original HPLC method. The final gradient was as follows: 0-1.9 min 0-70 % eluent B (linear), 1.9-3.6 min 70-100 % eluent B (curve 2), 3.6-4.4 min 100-0 % eluent B (linear), 4.4-7.6 min 0 % eluent B, with a flow rate of 5.5 ml/min. We could increase the flow rate even further to 5.5 ml/min, because at some point during the method development we changed the nut and ferrule connecting the column, and therefore the maximum pressure limit of the whole system could be raised from 850 bars to 1000 bars. The final gradient is graphically shown in figure 4, and a comparison of paprika carotenoids analyzed with this final gradient and the first version of the modified gradient is shown in figure 5. The column equilibration part of the chromatogram (last 3-3.5 minutes) is not shown in figure 5.

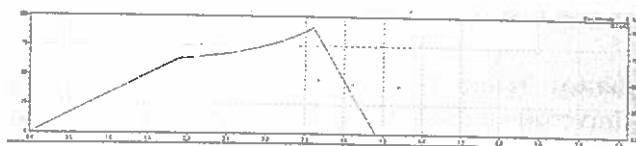


Figure 4. The final UHPLC gradient showing percentage of eluent B

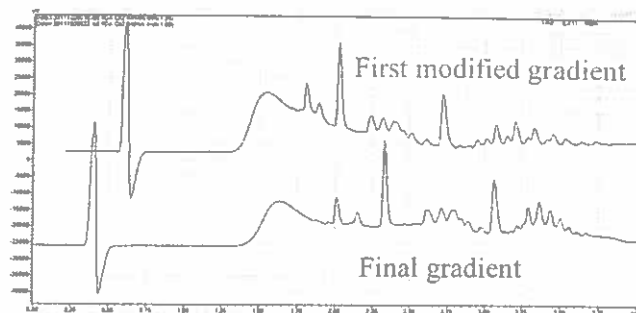


Figure 4. Paprika carotenoids analyzed with the first modified UHPLC gradient and the final UHPLC gradient

CONCLUSIONS

We managed to shorten the analysis time from 35 minutes to 7.6 minutes (22 % of the original), both times including equilibration time of the column. Solvent consumption (and hence also the waste production) was reduced from 17.5 ml to 4.2 ml per analysis (24 % of the original). These reductions result in significant savings both in the working time and reagent costs, as well as reducing the amount of harmful solvent waste produced. At the same time we managed to increase peak resolution slightly.

One important thing to remember when making conversions like this is to use proper samples and standards so that the new method is usable for all the samples that are to be analyzed with it. As seen with this work, linear conversion is not always enough to gain the best possible results, so further method development may be needed.

REFERENCES

Nguyen D. et al. (2006) *Fast analysis in liquid chromatography using small particle size and high pressure*. J. Sep. Sci. 29 1836-1848