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NEW CONSIDERATIONS IN DETECTOR–APPLICATION RELATIONSHIPS

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SUMMARY

This report relates new ways of using refractometer and ultraviolet (UV) detectors in liquid chromatography (LC) which involve less sample preparation and handling. A new cell design is proposed which extends the sensitivity of UV detectors by correcting for refractive index effects as light passes through a cell. Finally, a technique called "trace enrichment" is proposed for concentrating non-polar organics, in very low concentration, from polar sources (river water) for analysis by LC.

INTRODUCTION

The explosive growth and broad utilization of high-pressure liquid chromatography (LC) is due, at least in part, to the development of high-performance detectors typified by the high-sensitivity, selective ultraviolet (UV) detector and the high-performance universal differential refractometer. In this paper we will consider some of the factors which influence the choice of detector and also examine how the detector can help contribute to the desired separation. Furthermore, we will see examples of some original approaches to matching the application to the detector, as well as matching the detector to the application.

One of the key contributions of the detector concerns the analysis of a complex matrix. In modern high-pressure LC, the ability to make direct injections with only a modest degree of sample preparation is indeed a considerable advantage over earlier methodologies including even versatile gas chromatography (GC). GC is far less forgiving with respect to foreign materials injected into the instrument. However, this advantage can also lead to a problem because of the tendency of some compounds in the complex matrix to obscure or interfere with the compound of interest. A classical example is in the analysis of nutrients such as vitamins in food matrices.

EXPERIMENTAL

All work was performed on a Waters Associates' Model 244 liquid chromatograph consisting of a Model 440 Absorbance Detector, R401 Refractometer and M6000A Solvery Delivery System. In addition, for gradient and flow programming, a Model 660 Programmer and a second M6000A were used. All injections were made with the Model U6K variable volume injector. All separations were perfomred on

either a 4-mm × 30-cm μBondapak C_{18} column or a μBondapak/Carbohydrate column.

RESULTS

Vitamin analysis is an area which is receiving increasing attention due to new, stringent government regulations, especially in the United States. This frequently leads to the analysis of nutrients in products such as infant formula extract. The standard methods published in the government guidelines involve a rather extensive saponification in order to free the vitamins from the sterols present in the milk. Unfortunately, as shown in Fig. 1, there is still considerably carry-over of the sterols, which have elution characteristics comparable to some fat-soluble vitamins such as vitamin A palmitate. Since the sterols also absorb at the traditional monitoring wavelength of 254 nm, analysis is extremely difficult and quantitation is impossible.

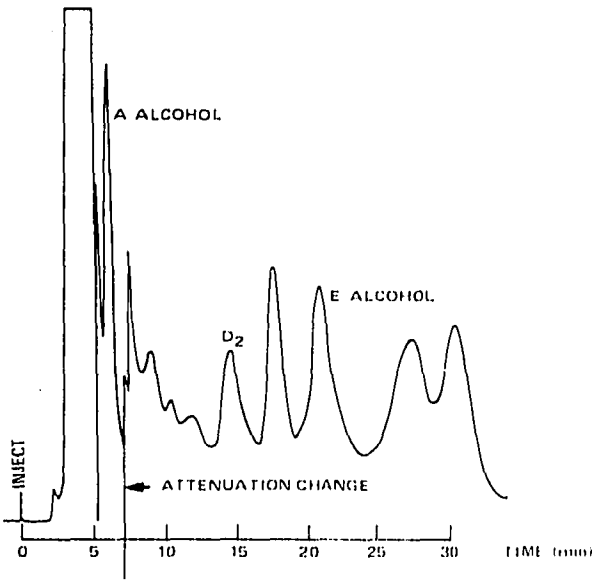

Fig. 1. Infant formula extract. The chromatograph was operated isocratically at 1 ml/min with methanol–water (95:5). Column: 4 mm × 30 cm μBondapak C_{18}. Sample: 90 μl extract in methanol. Detector, UV, 0.16–0.04 a.u.f.s. (254 nm).

One approach is to side-step the problem rather than pursuing the traditional sample work-up to its ultimate. After considering the characteristics of the interfering compounds as well as the compounds to be analyzed, another sample of the same infant formula extract was monitored at two different wavelengths. As shown in Fig. 2, at the traditional wavelength of 254 nm there is no response which can be correlated with vitamin A palmitate. However, at 334 nm, a monitoring wavelength close to the absorbance maximum of the A vitamins, but a wavelength at which the sterols tend to absorb weakly if at all, we see a strong response corresponding to vitamin A palmitate. There is complete circumvention of the interference due to the co-eluting

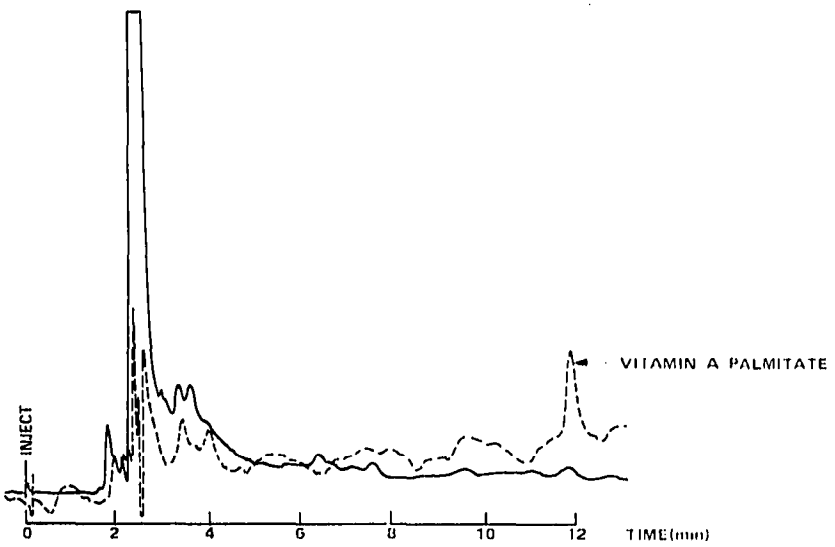

Fig. 2. Infant formula extract. The chromatograph was operated in the gradient mode. Solvents: (A) 1 % ammonium carbonate in water; (B) methanol. Linear gradient: 95% B to 100% B. Column: 4 mm × 30 cm μBondapak C_{18}. Sample: diethyl ether extract. Flow-rate: 1.5 ml/min. Detector: UV, 334 nm 0.04 a.u.f.s., 254 nm 0.16 a.u.f.s.

species. This is an example of selectively tuning out interfering species, providing the detector has that capability. Essentially we have achieved an effective separation, even though in the strictest physical sense the separation was not complete.

The next point to consider is the nature of the sample itself. There are many samples which tend to be better suited for one type of detection than for another. Consider, for example, the saccharides. Traditionally, these have been analyzed by GC, in which it has been necessary to exhaustively remove the water present and also to prepare derivatives in order to volatilize the saccharides. Unfortunately, this results in the formation of anomers and rearrangements so that we are no longer dealing with just the original compounds but also with many variations of them. This makes the subsequent interpretation of the chromatogram exceedingly complex. By contrast, using a sepcial-purpose LC column, μBondapak/Carbohydrate, it is now possible to separate the sugars by direct injection without ever having to remove them from their original aqueous matrix. There is no derivatization. As shown in Fig. 3, the simple saccharides are well resolved rapidly with direct injections. These are the lower sugars or simple saccharides typically present in hydrolyzed corn syrup. They are used in brewing to shorten the worting process since these are the fermentable sugars. The saccharides do not have any characteristic UV absorbance in the typical use range. Consequently, the differential refractometer is the appropriate detector for this particular sample. A high-sensitivity deflection-type refractometer is well suited to handling the usual concentrations and the higher flow-rates typically encountered with the sugar separations in order to achieve rapid analyses.

Going beyond the simple saccharides, there is a great deal of interest in the higher saccharides or the oligosaccharides produced in the hydrolysis of corn starch. In this case, Fig. 4, it is necessary to increase the flow-rate during the course of the

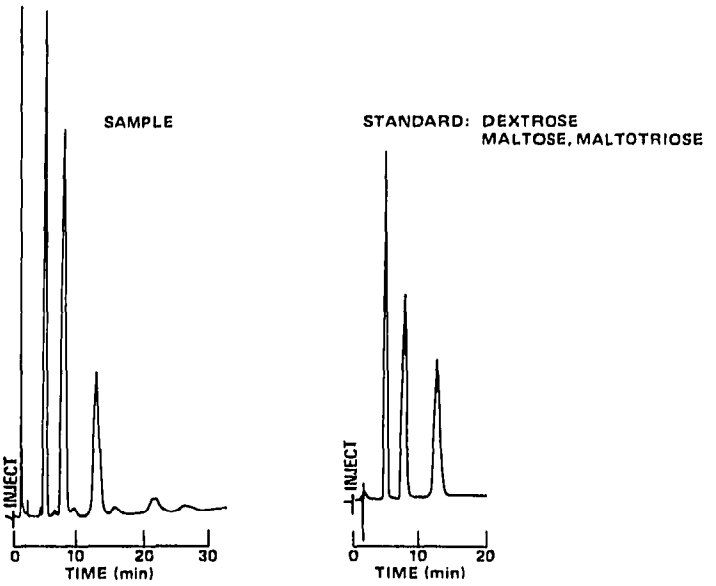

Fig. 3. Corn syrup. The chromatograph was operated isocratically at 2.0 ml/min using water–acetonitrile (35:65). Detector: RI, 8×. Column: 4 mm × 30 cm μBondapak/Carbohydrate. Sample: syrup.

separation in order to cause the higher-molecular-weight saccharides to elute within a reasonable period of time. Since changing the solvent strength (*i.e.*, gradient elution) is extremely difficult using a refractive index detector, the technique of increasing flow-rate (flow programming) greatly extends the usefulness of this detector to samples eluting over a wide range. In the example, the flow is increased up to a maximum of 4 ml/min according to the profile indicated on the chromatogram. The baseline is very stable throughout the entire run. In this example the high flow stability and sensitivity of the refractometer were well suited to the nature of the sample and requirements of the separation.

Another very important aspect of LC detection is the question of sensitivity. One limit on sensitivity is noise, presuming that a signal-to-noise factor of two is considered the minimum detectable limit for practical purposes. In the investigation of the sources of noise in absorbance detection, it has been often reported that various refractive index effects within the flow cell contribute to detector noise[1]. There are a number of ways in which this occurs. The fundamental mechanism involves the fact that any change in refractive index as light enters the flow cell or within the flow cell will cause the light in the cell to be bent. When this occurs, some of the light which would normally pass directly through the cell strikes the cell wall instead. Since there is very little reflection in the UV range, this light is lost. Unfortunately, the detector has no way of distinguishing between light which is lost (because it has been deflected enough to strike the cell wall) and that which is actually absorbed by some species present in the flow cell. The net result in either case is a displacement in the baseline signal. Since there are transient effects within the cell which give rise to different refractive index phenomena, the amount of light striking the cell wall fluctuates and therefore noise is generated.

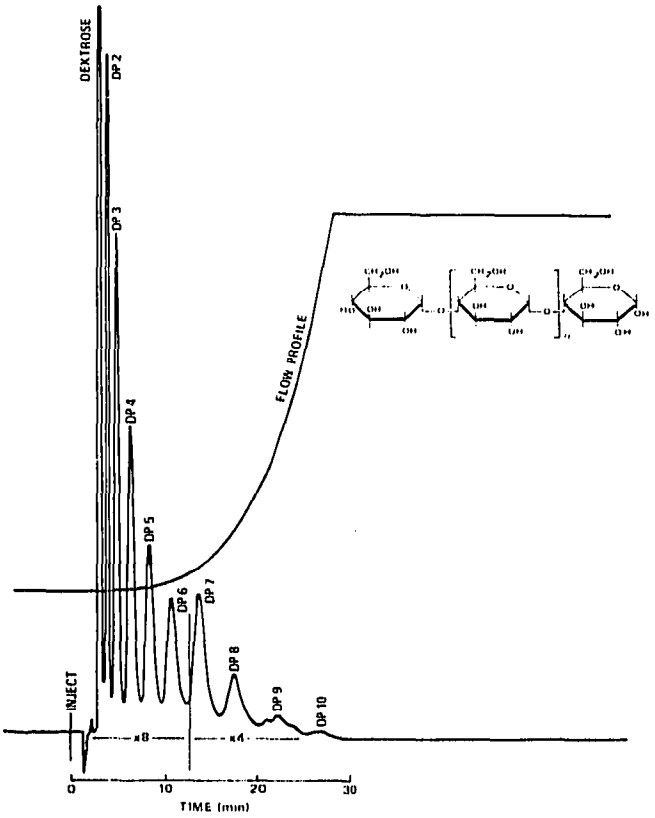

Fig. 4. Starch hydrolysate (oligosaccharides). The chromatograph was operated in the flow-programming mode. Solvent: water–acetonitrile (35:65), flow programmed from 2–4 ml/min by Curve 7 on the Model 660 programmer. Column: 4 mm × 30 cm μBondapak/Carbohydrate. Detector: RI, 8×, 4×.

As indicated in Fig. 5, there are two different domains within the cell where this bending and loss of light can occur. The first is where the light passes from the quartz window into a solvent of lower refractive index. Some light-bending occurs in this region, resulting in light striking the wall and being lost. This can sometimes be compensated by offset correction. The other refractive index transients occur within the cell itself. These are caused by variations in flow or pressure, temperature gradients, or solvent gradients due to incompletely mixed solvents or just the normal gradient profile of solvent programming. All of these effects can change the refractive index in the cell. Furthermore, the sample itself can also contribute to these refractive index transients. The net effect is to cause additional deflection of light onto the cell wall with a resulting contribution to noise. We term these various phenomena the "liquid lens" effect, because they all operate as if there were a lens actually placed in the liquid. These are transient effects. Although we have drawn the lens as a diverging lens, the effect would equally be disruptive if a converging lens was used to model the phenomenon. Then some of the light which "normally" should hit the wall will not

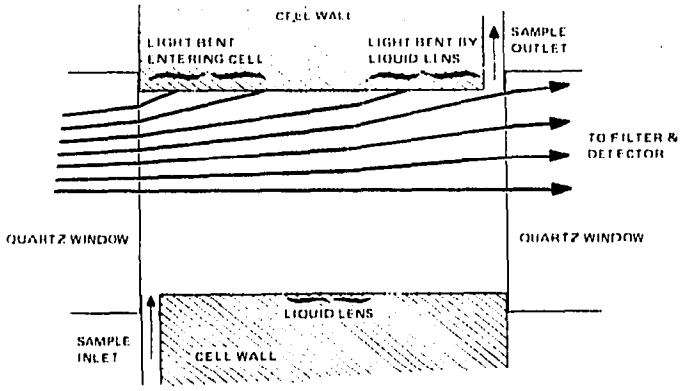

Fig. 5. Schematic of conventional flow cell.

and additional light reaching the detector has the same effect of disrupting the baseline and creating noise. These are phenomena which occur within the flow cell and which cannot be corrected merely by an offset.

Our research on studying these effects has led us to a configuration in which the walls of the cell actually diverge to accommodate the worst possible case of light-bending (Fig. 6). Consequently, with this configuration every bit of light which enters the flow cell will leave the flow cell unless there is actual true absorbance occurring. This provides a much more stable baseline and also permits truer operation of gradient in which pronounced extremes of refractive index change have traditionally caused strange baseline effects with attendant problems in the interpretation of the gradient. This configuration results in improved baseline stability and permits high-sensitivity monitoring as demonstrated by the separation of a linoleic reaction mixture monitored at 0.005 a.u.f.s. (Fig. 7).

An example of the manner in which this configuration can correct for refractive index phenomena is indicated in a blank gradient of methanol–water which was run without any column in place. In Fig. 8 the upper trace is from a conventional flow cell. It produces a very strange baseline, which actually tends to dip below the original

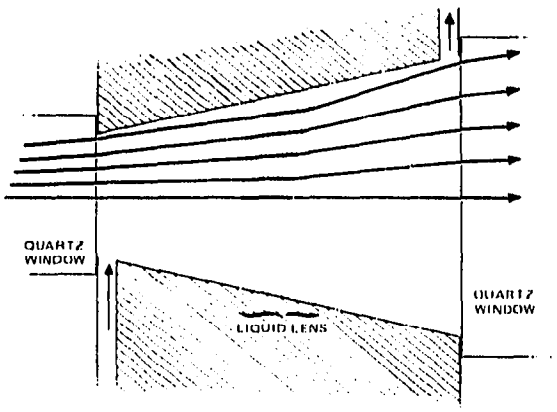

Fig. 6. Schematic of a tapered flow cell.

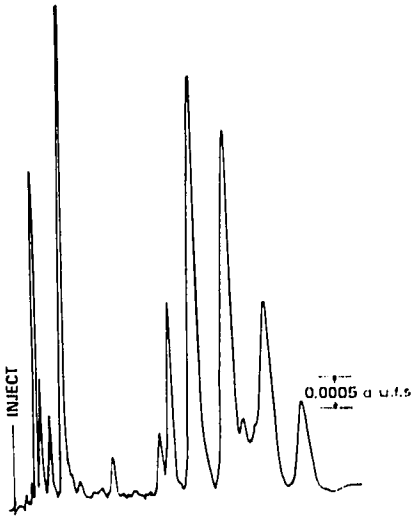

Fig. 7. Impurities in a commercial sample of linoleic acid. The chromatogram shows that the sample has decomposed to form UV-absorbing compounds, presumably aldehyde- or ketone-type. The chromatograph was operated isocratically at 1 ml/mm with acetonitrile–water (50:50). Column: μBondapak C_{18}. Detector: UV, 0.005 a.u.f.s.

starting point. This is due to the fact that the water–methanol refractive index does not follow the linear program of the mixing ratios but goes through a maximum due to partial molar mixing volumes. Under these circumstances the baseline is deflected and results in distorting the shape of the baseline, making it very difficult to interpret. In contrast, the smooth steady upward shape of the water–methanol baseline in the tapered configuration is changed only by the background absorbance in this sample of methanol rather than by any concurrent refractive index phenomenon which would tend to obscure the true baseline.

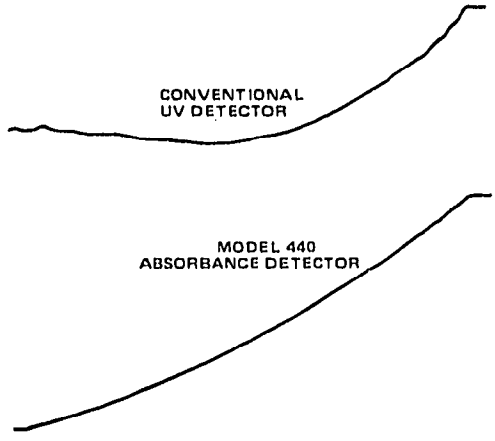

Fig. 8. Refractive index effects of water–methanol gradient. The chromatograph was operated in the gradient mode. Solvents: (A) water, (B) methanol, 0% B–100% B. Curve 6 (linear) for 30 min. Flow-rate: 3 ml/min. Detectors: conventional UV, 0.08 a.u.f.s.; tapered UV, 0.05 a.u.f.s. Column: 4 mm × 30 cm μBondapak C_{18}.

One of the chief objectives which have been uppermost in the minds of liquid chromatographers for many years has been the quest for higher and higher sensitivity. In some cases technical advances have contributed some gains. Based on this new phenomenon, there is an interesting alternative. Rather than trying to match the detector to the application by making it sensitive enough to detect these trace concentrations, we can match the application to the detector. In this case, the liquid chromatograph is used as an enriching device to concentrate these trace concentrations to the level at which we conveniently detect them with today's technology. We refer to this method as trace enrichment. It appears to have considerable promise, especially in areas such as pollution control, pesticide residue studies, and even affiliated programs concerned with metabolites.

In trace enrichment, we are dealing with organic compounds dissolved in very low concentration in some type of aqueous or other high-polarity medium. Generally, we are talking about water supplies or body fluids. The technique of trace enrichment consists of pumping a sufficient quantity of the appropriately filtered sample through a column. If a reversed-phase column of the type with C_{18} groups is used, organic compounds from the pumped sample tend to concentrate on the column. After the sample has been pumped through the column, one switches to a solvent or solvent mixture strong enough to cause these adsorbed organics to elute. Fig. 9 shows an example in which a sample of a local river in Massachusetts was pumped across the column. After 200 ml had been pumped across the column, the gradient was initiated, causing elution of the retained compounds. It is noteworthy that the baseline increased during the initial stage of pumping the 200 ml of sample across the column.

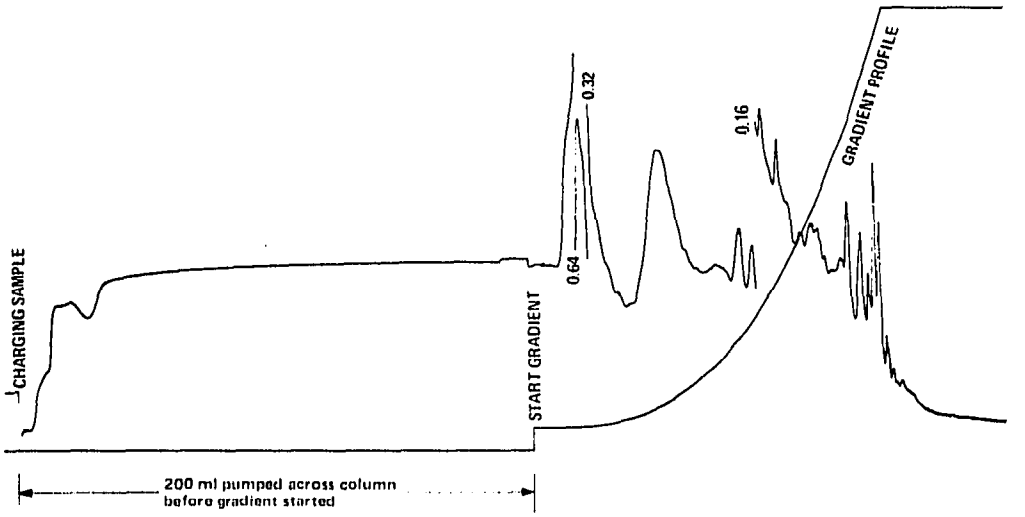

Fig. 9. Demonstration of trace enrichment with aromatics in river water. The compounds are very non-polar (probably contain only carbon and hydrogen) and are aromatic because of the strong UV absorption; presumably they are some form of polynuclear aromatics. The chromatograph was operated isocratically with river water for 200 ml, then gradient mode. Solvents: (A) water, (B) acetonitrile, 5% B to 95% B. Curve 8 on Model 660 Programmer for 30 min. Sample: Blackstone (Mass.) river water. Flow-rate: 2.0 ml/min. Detector: UV, 0.32 a.u.f.s. Column: 4-mm × 30-cm μBondapak C_{18}.

This is undoubtedly due to some very-high-polarity fractions dissolved in the water which apparently were not adsorbed by the μBondapak C_{18} column. In many cases where the concentration in contaminated water supplies is sufficiently high it may not even be necessary to pump as much water across the column. Instead, using only 0.5 ml to perhaps as much as 2 ml of sample and pumping that across the column is sufficient to concentrate enough organics for detection. The advantages of trace enrichment over the older methods of extraction or evaporation are obvious in terms of sample recovery and the lack of any phase change which might tend to cause rearrangement or degradation of some of the sample components when evaporated to dryness.

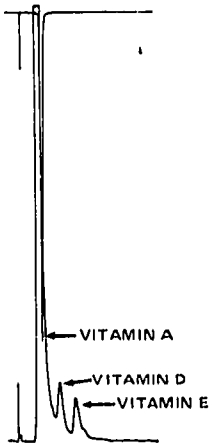

Fig. 10. Vitamin analysis. The chromatograph was operated isocratically at 2 ml/min using methanol–water (90:10). Detector: A 2.0 a.u.f.s., D and E 0.02 a.u.f.s. Column: 4-mm × 30-cm μBondapak C_{18}.

Many times one is faced with the problem of quantitating several species in a mixture which are present in widely different concentrations. One way of accomplishing this task is to monitor at two wavelengths or monitor at two attenuations simultaneously. As shown in Fig. 10, a vitamin tablet extract containing high concentrations of vitamin A, perhaps 10,000 international units, and small amounts of vitamins D and E, present in only a few hundred units, can be monitored simultaneously at two sensitivities for purposes of quantitation by peak height. By setting one of the channels at a relatively insensitive 2.0 a.u.f.s. and the other at the high sensitivity 0.02 a.u.f.s., both can be monitored simultaneously. The high sensitivity setting shows good response for the very low concentration of vitamins D and E while the vitamin A is off-scale. Conversely, the very low sensitivity setting keeps vitamin A on-scale while vitamins D and E are not detected. In summary, matching the detector to the application and the application to the detector can establish new standards of LC performance, enhancing detection limits while improving the ease and validity of interpretation.

REFERENCE

1 S. H. Byrne, Jr., in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 104.