Advance Analytical Course

Prof. Padma Vankar

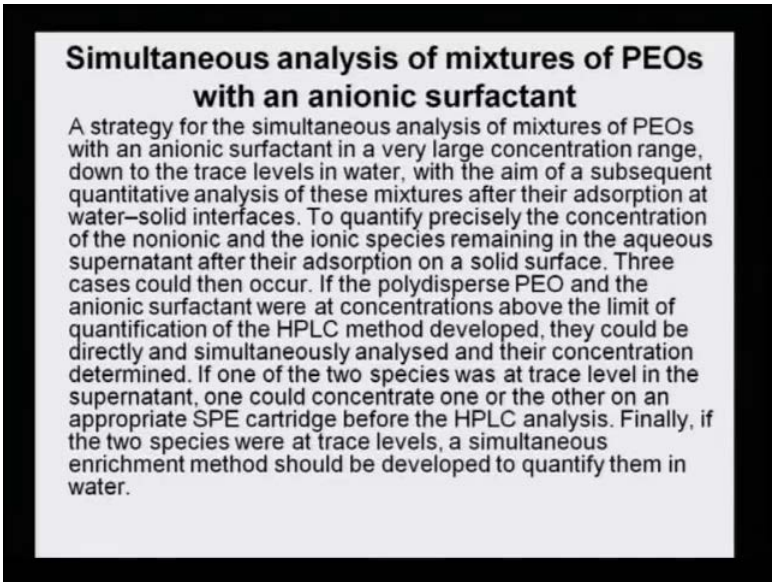
Department of Chemistry

Indian Institute of Technology, Kanpur

Lecture No. # 19

Simultaneous analysis of mixtures of PEOs with anionic surfactant – because as time and again I have told, that these soaps and detergents that are formulated are not with just one single surfactant; there is always a combination. So, the method of analysis also must be so that it is able to identify both the nonionic surfactant and the anionic surfactant simultaneously.

(Refer Slide Time: 00:50)

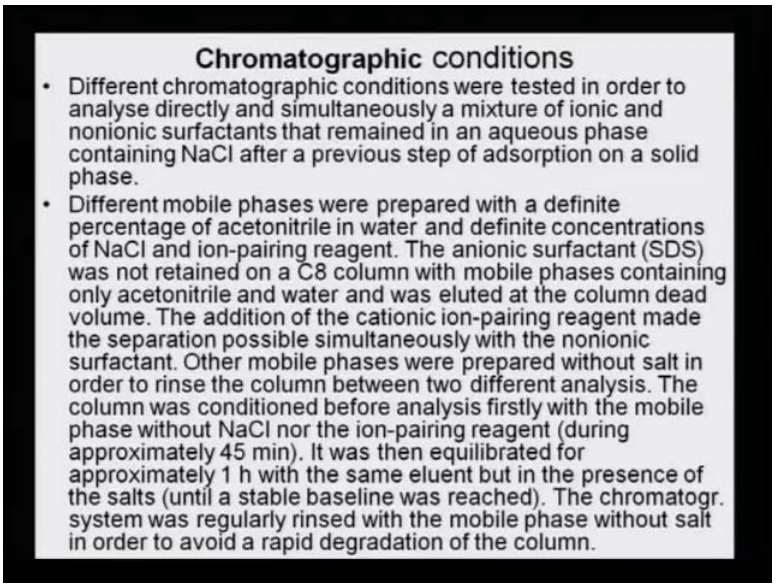


A strategy for the simultaneous analysis of mixtures of PEOs with an anionic surfactant in a very large concentration range, down to trace levels in water, is required; and, with an aim of a subsequent quantitative analysis of these mixtures after their absorption at water-solid interfaces must be taking place. To quantify precisely, the concentration of the noninoic and the ionic species remaining in the aqueous supernatant after their adsorption on the solid surface is what actually is carried out. Three cases could then occur. If the polydisperse PEO and the anionic surfactant were at concentrations above the limit of quantification of the HPLC method developed, they could be detected and

simultaneously analysed, and their concentrations can be determined. If one of the two species was at trace levels in the supernatant, one could concentrate one or the other on an appropriate solid-phase extracting cartridge before the HPLC analysis.

Finally, if the two species were at trace quantities, a simultaneous enrichment method could be developed to quantify them in water. So, now, there could be three possibilities. One possibility is that they are in large quantities and they can be identified by the normal procedure. Another is that if one of them is in substantial quantity, that is, PEO is in substantial quantity, and the anionic surfactant is in trace quantity, then there has to be a method of using different types of solid-phase extracting cartridges for separating them, because the quantities are very different. And, if both them, that is, the last option is that if both these types of surfactants are in trace quantities, even then, there has to be a special technique, which is the simultaneous enrichment method. That means they have to be repeatedly extracted till the concentrations are made enough, so that they can be analysed on the HPLC.

(Refer Slide Time: 03:45)



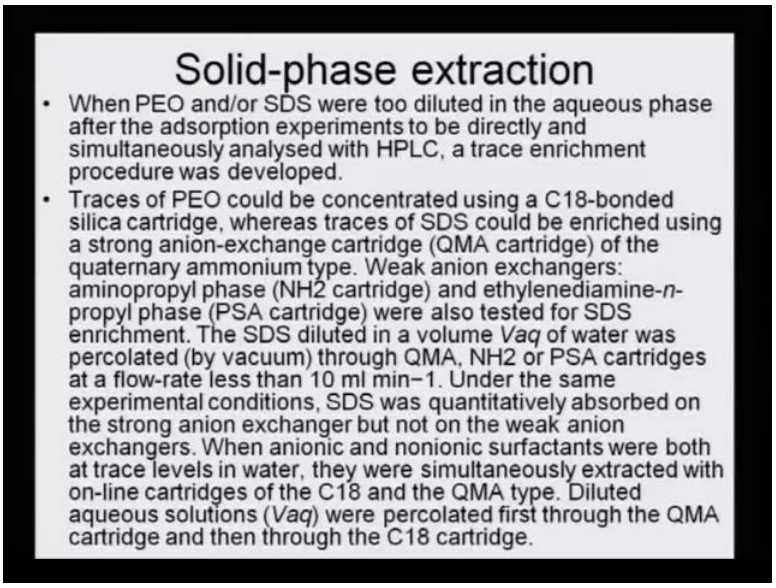
Chromatographic conditions also need to be very typical. As I told you, for every analysis, there is a requirement that a program should be made; that is the analytical program; not the analyzers program. Different chromatographic conditions were tested in order to analyse directly and simultaneously a mixture of ionic and nonionic surfactants

that remained in an aqueous phase containing sodium chloride after a previous step of adsorption on a solid phase.

Different mobile phases were prepared with a finite percentage of acetonitrile in water and definite concentrations of sodium chloride and ion-pairing reagent. The ionic surfactant, that is, the SDS was not retained on a C8 column with mobile phases containing only acetonitrile and water and was eluted at the column dead volume. The addition of a cationic ion-pairing reagent made the separation possible simultaneously with the nonionic surfactant, because ionic surfactant will only couple with cationic reagent. So, when this was done, they could be analysed at the same time.

Other mobile phases were prepared without salt in order to rinse the column between two different analyses. The column was conditioned before analysis firstly with the mobile phase without sodium chloride not the ion-pairing reagent during approximately 45 minutes. It was then equilibrated for approximately 1 hour with the same eluent, but in the presence of the salts until a stable baseline was reached. The chromatographic system was regularly rinsed with the mobile phase without salt in order to avoid a rapid degradation of the column. So, one has to remember, that no reagent can be allowed to stay in the column, because it is bound to degrade the column, and these columns are very expensive. So, they need to be maintained and washed and rinsed very thoroughly. And, between the experiments, that means, between different sampling, they need to be covered and cleaned and rinsed very regularly in order to be able to preserve the column for longer life time.

(Refer Slide Time: 06:41)



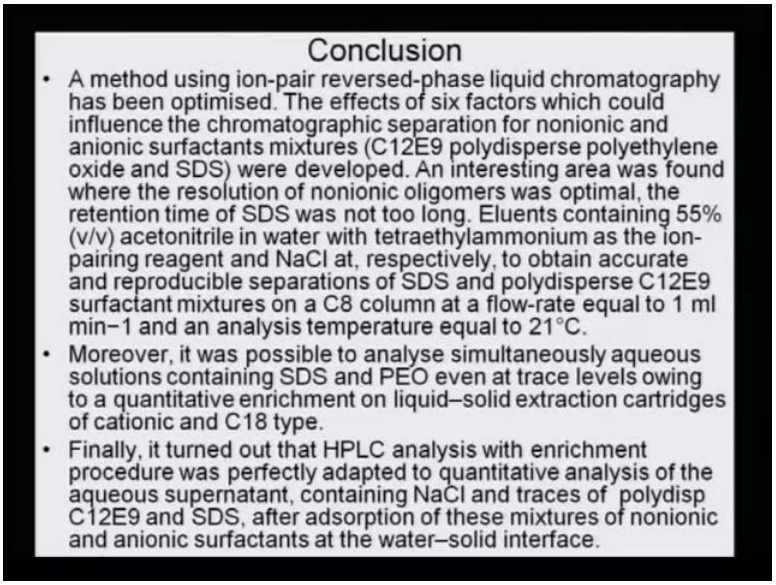
Solid-phase extraction – a little while ago, I was mentioning that when both these nonionic surfactant and anionic surfactant are present simultaneously, there are different solid-phase extractors to be able to extract them from the water medium. When PEO and/or SDS were two diluted in the aqueous phase after the adsorption experiments to be directly and simultaneously analysed with HPLC, a trace enrichment procedure was developed. So, I told you, that the last type of option, when both them are in trace quantities, there is a need for further more adaptation. And, what is that adaptation?

Traces of PEO could be concentrated using a C 18-bonded silica cartridge, because C 18 is a non-polar; and, we are talking about PEO, which is also non-polar. That is why they are compatible; whereas, traces of SDS could be enriched using a strong anionic-exchange cartridge, which is a QMA cartridge of the quaternary ammonium type. Weak anionic exchangers like aminopropyl phase, that is, NH2 cartridge and ethylenediamine-n-propyl phase cartridge, that is, PSA cartridge were also tested for SDS, because SDS is anionic in nature. That is why we needed these different types of cartridge and because PEO is non-ionic. I am again repeatedly telling, because this should be made very clear why these two different types of cartridges are being used.

The SDS diluted in a volume of aqueous water was percolated by vacuum through QMA, NH2 or PSA cartridges at a flow-rate less than 10 ml per minute. Under the same experimental conditions, SDS was quantitatively absorbed on the strong anionic

exchanger, but not on the weak anionic exchanger. When anionic and nonionic surfactants were both at trace levels in water, they were simultaneously extracted with on-line cartridges of the C 8 and the QMA type. Diluted aqueous solution, which is referred as Vaq were percolated first through the QMA cartridge and then through the C 18 cartridge. So, one-by-one, they are passed through it, and they will specifically only adsorb either the anionic one or the ionic.

(Refer Slide Time: 09:22)



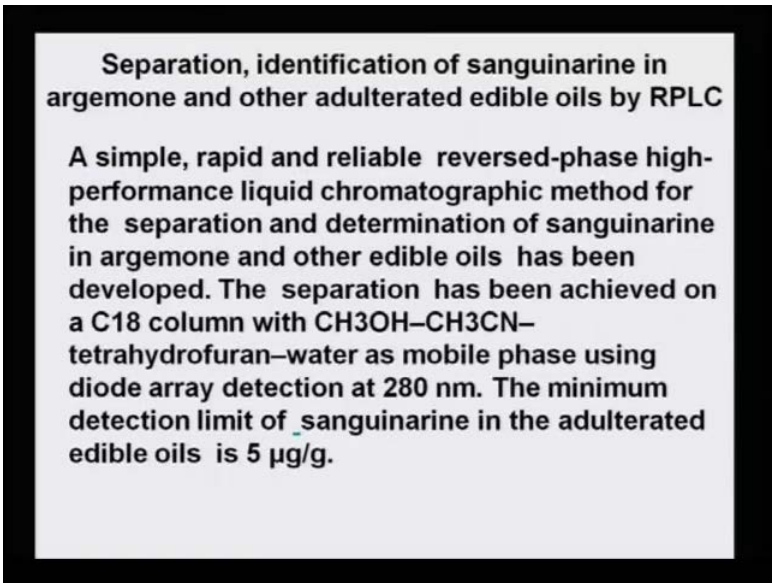
Conclusion – a method using ion-pair reversed-phase liquid chromatography has been optimised. The effects of six factors which could be influence the chromatographic separation for nonionic and anionic surfactants mixtures have been discussed very well. Moreover, it was possible to analyze simultaneous aqueous solution containing SDS and PEO even at trace levels owing to a quantitative enrichment on liquid-solid extraction cartridges of cationic and C 8 type. Just now, I mentioned that why these two different types of cartridges are required, because one would specifically only adsorb the PEO, the other one would specifically adsorb the SDS; and, that is why.

An interesting area was found where the resolutions of nonionic oligomers were optimal; the retention time of SDS was not too long. Eluents containing 55 percent, that is, volume by volume acetonitrile in water with tetraethylammonium as the ion-pairing reagent and sodium chloride at, respectively, to obtain accurate and reproducible separations of SDS and polydisperse C12E9 surfactant mixtures on a C8 column at a

flow-rate equal to 1 ml per minute and an analysis temperature equal to 21 degree centigrade could be achieved. These are so many factors, which need to be considered, because each compound is very specific and needs to be separated from the other compounds. Because the water has an array of different types of surfactants, they are broadly classified under two classes, and these classes need to be separated by different means.

Finally, it turned out that HPLC analysis with enrichment procedure was perfectly adapted to quantitatively analyse the aqueous supernatant, containing sodium chloride and traces of polydisperse nonionic surfactants, that is, C12E9 and SDS, which is an anionic surfactant, after adsorption of these mixtures of nonionic and anionic surfactants at the water-solid interface. So, where the water is coming in conjunction with the solid interface, only there, this extraction procedure takes place, and then finally, it is analysed on the HPLC machine; because time and again I have mentioned, that extraction must be very efficient for the extracted material to be analyzed and data to be derived.

(Refer Slide Time: 12:40)

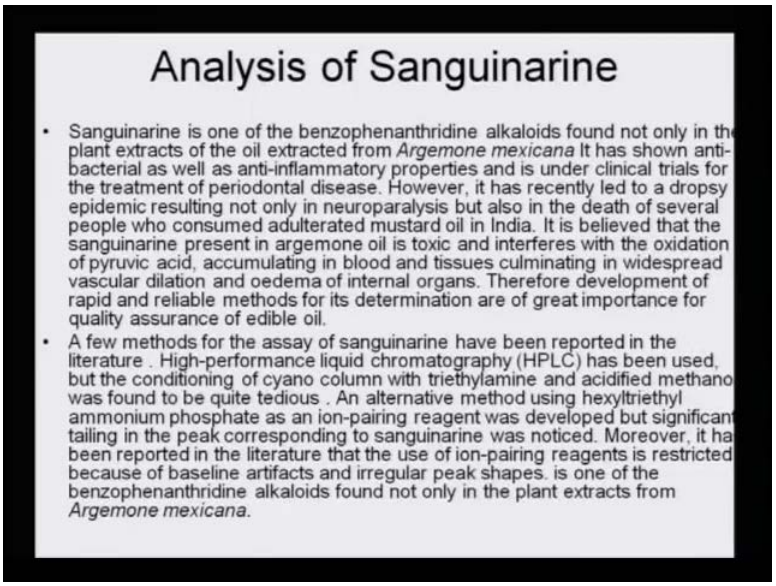


Similarly, there are methods, which need to be developed for very special techniques. Separation identification of sanguinarine in argemone and other adulterated edible oils by RPLC – this is a very crucial and important type of analysis, and I thought that I must bring to your notice that how this argemone oil was added to mustard oil and that created a lot of health problems to the people who consumed this contaminated or adulterated

oil. Now, this was a big issue, because a lot of people fell sick, some were even dead, because of the consumption of this adulterated oil. And, the culprit molecule in this adulterated oil was sanguinarine, which came from the argemone. And, the argemone seed and mustard seed look very alike. So, they were ground together and that is how people who do this kind of adulteration business can cause havoc to the society.

A simple, rapid and reliable reversed-phase high performance liquid chromatographic method for the separation and determination of sanguinarine in argemone and other edible oils has been developed. So, there was a need that how to identify this particular crucial compound. The separation has been achieved on a C18 column, which is reversed-phase column with solvent system, such as methanol and acetonitrile, that is, CH3OH and CH3CN with tetrahydrofuran and water. That means there are four solvents and combination of these four solvents was able to separate the sanguinarine very clearly using diode array detector at 280 nanometer. The minimum detection of limit of sanguinarine in the adulterated edible oil was 5 microgram per gram. So, as small as this quantity also could be traced and analysed by this RPLC method.

(Refer Slide Time: 15:17)

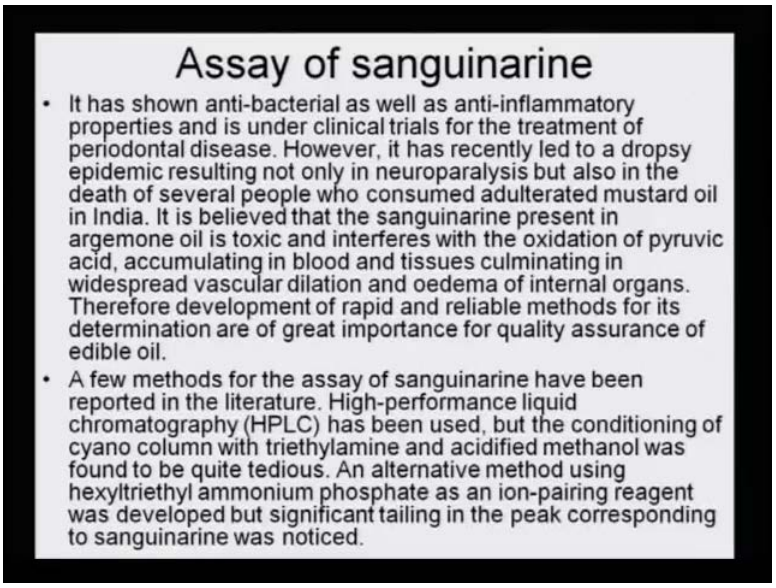


I will give a little detail about this analysis, because sanguinarine is very crucial compound. Sanguinarine is one of the benzophenanthridine alkaloids found not only in the plant extracts of the oil extracted from Argemone mexicana. It has shown anti-bacterial as well as anti-inflammatory properties and is under clinical trials for the

treatment of periodontal diseases. However, it has recently led to a dropsy epidemic resulting not only in the neuroparalysis, but also in the death of several people who consumed adulterated mustard oil in India. I was just telling you a while ago this whole story. It is believed that the sanguinarine present in argemone oil is toxic and interferes with the oxidation of pyruvic acid, accumulating in blood and tissues culminating in widespread vascular dilation and oedema of internal organs. Therefore, development of rapid and reliable methods for its determination is of great importance and quality assurance of edible oil is of prime importance.

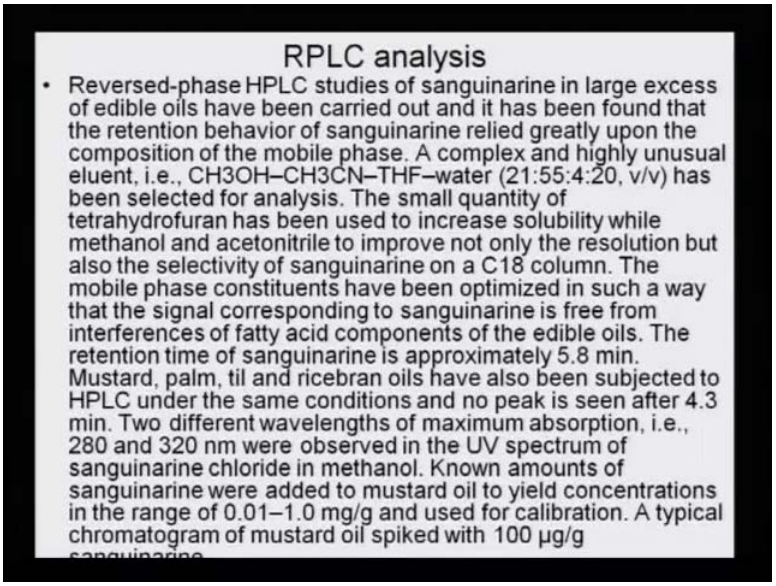
A few methods for the assay of sanguinarine have been reported in the literature. However, high-performance liquid chromatography, that is, HPLC has been used, but the conditioning of cyano column with triethylamine and acidified methanol was found to be quite tedious. An alternative method using hexyltriethyl ammonium phosphate as an ion-pairing reagent was developed, but significant tailing in the peak corresponding to sanguinarine was noticed. So, these were some of the earlier method, which were first tried out for sanguinarine analysis, but it was found that they are not really befitting the need and the accuracy for which sanguinarine needs to be analysed. Moreover, it has been reported in the literature that the use of ion-pairing reagents is restricted because of the baseline artifacts and irregular peak shapes, is one of the benzophenanthridine alkaloids found not only in the plant extracts from the argemone. So, it was not a very identifiable kind of peak. Again I will repeat that for a pure compound, the peak that should be shown by either the GC chromatograph or the HPLC chromatogram should be very sharp. Only then, it will be considered that it is representing one compound; otherwise, it will not be appropriate to say whether it is a signal that has come from one compound or from several compounds that have given a broad peak.

(Refer Slide Time: 18:44)



Assay of sanguinarine – assay means testing procedure. It has shown anti-bacterial as well as anti-inflammatory properties and is under clinical trials for the treatment of periodontal disease. However, it has recently led to a dropsy epidemic resulting not only in neuroparalysis, but also in the death of several people who consumed it. A few methods of the assay of sanguinarine have been reported in the literature. However, the method that will be described is of the latest type and most accurate for sanguinarine.

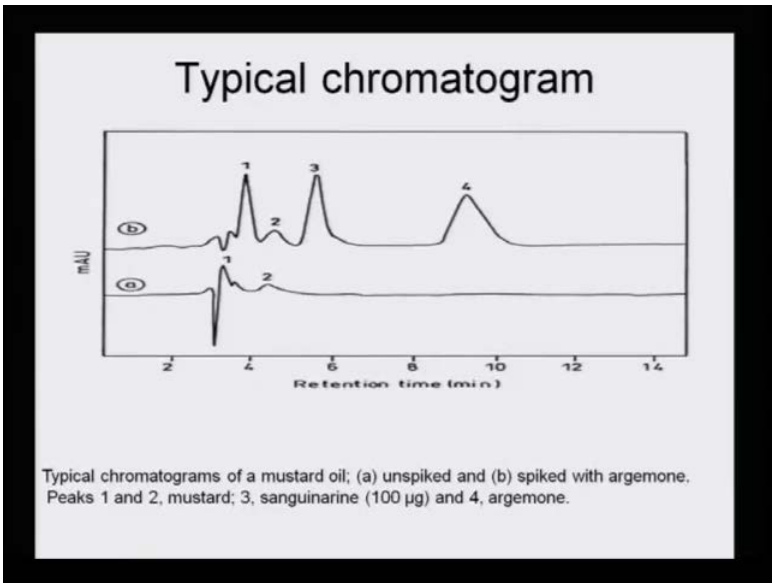
(Refer Slide Time: 19:22)



RPLC analysis – the latest method shows that reversed-phase high pressure liquid chromatographic studies on sanguinarine in large excess of edible oils have been carried out and it has been found that the retention behavior of sanguinarine relied greatly upon the composition of the mobile phase. As what I was talking a little while ago, that the mobile phase is a very typical quaternary system of mobile phase, which includes methnol, acetonitrile, tetrahydrofuran and water in the percentage composition of 21 is to 55 is to 4 is to 20 volume by volume, and that is what the mobile phase has been selected for. The small quantity of tetrahydrofuran has been used to increase the solubility while methnol and acetonitrile to improve not only the resolution, but also the selectivity of sanguinarine on to the column of C18.

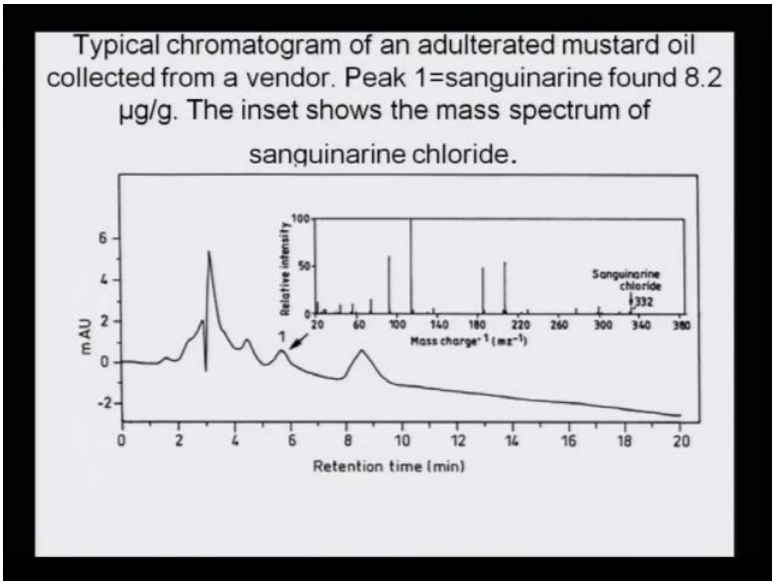
The mobile phase constituents have been optimized in such a way that the signal corresponding to sanguinarine is free from interferences of other fatty acid components of the edible oils. The retention time of sanguinarine is approximately 5.8 minutes. Mustard, palm, til, ricebran oils have also been subjected to HPLC under the same conditions and no peak is seen after 4.3 minutes. Two different wavelengths of maximum absorption, that is, at 280 and 320 nanometers were observed in the UV spectrum of the sanguinarine chloride in methanol. Known amounts of sanguinarine were added to mustard oil to yield concentrations in the range of 0.01 to 1.0 milligram per gram and used for calibration. So, as I told you, that when we are analyzing on any calibration method, there has to be a calibration graph that needs to be drawn. Calibration graphs are prepared by making standards of known concentrations and then trying to see whether they are showing a linear response on the machine. And, that also reflects whether the analysis is been carried out properly; it also reflects that the samples were prepared accurately, because it is a very simple fundamental idea that the concentration is directly proportional to the absorbance. And so, if the UV absorbance shows certain concentration, that will be reflecting from the molecular species that is present in the standard solution. A typical chromatogram of mustard oils spiked with 100 microgram per gram of sanguinarine can then be tested as a test sample.

(Refer Slide Time: 22:52)



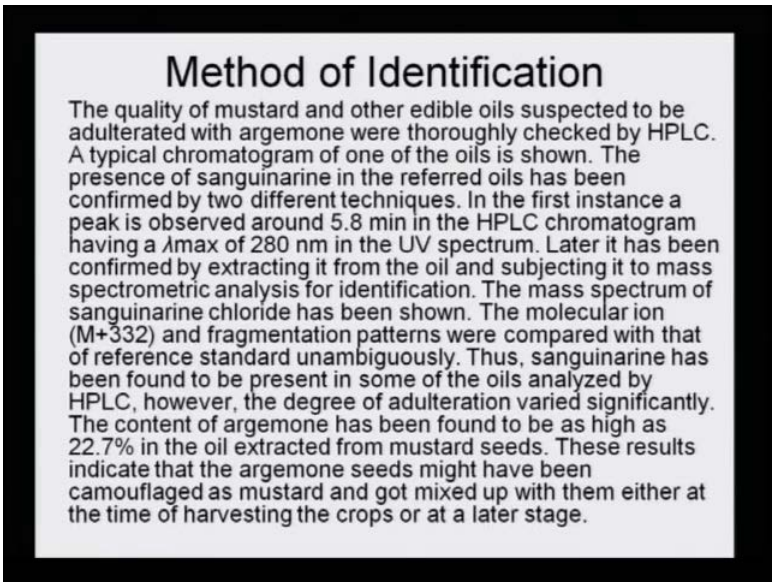
Now, you see that this is a typical chromatogram of mustard oil. The first one shows, that is, the a shows that it is unspiked. Sanguinarine has not been added additionally to it. And, the second one, that is, the b chromatogram shows that it has been spiked with argemone. That means sanguinarine has been added, and therefore, it shown very distinct peaks as what you can see in the slide; that peak 1 and 2 show mustard oil, and peak 3 is a very significant peak of sanguinarine, which has been coming from the spiked 100 microgram to that. And, fourth is the peak that comes from the argemone compound.

(Refer Slide Time: 23:48)



Typical chromatogram of an adulterated mustard oil collected from a vendor. Peak 1 shows the sanguinarine found in concentration at 8.2 microgram per gram. And, in the inset, there is a mass spectrum of sanguinarine chloride. So, this is a very typical chromatogram of the sanguinarine compound, and in the inset, is the mass spectral data.

(Refer Slide Time: 24:21)

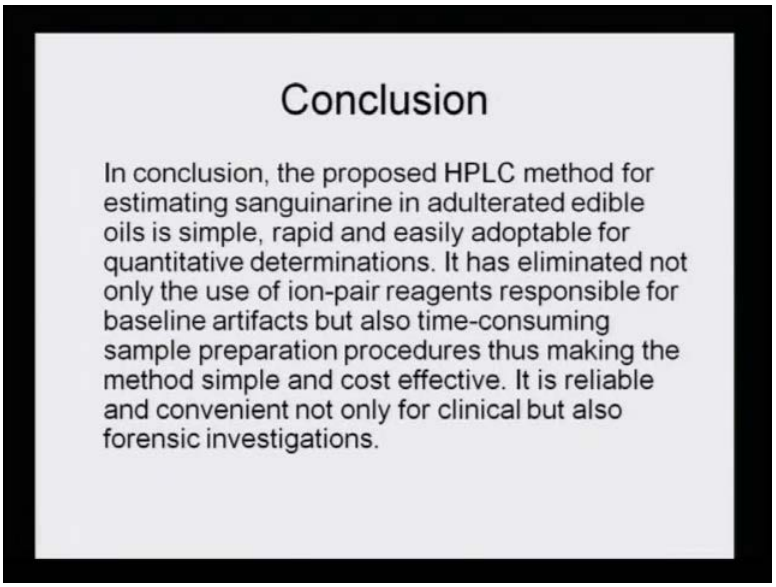


Method of detection and identification – the quality of mustard oil and other edible oils suspected to be adulterated with argemone were thoroughly checked by HPLC. A typical chromatogram of one of the edible oils is shown. The presence of sanguinarine in the referred oil has been confirmed by two different techniques. I will like to draw your attention, that suppose in one method, one tries to find out that there is some kind of peak that is appearing quite similar to that of the adulterated molecule, but that may not be sufficient to validate the process. For validation or confirmation of presence of that particular adulterant molecule, it is necessary to have two methods to testify it. That would be the best way and the most accurate and acceptable way. In the first instance, a peak is observed around 5.8 minutes in the HPLC chromatogram having a lambda max of 280 nanometer in the UV spectrum.

Later, it has been confirmed by extracting it from the oil and subjecting it to mass spectrometric analysis for identification. So, you see, that mass spectrometry is always the most conclusive and decisive method to be able to identify the compound completely. Otherwise, there is always a doubt in the mind of analyzer that it may be

sanguinarine or it may not be sanguinarine, because the peak does not come with any kind of labelling. It is just that under the same condition, the retention time, that is, the appearance of the peak time in a chromatogram is similar. That is the only similarity. But, based on that similarity, one cannot say that this is the compound. So, for confirmation, MS is the only answer. The mass spectrum of sanguinarine chloride has been shown. The molecular ion peak shows M plus 332 and fragmentation patterns were compared with that of the reference standard unambiguously. Thus, sanguinarine has been found to be present in some of the oils analyzed by HPLC, however, the degree of adulteration varied significantly. The content of argemone has been found to be as high as 22.7 percent in the oil extracted from mustard seeds. These results indicate that argemone seeds might have been camouflaged as mustard and got mixed up with them either at the time of harvesting crops or at a later stage or may have been deliberately added.

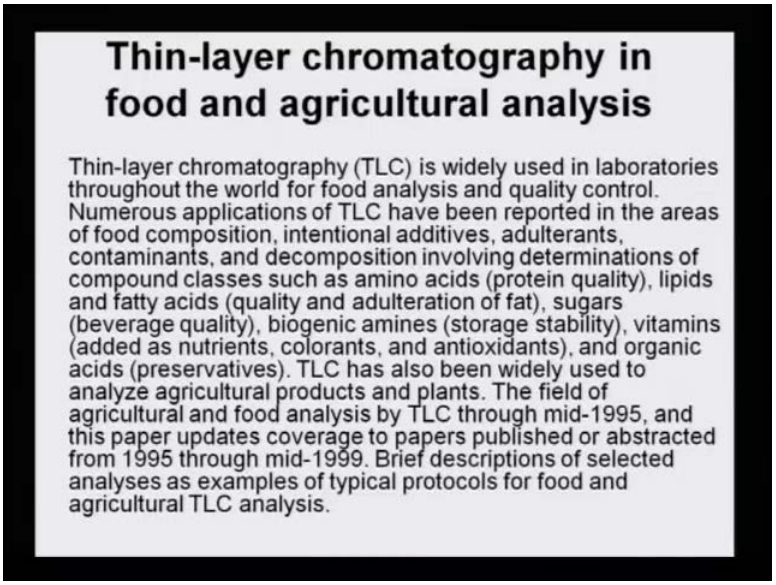
(Refer Slide Time: 27:43)



So, to conclude this particular procedure, in conclusion, the proposed HPLC method for estimating sanguinarine in adulterated edible oils is simple, rapid and easily adaptable for quantitative determination. It has been eliminated not only the use of ion-pair reagents responsible for baseline artifacts, but also time-consuming sample preparation procedures thus making the method simple and cost effective. It is reliable and convenient not only for clinical purposes, but also for forensic investigations. Now, I am telling you time and again, different types of methods, because it is important for you to

understand that how important it is to analyse each compound, and each compound needs different treatise and a different kind of experimental and machine parameters, so that they can be detected even in trace quantities most effectively.

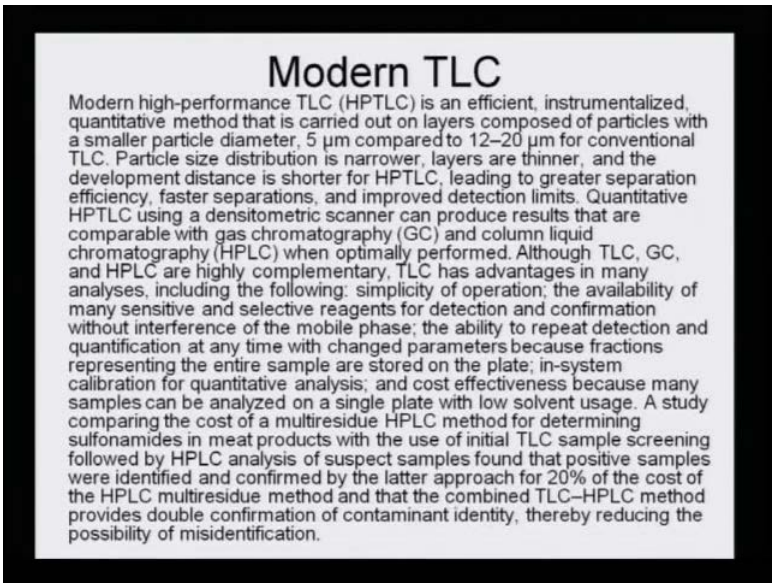
(Refer Slide Time: 29:24)



Thin layer chromatography – how does it play a role in food and agricultural analysis. Though we have talked a little bit about thin layer chromatography in the past, I would like to draw your attention to the fact that thin layer chromatography or TLC is widely used in the laboratories throughout world for food analysis and quality control. Numerous applications of TLC have been reported in the areas of food composition, intentional additives, adulterants, contaminants, and composition involving determinations of compound of classes, such as amino acids, that is, protein quality, lipids and fatty acids (quality and adulteration of fat), sugars, that is, beverage quality, biogenic amines, that is, storage stability, vitamins (added as nutrients, colorants and antioxidants), and organic acids (preservatives).

TLC has also been widely used to analyze agricultural products and plants. The field of agricultural and food analysis by TLC was invented way back in 1995, and this paper updates coverage to papers published or abstracted from 1995 through mid of 1999. From that era, these papers have been taken only to introduce you to the fact that how TLC can help in food and agricultural analysis. Brief descriptions of some of methods and typical protocols for food and agricultural TLC analysis will be discussed.

(Refer Slide Time: 30:47)



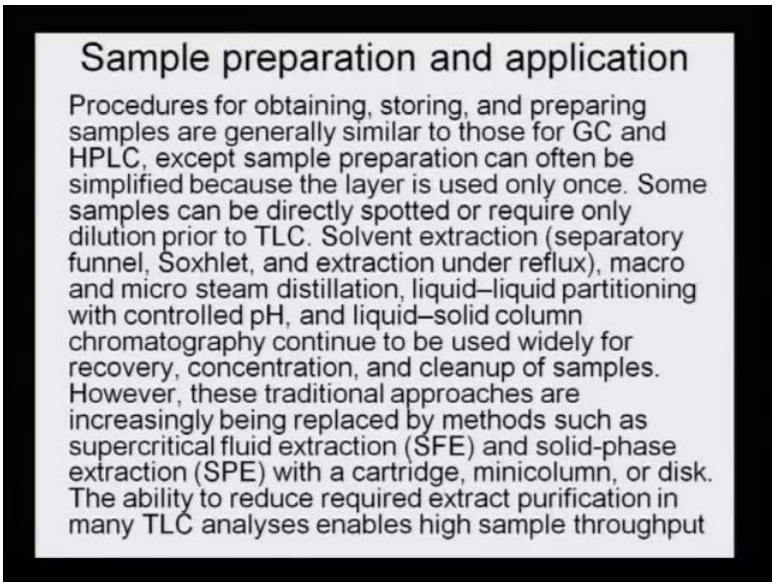
Modern TLC – modern high-performance TLC or HPTLC is an efficient instrumentalized, quantitative method that is carried out on layers composed of particles with a smaller particle diameter, that is, 5 micrometer compared to 12 to 20 micrometer for conventional TLC. Particle size distribution is narrower, layers are thinner, and the development distance is shorter for HPTLC, leading to greater separation efficiency, faster separations, and improve detection limits. Quantitative HPTLC using a densitometer scanner can produce results that are comparable with the gas chromatography. So, you see that this modern TLC, which will be called as high pressure thin layer chromatography is as at par with GC, and it can give as efficient result, because of this densitometer scanner; and, liquid chromatography also can be compared with optimal perform.

Although TLC, GC and HPLC are highly complementary, TLC has advantages in many analyses, including the following: simplicity of operation; the availability of many sensitive and selective reagents for detection and confirmation without interferences of the mobile phase; the ability to repeat detection and quantification at any time with changed parameters, because fractions representing the entire sample are stored on the plate; in-system calibration for quantitative analysis; and cost effectiveness because many samples can be analyzed on a single plate with low solvent usage. So, it has so many advantages; that it is very reliable, first thing; and second thing, it is cost effective; it can do quantitative calibrations also. So, one can not only… Earlier, when I was

talking about simple TLC, I had mentioned very clearly that TLC is a qualitative method. But, here with the help of these, densitometer scanner and this specialization of its preparation and its miniaturization and its mechanisation, it has been converted into a full-fledged instrument rather than a small plate. And, that has brought about so much of its efficiency and its result bearing factor.

A study comparing the cost of multiresidue HPLC method for determination of sulphonamides in meat compounds with the use of initial TLC sample screening followed by HPLC analysis of suspect samples found that positive samples were identified and confirmed by the latter approach for 20 percent of the cost of the HPLC multiresidue methods and that the combined HPTLC and HPLC method provides double confirmation of the contaminant identity, thereby reducing the possibility of misidentification. So, you see that if it is combined, if an HPTLC and HPLC are carried out one after the other, it completely removes any doubt of any misidentification, and thus, it is of utmost importance when screening needs to be done, when very fast analysis has to be done. And, other beauty of this HPTLC is that once separated, the plates can be stored, and they can be analyzed on different wave lengths if required if the optimisation of the results needs to be carried out.

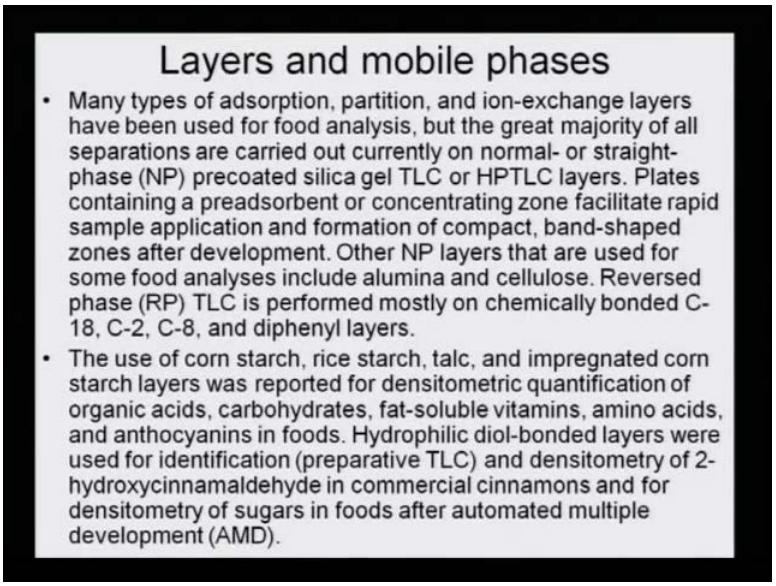
(Refer Slide Time: 35:27)



Sample preparation and application – as what I would again and again say that every procedure has a very typical method of sample preparation. Procedures for obtaining,

storing and preparing samples are generally similar to those for GC and HPLC, except sample preparation can often be simplified because the layer is used only once. Some samples can be directly spotted or require only dilution prior to TLC. Solvent extraction, that is, separatory funnel, Soxhlet, and other types of extraction under reflux can be carried out; macro or micro steam distillation also can be carried out; liquid-liquid partitioning at controlled pH, and liquid-solid column chromatography continue to be used widely for recovery, concentration, and cleanup of the samples. However, these traditional approaches are increasingly being replaced by methods, such as supercritical fluid extraction, that is, the SFE, the solid-phase extraction, that is, the SPE with the cartridge, minicolumn, or disk. The ability to reduce required extract purification in many TLC analyses enables high samples throughput.

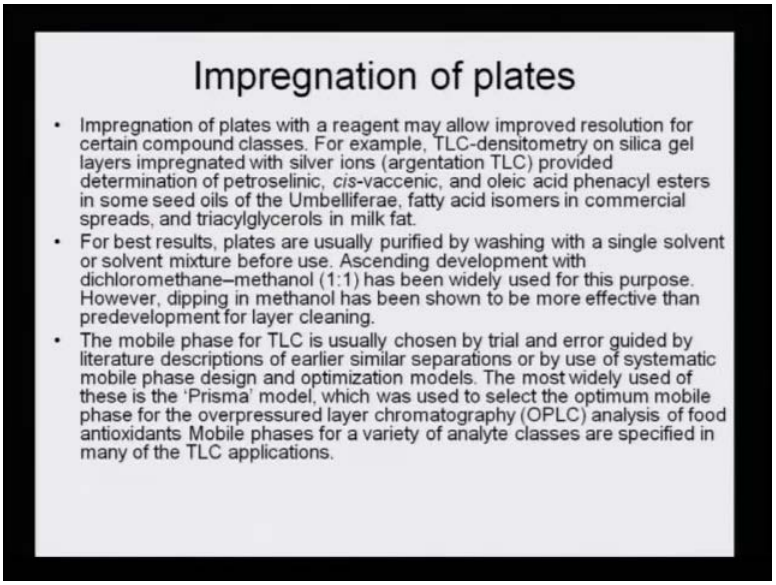
(Refer Slide Time: 36:37)



Layers and mobile phases – obviously, the entire functioning takes place in these layers and mobile different phases. Many types of adsorption, partition and ion exchange layers have been used for food analysis, but the great majority are separations are carried out currently on normal- or straight-phase precoated silica gel TLC or HPTLC layers. Plates containing a preadsorbent or concentrating zone facilitate rapid sample application and formation of compact, band-shaped zones after development. Other normal-phase layers that are used for some food analyses include alumina and cellulose. Reversed phase, that is, the RP TLC is performed mostly on chemically bonded C-18, C-2, or C-8, diphenyl layers.

The use of corn starch, rice starch, talc and impregnated corn starch layers was reported for densitometric quantification of organic acids, carbohydrates, fat-soluble vitamins, amino acids, and anthocyanins in foods. Hydrophilic diol-bonded layers were used for identification of preparative TLC and densitometry of 2-hydroxycinnamaldehyde in commercial cinnamons and for densitometric identification of sugars in food after automated multiple development, that is, the AMD. So, there are many layers that are used; there are many mobile phases that need to be very compound specific. And, the impregnation of the plates is also a very special technique that needs to be understood.

(Refer Slide Time: 38:36)



Impregnation of plates with a reagent may allow improved resolution for certain compound classes. For example, TLC-densitometer on silica gel layers impregnated with silver ions, that is, the argentation of TLC, provided determination of petroselinic, cis-vaccenic, and oleic acid phenacyl esters in the same oil of the Umbelliferae, fatty acid isomers in commercial spreads, and triacylglycerols in milk fat. So, there are many methods that need to be modified and there are a variety of analytical classes, where TLC applications are very successfully done.