P1301-M
On-line two-dimensional liquid chromatography by a single column

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Summary:

The purpose of the presentation is to establish an on-line two-dimensional liquid chromatography by one column expressed as (2DLC-1C). With instrumentation improvement, all of chromatographic operation, such as sample collection and re-injection, buffer exchange and so on can be carried out in a closed system by means of on-line manner. An example of human serum separation into 73 fractions of 2DLC-1C was obtained. The 2DLC-1C not only has resolution as good as the two pieces of column of HIC and WCX employed independently, but it also change the selectivity of 2DLC-1C, respectively.

Introduction:

The isolation of native proteins from natural products and the development of the pre-fractionation of intact proteins required for the "top-down" method in proteomics need to explore a new approach for fast protein separation. An improvement MS for measuring an intact protein with a very high mass of 229kDa also requires a very fast and efficient separation method of intact protein by liquid chromatography (LC)[Science, **314:**109-112; 2006]. To A new approach of 2DLC~1C (HIC~WCX) for the fast separation of native proteins was recently reported. [Chin. Sci. Bull. (B), 2008, **53:** 113~117].

Resolution and selectivity of the (HIC~WCX) column

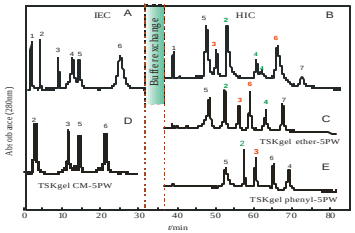


Figure 1 Chromatogram of standard proteins by (HIC~WCX) column and comparison with three TSK gel columns (two pieces for HIC and one piece for WCX).

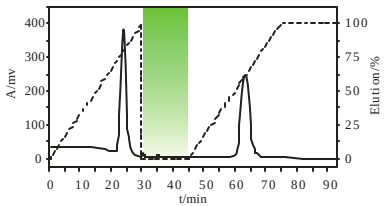


Figure 2 Chromatogram of standard proteins by on-line 2DLC(WCX~HIC$_2$) mode

Principle

The excellent resolution of intact proteins by the (HIC~WCX) column mode can be explained by the reported "U" shape elution of proteins in literature. The interaction of both electrostatic and hydrophobic forces is shown in Figure 3.

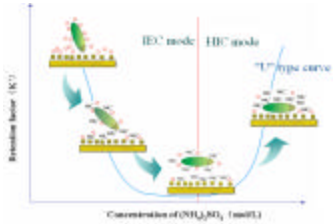


Figure 3 Scheme of principle of protein separation by 2DLC(IEC-HIC) corresponding to the "U" shape of elution curve.

Column type: A and B: The single silica-based (HIC~WCX) column (made by Xi'an Aulan Scientific & Technology Development Co); D, TSKgel CM-5PW for WCX ; C and E : TSKgel ether-5PW and phenyl-5PW.

Column size 7.5mm x 75mm;

Proteins: 1, solvent peak; 2, myoglobin (Myo); 3, ribonuclease A (Rnase A); 4, α-chmotripsin (α-Chy); 5, cytochrome c (Cyt-C); 6, lysozyme (Lys) 7, insulin (Ins).

Column capacity (HIC~WCS) column, >1,220 for RNase A, linear gradient, 30 min)

Selectivity: Two-pair proteins, Cyt-C-RNase A(and Myo-α-Chy (green) change their elution orders, respectively.

Other parameters of the (HIC~WCX) column: Mass recovery; adsorbed amount and break-through amount were measure to be comparable with commercial products. Also, this column can be permitted to work under a broad range of column pressure up to 500 mPa .

Figure 2 shows that three of seven standard proteins (See green below) could not be completely separated and eluted together with solvent) and other two of them (See red below) proteins also could not completely separated by IEC mode. Collecting the two fractions and separately re-injecting them the same column and separated by HIC mode. This protocol is expressed as (WCX~HIC$_2$).

Column(4.0mm x 150mm), peaks: 1, Solvent peak + α-Amy + Ins; 2, Myo; 3, RNase; 4, 4' Cyt -C; 5, 5, α-Chy; 6, Lys; 7', α-Amy; 8', Ins.

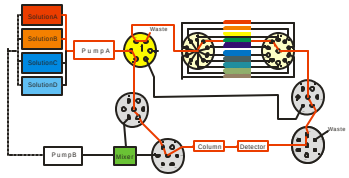
(1) Non-linear gradient elution program expressed as dash line in the chromatogram. resulting in that the seven standard proteins could be completely separated.

Figure 3 shows proteins can be adsorbed either in low salt concentration, based on WCX mode (left side), or in higher salt concentration due to HIC mode (right side). In the middle concentration of salt, both WCS and HIC modes compete adsortion occur on the stationary phase of the (HIC~WCX) column (bottom).

Instrumentation for the on-line buffer exchange and continuous sample injection:

For on-line buffer exchange (1) When same mobile phase is employed for the two modes, it is just done by switching the weak and strong solutions of gradient elution by using a two-way pump system. (2) When different mobile phases are used for the two modes, the gradient elution can be carried out by using a four-way pump system of usual chromatograph without any instrument improvement, or using a two-way pump system plus an extra valve,

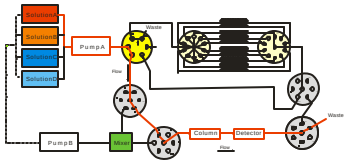
For on-line continuous sample injection: With a little improvement of instrument, whole chromatographic operations can be done in a closed system shown in Figures 4 and 5.



First Run– Peak Collection

Figure 4 Scheme of on-line 2DLC for equilibrium (red), collecting fraction (green), and re-injection.

The sample collection and re-injection can be done with a sample injector with ten position, eight sample loops of 5.0ml and controlled by a series of two way valves which are set up in a box with temperate controlled at 4° C as well two pump systems. A pump have four fluid channels connecting to two-pair of mobile phase for gradient elution (A-B, C-D),



Second Run --Injection

Figure 5 On-line continuous sample re-injection.

A pump here is only employed for continuous sample injection and/or buffer exchange. The second pump has two fluid channels. One of the fluid channel is connected to any required of the four solutions and other fluid channel is connected to the any one of the sample loops. A fast flow rate would shorten the time for changing buffer solution and continuous sample injection. Chromatographic cake is favorable for accomplishing this purpose, because of employing a very high low rate of fluid.

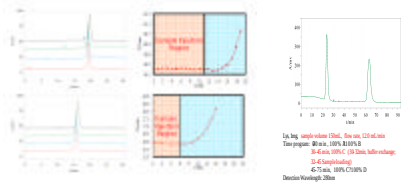


Figure 6 Scheme of principle for on-line buffer exc... and continuous sample injection. Lys retention under a linear gradient elution with non-synchronous sample injection.

...-line buffer exchange and continuous sample injection

Figure 7 indicates that the 1st fraction of 150 mL of Lys from HIC mode was collected, and on-line buffer exchange, and then on-line continuous sample injection into the 2nd WCX mode by the chromatographic cake (2.0mm × 20 mm) under flow rate of 12.0 mL/min, but under 1.0 mL/min for protein separation(See: HP2008-P2208-Th)

On-line pre-fractionation of human serum in whole-range screening by 2DLC-1C

Human serum is a very complex sample containing thousands of proteins. Disregarding with peaks, the original sample of 5.0 mL was divided into eight fractions and one more solvent fraction by the 1st LC of WCX mode separation shown in Figure 8. Subsequently each of the collections was re-injected into the 2nd LC of HIC mode separation, also eight fractions. To make the whole chromatogram be clear, the first three of them on the bottom of this figure, while the other five of them were zoom in the top box are shown in it. The 72 (8 × 9) sub-fractions, plus one more of fraction the second solvent peak fro HIC mode, totally 73 fractions. The whole operation took less than 400 min. This is an on-line pre-fractionation of proteins by means of whole-range screening by 2DLC-1C.

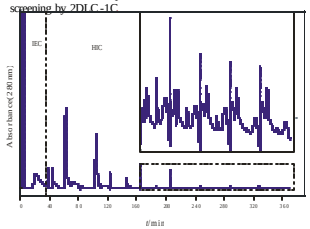


Figure 8 Scheme of human serum on-line pre-fractionation by whole-range screening into 73 fpsub-fractions with 2DLC-1C(WCX~HIC$_8$) mode.

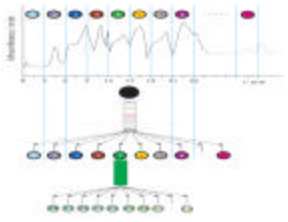


Figure 9 Scheme of the protocol of pre-fractionation of proteins dividing hundreds to thousands of sub-fractionation by whole-range screening by means of on-line 2DLC-1C in future

If it is necessary, a series of ten-way valve, even more than them of sample injection, the sample can be divided into twenty, even hundred sub-fractions for each of either the 1st , or the 2nd mode, totally thousands to ten thousands of final fractions shown in Figure 8.

Conclusion: The (HIC~WCX) column not only has resolution as good as two pieces of commercial columns of HIC and WCX independently, but also changes the selectivity of both modes, respectively, providing a new choice for chromatographers. With instrumentation improvement, buffer exchange, sample collection, and re-injection can be accomplished by on-line 2DLC-1C and in a closed system, as well thus all components in original sample can be quantitatively transferred to the subsequent operation. A very complex original sample can be easily divided into many fractions, greatly simplifying its complexity. This purified proteins are not only intact ones and provides more exact information about protein, but also have exactly three-, or four-dimensional molecular structure of proteins in preparative scale, providing both substances and information.