Advance Analytical Course

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Lecture No. # 18

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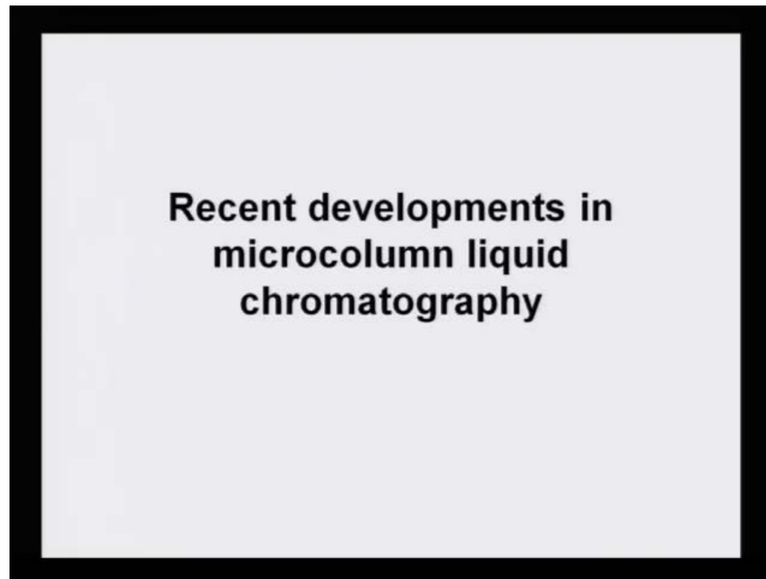

Recent development in micro column liquid chromatography - I am again referring to liquid chromatography, because this aspect of chromatography was not covered in the previous lecture.

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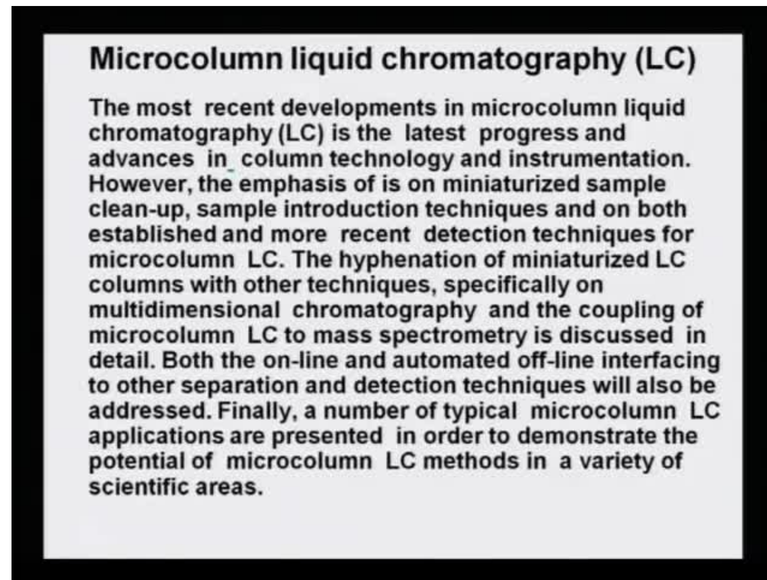

Recent developments in micro column liquid chromatography or LC as what we would popularly call it - the most recent developments in micro column liquid chromatography is the latest progress and advances in column technology and instrumentation. However, the emphasis of is on the miniaturization of the sample, clean-up method, sample introduction techniques and on both established and more recent detection techniques to be used for micro column LC.

Now, you see as the word micro has been introduced with LC, it means that everything must be made smaller and smaller and more precise because that is where the new technology has come into picture; otherwise, it is like any other normal liquid chromatography. The hyphenation of miniaturized liquid chromatography columns with other techniques, specifically on multi-dimensional chromatography and the coupling of micro column LC to mass spectrometry are discussed in this lecture and we will talk in detail how this helps even the smallest of the smallest and the trace quantities of analyte can also to be analysed on LC by simply miniaturizing it.

Both the on-line and automated off-line interfacing to other separation and detection techniques will also be covered. Finally, a number of typical micro column LC applications are presented in order to demonstrate the potential of micro column LC methods in a variety of scientific areas.

So, as what I have tried to introduce you all to the fact that where all which type of analysis and what modification of the analytical tool must be taken into account for a very specific kind of analyte.

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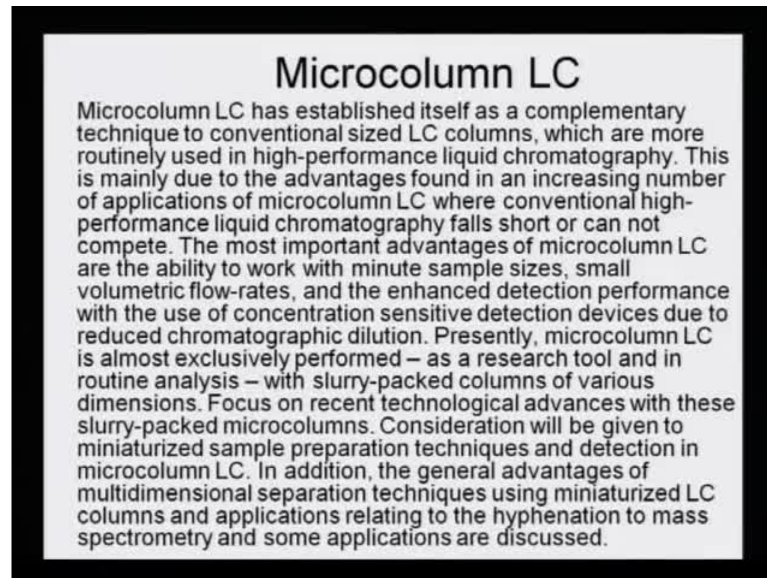

Micro column LC - micro column LC has established itself as a complementary technique to conventional sized LC columns, which are more routinely used in high-performance liquid chromatography. is mainly due to the advantages found in an increasing number of applications of micro column LC, where conventional high-performance liquid chromatography falls short or cannot compete.

The simple reason that micro column was brought into because the conventional liquid chromatography was not a very appropriate machine. The most important advantages of micro column LC are the ability to work with minute sample sizes, small volumetric flow-rates and the enhanced detection performance with the use of concentration sensitive detection devices due to reduced chromatographic dilution.

Presently, micro column LC is almost exclusively performed - as a research tool and in routine analysis - with slurry-packed columns of various dimensions. Focus on recent technological advances with these slurry-packed micro columns has been brought about. Consideration will be given to miniaturization of the sample preparation techniques and detection in micro column LC.

In addition, the general advantages of multidimensional separation techniques using miniaturized liquid chromatographic columns and applications related to hyphenation to mass spectrometry and some applications will also be discussed. Now, one very important thing that needs to be remembered when we are working with micro column is that we are working on slurry-packed columns - first thing. Second thing - that the sample sizes are very small or minute and the flow–rates are also very small volume and thus it has to have an enhanced detection device or performance.

Extra column band broad link – Now, when we are working on smaller system, there has to be a lot of other considerations, which need to be addressed and if we forget to do that then the data that is derived from the analysis does not seem to be correct.

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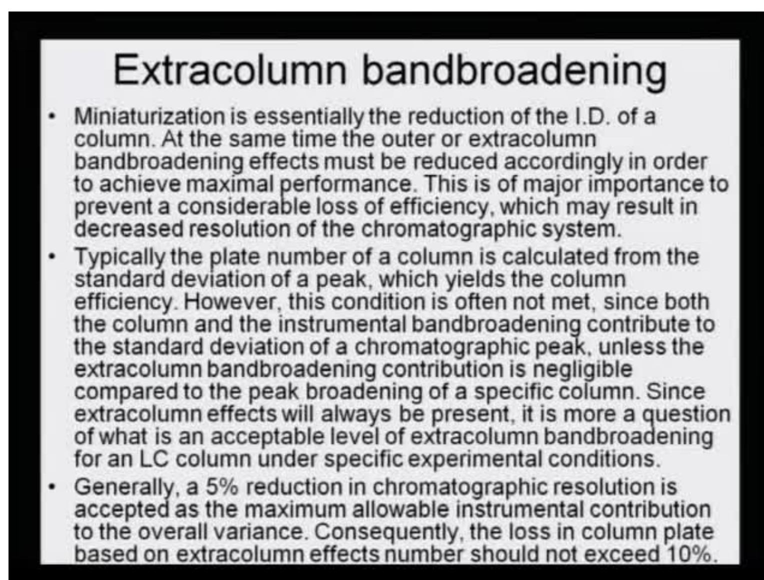

Miniaturization is essentially the reduction of the internal diameter or the I.D. of a column. At the same time, the outer or extra column band broadening effects must be reduced accordingly, in order to achieve maximum performance. This is of major importance to prevent a considerable loss of efficiency.

So, we have to keep in mind that all other parameters must also be reduced considerably for a good performance. This is of major importance to prevent a considerable loss of efficiency, which may result in decreased resolution of the chromatographic system.

Typically, the plate number of the column is calculated from the standard deviation of a peak, which yields the column efficiency. However, this condition is often not met, since both the column and the instrumental band broadening contribute to the standard deviation of a chromatographic peak, unless the extra column band broadening contribution is negligible compared to the peak broadening of a specific column.

I have been talking about one very specific thing and that is the peak, whether it is GC or HPLC, must be very sharp in order to represent one single compound and if because of some kind of a non-manipulation of the technique and its parameter, if the peaks come out as broad, then that is not considered to be a good analysis. Since extra column effects will always be present, it is more a question of what is an acceptable level of extra column band broadening for the LC column under specific experimental conditions.

There are bound to be some solvent effects, which cause the extra column band broadening, which cannot be completely eliminated. So, one has to optimize the experimental condition in order to be able to minimize the extra column band broadening.

Generally, a five percent reduction in chromatographic resolution is accepted as the maximum allowable instrumental contribution to the overall variance. Consequently, the loss in column plate based on extra column effect number should not exceed ten percent. Now, between five to ten percent, one can still make some allowances and accept the analytical data. However, beyond that, it will all go haywire.

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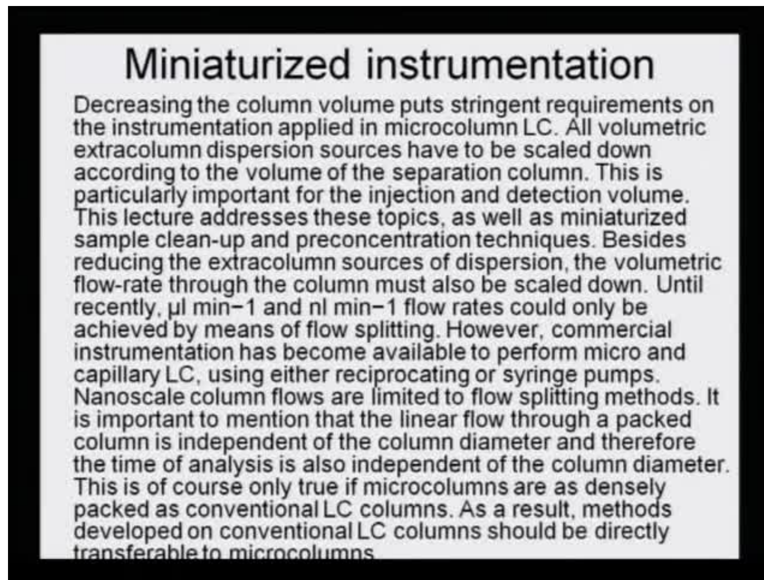

Miniaturized instrumentation - decreasing the column volume puts stringent requirements on the instrumentation applied in micro column LC. Time and again, I am repeating the word micro, which means we are making it smaller. All volumetric extra column dispersion sources have to be scaled down according to the volume of the separation column.

This is particularly important for the injection and detection volume. This lecture addresses these topics as well as miniaturized sample clean-up and pre-concentration techniques. Besides reducing the extra column sources of dispersion, the volumetric flow-rate through the column must also be scaled down. Now, we cannot have the same flow-rate as what we used for normal LC.

Obviously, because here the column is small and so the flow-rate also has to be optimized accordingly. Besides reducing the column sources of dispersion, the volumetric flow-rate also must be scaled down. Until recently, 1 microlitre per minute or 1 nanolitre per minute flow rates could only be achieved by means of flow splitting.

However, commercial instrumentation has become available to perform micro and capillary liquid chromatography using either reciprocating or syringe pumps. Nanoscale column flows are limited to flow splitting methods. It is important to mention that linear flow through a packed column is independent of the column diameter and therefore, the time of the analysis is also independent of the column diameter. This is of course, only

true, if microcolumns are as densely packed as the conventional liquid chromatographic columns.

As a result, methods developed on convenient liquid chromatographic columns should be directly transferable to micro columns. Now, one thing has to be understood that because of the internal diameter and the size of the column being different in the case of micro LC and normal LC, there have to be a lot of adjustments in the case of micro LC. It is shear logic that one cannot do a macro scale analysis on a micro machine. So everything has to be miniaturized.

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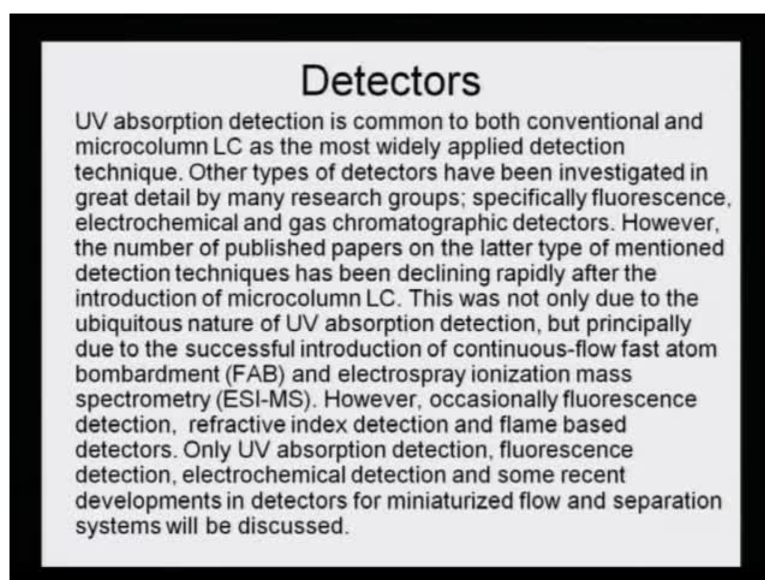

Detectors - UV absorption detection is common to both conventional and micro column LC as the most widely applied detection technique. Other types of detectors have been investigated in great details by many research groups; specifically the fluorescence, electrochemical and gas chromatographic detectors. However, the number of published papers on the latter type as mentioned in the detection techniques series, has been declining rapidly after the introduction of the micro column LC.

Now, research is what makes new and new trend come into the market for simple reason that there was a need that was felt that there should be a more advanced technique and so, micro column technique is one of the latest.

This was not only due to the ubiquitous nature of the UV absorption detection, but principally due to the successful introduction of continuous flow fast atom bombardment, that is, FAB and electrospray ionization mass spectrometry, that is, ESI-MS.

However, occasionally fluorescence detection, refractive index detection and flame based detectors have also been used. Only UV absorption detection, fluorescence detection, electrochemical detection and some recent developments in detectors for miniaturized flow and separation systems will be discussed here. Why, because we have already discussed the other detectors of the GCs and in the previous lectures, but this is we are talking about liquid chromatography and it is a miniaturized form of liquid chromatography.

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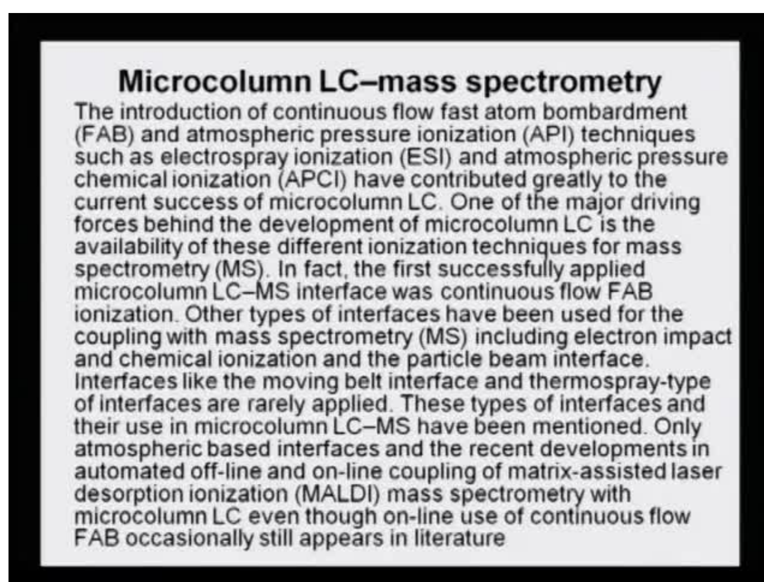

Microcolumn LC with the mass detector - that means, it is a micro column LC mass spectrometry. The introduction of continuous flow fast atom bombardment and atmospheric pressure ionization, that is, API techniques such as electro spray ionization, that is, ESI and atmospheric pressure chemical ionization (APCI) have contributed greatly to the current success of micro column LC.

Now, these are methods; that is, FAB method, the API method, the ESI method and the APCI method are different methods of ionization; once the separation in the micro column has taken place, and these take place in the detector. So, this should be

understood very clearly that we can have a FAB mass for a GC-MS and we can have a FAB mass for a LC-MS. So, that should not be a confused. Mass detector remains common for both of them.

One of the major driving forces behind the development of micro column LC is the availability of these different ionization techniques for mass spectrometry. In fact, the first successful applied micro column LC-MS interface was continuous flow FAB ionization.

Other types of interfaces have been used for the coupling with mass spectrometry including electron impact and chemical ionization and the particle beam interfaces. Interfaces like the moving belt interface and thermo spray type of interfaces are rarely applied. These types of interfaces and their use in micro column LC-MS have been mentioned very fleetingly. They are not very popular.

Only atmospheric based interfaces and the recent developments in automated off line and on line coupling of matrix assisted laser desorption ionization (MALDI) - just a while ago, I had mentioned about this in one of the lectures - mass spectrometric technique with micro column LC, even though on line use of continuous flow FAB occasionally still appears in literature. So, now the latest trend has been, although it started from the continuous flow FAB detector type of mass machine, but now the most common machine is the MALDI machine.

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Applications

Microcolumn LC is applicable over a variety of different fields. Presently, its main application areas are bioanalysis, neuroscience and protein/peptide research. Sample availability and mass spectrometric compatibility are the main reasons behind the continued success. Other application areas are chiral separations and the analysis of industrial samples, such as polymers and additives and the quantitative analysis of pesticides by solid-phase extraction-capillary LC has been reported.

Applications - micro wave column LC is applicable over a variety of different fields. Presently, its main application areas are bio-analysis, neuroscience and, protein and peptide research.

All in the biological system; why, because very trace quantities of analytes are available for analysis and for that matter, one big LC will not be a good idea and therefore, miniaturization or micro LC is the answer for such applications. Sample availability and mass spectrometric compatibility are the main reasons behind the continuous success. Other application areas are chiral separations and the analysis of industrial samples such as polymers and additives, and the quantitative analysis of pesticides by solid-phase extraction capillary LC; that also has been reported many a times.

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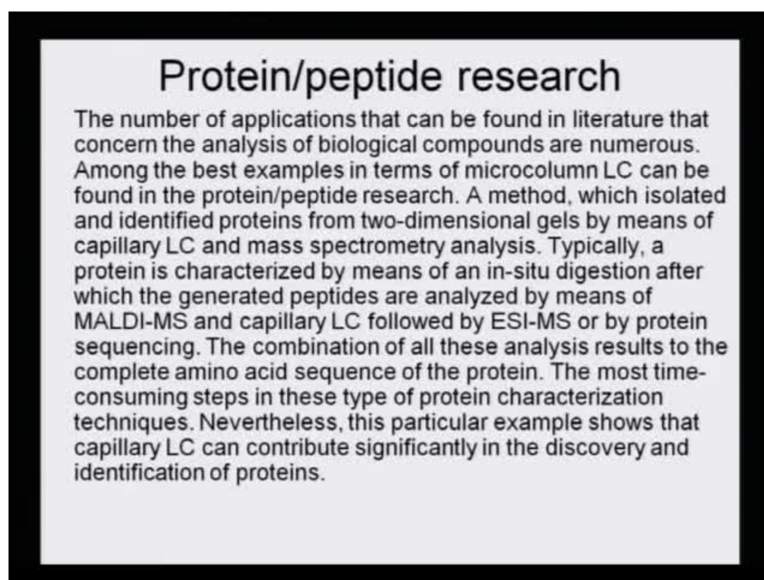

Protein peptide research - how this analysis is carried out, I will give a little bit of detail because for students of biotechnology and biochemistry and also microbiology, this will be of great interest to know where all they can use the micro LC. The number of applications that can be found in literature that concern the analysis of biological compounds are numerous. Among the best examples in terms of micro column LC can be found in the use of protein and peptide research.

A method, which isolated and identified proteins from two-dimensional gels by means of capillary LC and mass spectrometric analysis. Typically, a protein is characterized by

means of an in-situ digestion, after which the generated peptides are analysed by means of MALDI-MS and capillary LC followed by ESI-MS or by protein sequencing.

The combination of all these analyses results to the complete amino acid sequence of the protein. The most time consuming test or rather, the most time consuming steps in these type of protein characterization techniques is the sequencing of those amino acids. Nevertheless, this particular example shows that capillary LC can contribute significantly in the discovery and identification of proteins.

I would like to draw your attention and for your information, I want to tell you how these proteins cannot be directly analysed. They need to be pre-digested before putting into the capillary column of the LC and then, when they are separated, the peptides are separated. There are further more hydrolyses that take place on the column and they are further separated and then a whole series of amino acids are derived from this main one protein. So that The way they come to the detector, which is the MALDI-MS detector, it is analysed through this very special type of a matrix-assisted desorption method of mass spectrometry.

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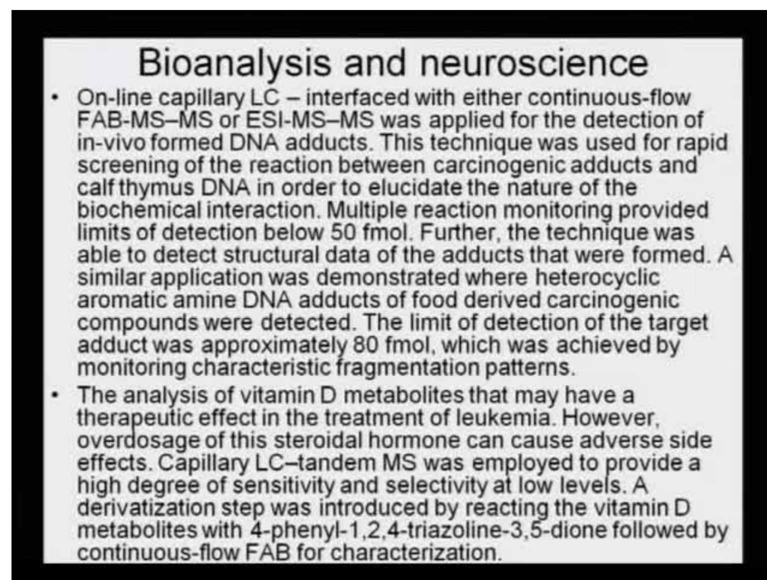

Similarly, in bio-analysis and neurosciences, on line capillary LC interfaced with either continuous flow FAB-MS-MS or ESI-MS-MS - now, you see there are two mass spectrometry; so, MS-MS; they are hyphenated as the name is showing - was applied for the detection of in-vivo formed DNA adducts. This technique was used for rapid

screening of the reaction between carcinogenic adducts and the calf thymus DNA in order to elucidate the nature of the biochemical interaction. So, even biochemical interaction can be studied through this capillary LCs and micro LCs. Multiple reaction monitoring provided limits of detection below even 50 ficomol. So, you see it is such a small; it is 50 fentomol.

Further, the technique was able to detect structural data of the adducts that were formed. A similar application was demonstrated where heterocyclic aromatic amine DNA adduct of the food derived carcinogenic compounds were detected. The limit of detection of the target adduct was approximately 80 fentomol, which was achieved by monitoring characteristic fragmentation pattern. Fentomol is a very, very, very small molecular moiety and you imagine that we were talking about the analysis of banned amines yesterday and these banned amines, how they act as carcinogen has also been studied because they interfere with the DNA material of the cell and that is how the adduct of this amine and the DNA have been even identified, which have a molecular size of 80 fentomol.

The analysis of vitamin D metabolites that may have the therapeutic effect in the treatment of leukemia can also be studied through this. However, over dosage of this steroidal hormone can cause adverse side effects. Capillary LC when it is tandem with MS was employed to provide a high degree of sensitivity and selectivity at low levels.

A derivatization step was introduced by reacting the vitamin D metabolites with 4-phenyl-1, 2, 4-triazoline-3, 5-dione followed by continuous flow FAB for characterization. So, you see that vitamin B metabolite when it is in small quantity, it has a therapeutic effect, but if this hormone is given in high doses, it can become a toxin and so, it is analysed whether it has exceeded the limit or not through this reagent, which is 4-phenyl-1, 2, 4-triazoline-3, 5-dione so that it forms an adduct and this adduct is very easily recognized by the MS continuous flow FAB method.

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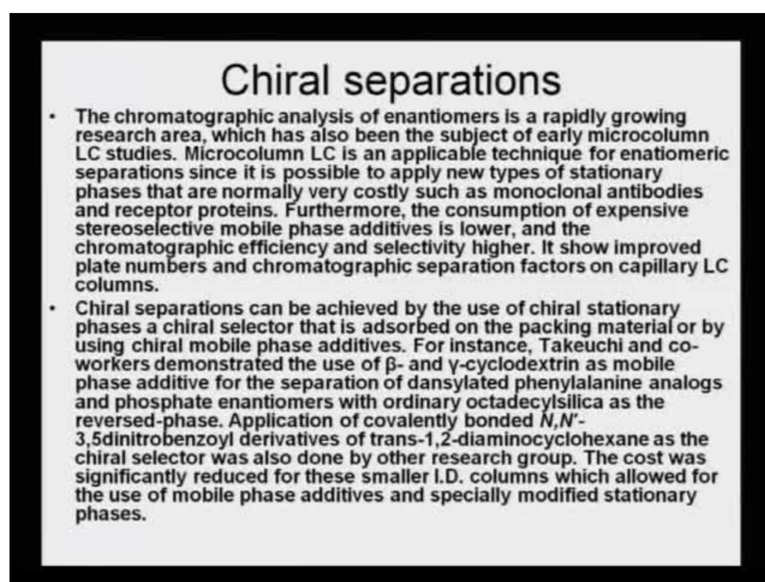

Even chiral separations can be carried out on the micro LC. The chromatographic analysis of enantiomers is a rapidly growing research area, which has also been the subject of early micro column LC studies. Micro column LC is an applicable technique for enatiomeric separations, since it is possible to apply new types of stationary phases that are normally very costly such as monoclonal antibodies and receptor proteins.

Furthermore, the consumption of expensive stereo selective mobile phase additives is lower, and the chromatographic efficiency and selectivity is very high. Remember that the moment we miniaturize, we have an advantage that we are using lesser reagents for the separation process. It shows improved plate numbers and chromatographic separation factors on capillary LC columns.

Chiral separations can be achieved by the use of chiral stationary phases, a chiral selector that is adsorbed on the packing material or by using chiral mobile phase additives. For instance Takeuchi and co-workers demonstrated the use of beta and gamma cyclodextrin as mobile phase additives for the separation of dansylated phenylalanine analogs and phosphate enantiomers with ordinary octadecylsilica as the reversed phase. Application of covalently bonded N,N-3,5dinitrobenzoyl derivatives of trans-1,2-diaminocyclohexane as the chiral selector was also done by other research group.

The cost was significantly reduced for these smaller internal diameter columns, which allow for the use of mobile phase additives and specially modified stationary phases. So,

you see that by adding some additives to the mobile phase, the process can be made more efficient. So, either, we add a mobile phase additive or we make some modification in the stationary phase, either it is slurry packed, but everything is made into smaller model so that the solvent that is required, the modifiers that are required are used in smaller quantities and the separation is done in a most effective manner.

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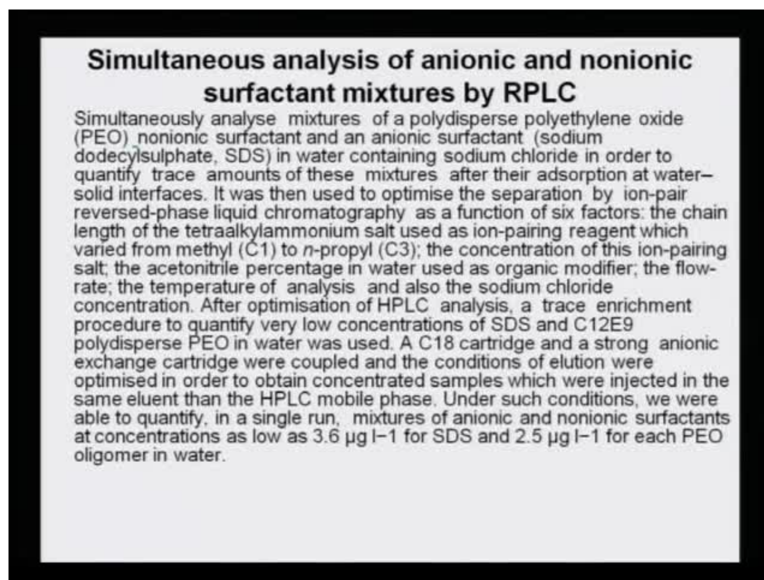

Simultaneous analysis of ionic and nonionic surfactants mixtures by RPLC - simultaneous analysis mixtures of a polydisperse polyethylene oxide, non-ionic surfactant and that is, sodium dodecylesulphate (SDS) in water containing sodium chloride in order to quantify trace amounts of these mixtures after their absorption at water solid interfaces.

It was then used to optimize the separation of ion-pair reversed phase liquid chromatography as a function of six factors: the chain length of the tetraalkylammonium salt used as ion-pairing reagent which varied from methyl one to n-propyl, that is, C3; the concentration of this ion-pairing salt; the acetontrile percentage in water used as organic modifier; the flow rate; the temperature of the analysis and also the sodium chloride concentration.

After optimisation of HPLC analysis, a trace enrichment procedure to quantify very low concentrations of SDS, that is, the sodium dodecylesulphate and C12E9 poly disperse PEO in water was used. A C18 cartridge and a strong anionic exchange cartridge were

coupled and the conditions of elution were optimised in order to obtain concentrated samples which were injected in the same eluent than the HPLC mobile phase. Under such conditions, we were able to quantify in a single run, mixtures of anionic and nonionic surfactants at concentrations as low as 3.6 microgram per litre for SDS and 2.5 microgram per litre for each of the PEO oligomers in water.

Now, this is a very, very important analysis from the point of view of water. You may have known that many times mu and nu types of soap qualities come into picture. Now, they have different types of surfactants and these surfactants are then run into water. Now, from the water body, they get contaminated and there is no way to remove.

So, it is important to know whether these surfactants are actually getting higher in their concentration and for that matter, one needs to analyse them. The best method to analyse these anionic and non-ionic surfactants is by means of RPLC, that is, the reversed phase liquid chromatography. So, common compounds like polydisperse polyethylene oxide (PEO), which is a non-ionic surfactant and surfactants, which are anionic in nature like sodium dodecylesulphate (SDS) can be analysed by reversed-phase liquid chromatography.

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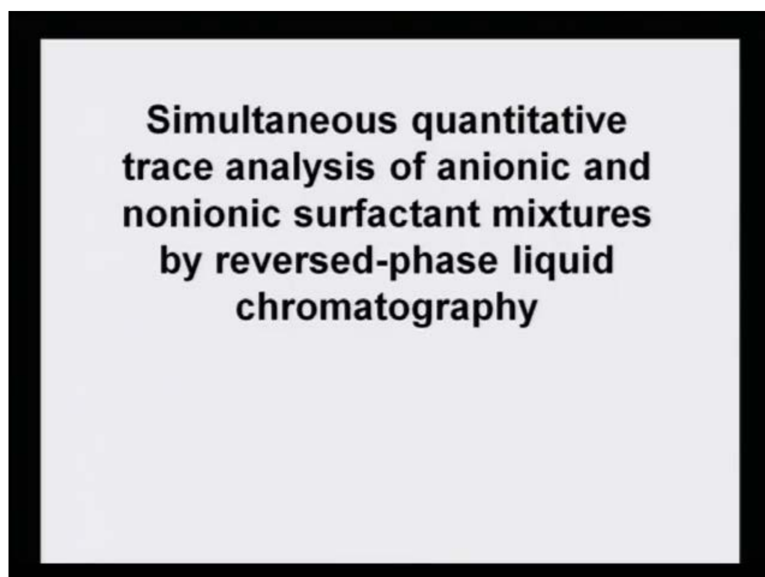

In continuation with simultaneous quantification of trace analysis of anionic and ionic surfactant mixture by reversed-phase liquid chromatography, we continue to do this a

little more in detail because it is important to know what the parameters are that need to be a taken into account.

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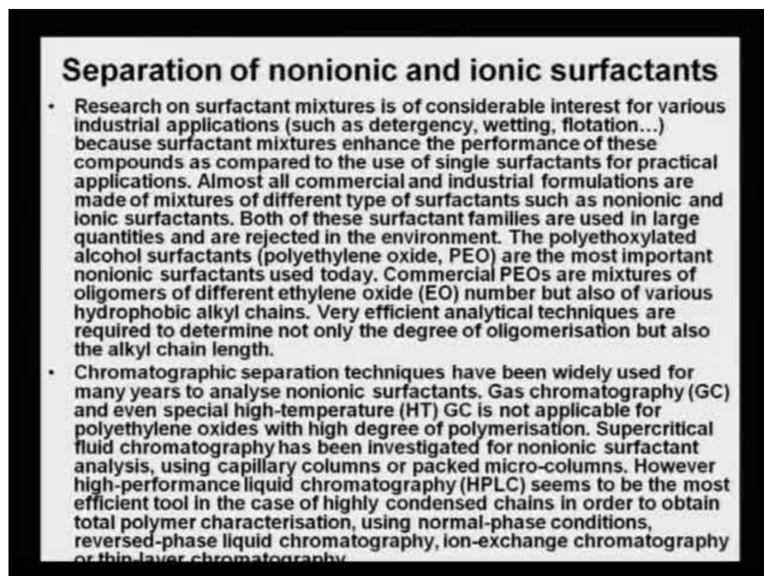

Separation of nonionic and ionic surfactants - why there is a need, what are these surfactants? I want to give you a focused information about various types of analytes. Because this is an advanced analytical chemistry course, you should be able to know all the different types of analytes that need to be addressed.

Research on surfactant mixtures is of considerable interest for various industrial application such that detergency, wetting, flotation because surfactant mixtures enhance the performance of these applications compounds as compared to the use of the single surfactant for practical applications.

Almost all commercial and industrial formulations are made of mixtures of different type of surfactant families and are used in large quantities and are rejected in the environment. Just a while ago, I was telling you that how the water bodies are getting contamination contaminated because these surfactants are run into the water and the environment is getting polluted, and different types of surfactants are being used by the different companies and not one variety, but several combinations also, they are using at times. The polyethoxylated alcohol surfactants or the PEO, as what we will refer from now onwards, are the most important non-ionic surfactants used today.

Commercial PEOs are mixtures of oligomers of different ethylene oxide, that is, the EO number, but also various hydrophobic alkyle chains. Very efficient analytical techniques are required to determine not only the degree of oligomerisation, but also the alkyle chain length.

Chromatographic separation techniques have been widely used for many years to analyse non-ionic surfactants. Gas chromatography and even special high temperature, that is, HTGC is not applicable for ethylene oxides with high degree of polymerisation. So, as I was telling you that there is always a need of a new method to be developed because the existing methods cannot cater to the needs, and the environmental conditions and parameters that are required for various different kinds of analytes.

Super critical fluid chromatography have been investigated for non-ionic surfactants analysis using capillary columns or packed micro columns. However, high performance liquid chromatography, that is, the HPLC seems to be the most efficient tool in the case of highly condensed chains in order to obtain total polymer characterisation using normal phase conditions or reversed phase liquid chromatographic conditions or it could also use ion exchange chromatography and sometimes even by mode of thin layer chromatography.

Identification can be enhanced by thin layer chromatography. However, that is not one of the separating methods of a substance, in the sense that one does not get the sample in hand to do further analysis on the spectroscopic machine.

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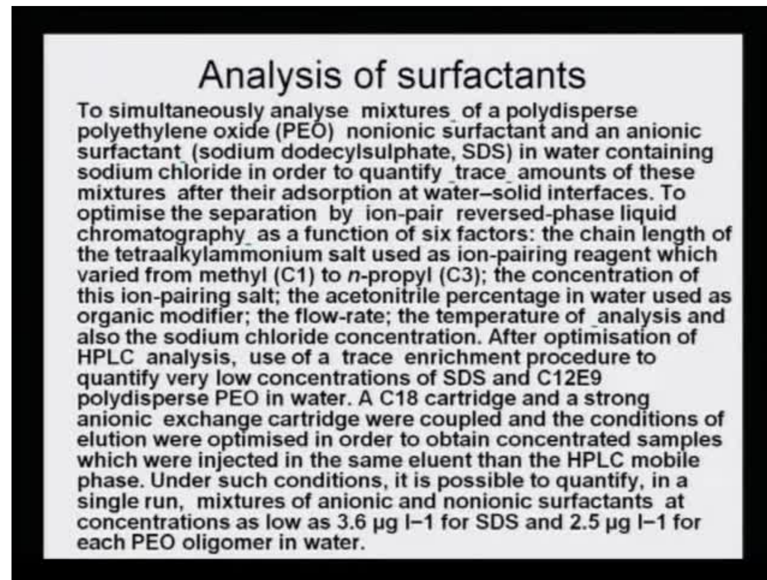

Analysis of surfactants - to simultaneously analyse mixtures of the polydisperse polyethylene oxide, that is, the PEO, non-ionic surfactant and an anionic surfactant such as sodium dodecylesulphate in water containing sodium chloride in order to quantify trace amounts of these mixtures after their absorption in water solid interfaces. To optimise this separation, ion-pair reversed-phase liquid chromatography as a function of a six factors was used and this method I have already discussed in my previous slide. So, I will not go into the detail of this furthermore.

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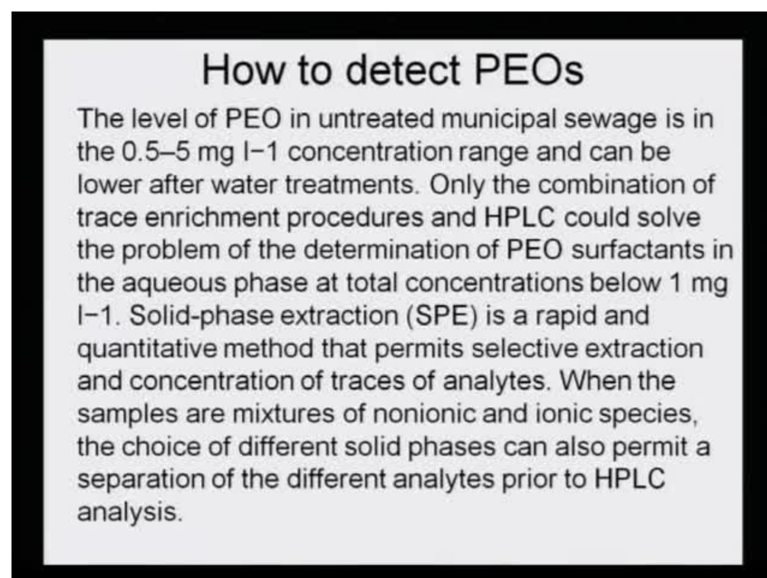

How to detect PEO? Now, let us look at the levels. The level of PEO in untreated municipal sewage is in the range of 0.5 to 5 milligram per litre concentration range and can be lower after water treatment. Only the combination of trace enrichment procedures and HPLC could solve the problem of determination of PEO surfactants in the aqueous phase at total concentration below 1 milligram per litre. Solid phase extraction is a rapid and quantitative method that permits selective extraction and concentration of traces of analytes. When the samples are mixtures of nonionic and ionic species, the choice of different solid phases can be also permitted to separate different analytes prior to HPLC analysis.

You have already learnt about the SP extraction method which means that different types of solid phase extractors could be used for ionic and nonionic surfactants and then they are put into the a this is of course, the extraction method. Then they are put into the HPLC machine for further analysis and therefore, it is possible to analyse the PEOs from the untreated municipal sewage very efficiently, although they are only in quantities as low as 0.5 to 5 milligram per litre.

So, you see that there is a liquid chromatographic technique available specifically for the analysis of these polyethylene compounds, which are very notorious nonionic surfactants.