

# Top 10 Articles

*Analytical Chemistry* looks at 10 seminal papers.

James Riordon, Elizabeth Zubritsky, and Alan Newman

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**"Colorimetric Method for Determination of Sugars and Related Substances" by Michel Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and Fred Smith, University of Minnesota; 1956, 28, 350–356.**

*Analytical Chemistry* papers. Rebers suspects that few of the many graduate students who contributed to developing the phenol-sulfuric acid test, as the method is generally known, realized the significance of their work in the mid-fifties. "There was at least one person who understood its importance," says Rebers, "and that was Fred Smith."

According to Rex Montgomery, a University of Iowa biochemist and close friend of Smith's until his death in 1965, the reason the phenol-sulfuric acid procedure became so popular is clear. "It's such a simple procedure," says Montgomery, "that it caught the attention of a lot of people." Mix 0.05 mL of 80% phenol in water with a 2-mL solution containing between 10 and 70  $\mu\text{g}$  of sugar, then rapidly add 5 mL of concentrated sulfuric acid, agitate, let stand in a warm bath for several minutes, and the characteristic yellow-orange color will be stable for hours. Other than a few details and some words of caution included in the original paper, that's it.

Of course, it didn't seem so simple at the time. In Smith's lab, only Rebers managed to perform the test reliably. Smith quickly unearthed the secret of Rebers' success, and he suggested that Rebers use the procedure

When Paul Rebers reflects on his days as a graduate student nearly 50 years ago, he recalls that luck played an important role in the research that led to the publication that tops the list of most cited

as part of his Ph.D. thesis. "I felt guilty in taking credit for his discovery," says Rebers, "but I knew better than to argue with my major professor."

The phenol-sulfuric acid method was only one of the notable achievements that marked Smith's distinguished career. His work on the composition of vitamin C led to a doctorate at the University of Birmingham (U.K.) and helped earn a Nobel Prize on ascorbic acid synthesis for his adviser, Sir W. N. Haworth. Smith also originated an important polysaccharide degradation reaction that now bears his name, and he applied his fluorine chemistry expertise to the separation of uranium isotopes—an important step in developing the first atomic bomb.

While working on uranium separation in Oak Ridge, TN, Fred Smith met Katherine Grandy, his future wife. And it's the traditional English teas that Katherine hosted at the Smith house near Lake Minnetonka that quickly come to Paul Rebers' mind when he speaks of his adviser at the University of Minnesota. That and Smith's fondness for gardening—a hobby that he encouraged his many graduate students to take up. "We'd all work in their garden," Rebers reminisces, "then there would be a big feast, and we'd discuss politics, religion, and science."

It seems anticlimactic when Rebers, now retired from his faculty position at Iowa State University in Ames, suddenly reveals the secret to his success with the phenol-sulfuric acid test: "The others were using distilled water that contained cotton fibers; I wasn't, by sheer luck." Rebers' auspicious and vital contribution is immortalized on the second page of the paper in a suggestion for avoiding cellulose lint contamination in test reagents.

For more than 70 years, *Analytical Chemistry* has been publishing key papers that have revolutionized the measurement sciences. What follows is one small sampling of seminal papers that were published during the last half of the twentieth century. Using citation indexes, we have collected the 10 most-cited papers from 1945 to 1999. Because the approach is based on total citations, it favors "older" papers. Nevertheless, it represents an interesting cross section of analytical science.

Rather than focus on the science, which is already well known, we have tried to reveal some of the human aspects that led to these advances. Full copies of the articles can be found on the Web at <http://pubs.acs.org/ac>.

"Automatic Recording Apparatus for Use in the Chromatography of Amino Acids" by Darrel H. Spackman, William H. Stein, and Stanford Moore, Rockefeller Institute for Medicine; **1958**, **30**, 1190–1206. "Chromatography of Amino Acids on Sulfonated Polystyrene Resins" by Stanford Moore, Darrel H. Spackman, and William H. Stein; **1958**, **30**, 1185–1190.

Although history will probably identify William Stein and Stanford Moore as protein chemists—Nobel-Prize-winning protein chemists—much of their careers were devoted to solving analytical problems.

In these two seminal papers,

they and post-doctoral student Darrel Spackman laid the groundwork for the automated amino acid analyzer. "It was obvious that if [the amino acid analyzer] was successful, it would speed up the work in protein structure analysis," recalls Spackman. "At that point [in history], protein structure was about at the same stage of development as nucleic acid work today."

The story begins in 1938 and 1939 when Stein and Moore separately arrived at what is now known as Rockefeller University to work as post-doctoral students with the prominent protein chemist Max Bergmann. World War II drew Stein and Moore into military service. Bergmann died in 1944, but after the war, the University decided to retain the young scientists and provided them with lab space to continue their studies.

Thus begins a remarkable collaboration between the two that continued until the 1980s. "They formed a team," recalls fellow faculty member Bruce Merrifield. "It turned out that they were promoted to full professor on the same day." Even at Rockefeller, which is known for its unique style, such teaming was unknown.

Some of their first successes came by using column chromatography with potato starch to separate amino acids for protein analysis. They developed a photometric measurement method based on the ninhydrin reaction to quantify their results. In

addition, they built an advanced fraction collector, which, says Merrifield, was later commercialized. Their new method required only milligram amounts of protein and offered precisions of better than 5%.

However, the method was slow and tedious, requiring weeks to do a total protein analysis. "It was a terrible ordeal to do all that," recalls Merrifield. Stein and Moore began experimenting with ion-exchange resins, which reduced the time to a week. But they were still manually measuring concentrations. "They needed a faster way, so they envisioned that if they could make an amino acid analyzer that could do this automatically, this would be a great step forward," says Spackman.

When Spackman arrived in 1956, Stein and Moore gave him the task of building an automated amino analyzer. "Spackman was really the mechanical guy who put the machine together," says Merrifield. According to Spackman, "We found out that we could use very fine-bore plastic tubing, run the effluent through that, and develop the color that came off. [As a result,] the amino acids that came off one after the other didn't get mixed up with each other and could be readily identified." At the same time, they improved the ion-exchange resin by finely pulverizing Amberlite IR-120, which offered better reproducibility. After that, analyses took about two days.

Spackman went on to Beckman Instruments and helped commercialize the analyzer. Five years later, he joined University of Washington's Department of Obstetrics and Gynecology. Today, he is retired and lives in Bellevue, WA.

Armed with the right analytical technology, Stein and Moore delineated the complete amino acid sequence of ribonuclease—the first enzyme fully sequenced—and won the Nobel Prize in 1972 (Merrifield would then synthesize ribonuclease, earning a Nobel Prize in 1985). Stein remained an active researcher, even after being confined to a wheelchair in the 1970s because of Guillain-Barré syndrome. He died in 1980. Moore passed away two years later.

Table II. *Chromatography of Amino Acids*

Added activity d.p.m.	Observed activity c.p.m.	Efficiency %
$1.4 \times 10^5 \text{ } ^3\text{H}$	$1.4 \times 10^4$	10
$1.4 \times 10^5 \text{ } ^3\text{H}$	$1.4 \times 10^4$	9
$1.4 \times 10^5 \text{ } ^3\text{H}$	$0.95 \times 10^4$	7
$1.4 \times 10^5 \text{ } ^3\text{H}$	$0.95 \times 10^4$	9
$1.4 \times 10^5 \text{ } ^3\text{H}$	$1.08 \times 10^4$	8
$1.4 \times 10^5 \text{ } ^3\text{H}$		10
$1.41 \times 10^5 \text{ } ^3\text{H}$	$1.39 \times 10^4$	10
$1.4 \times 10^5 \text{ } ^3\text{H}$	$0.95 \times 10^4$	7
None		52
2035-C	1377	68
2035-C <sup>14</sup>	1207	59
2035-C	1317	62
2035-C <sup>14</sup>	1317	65
2035-C <sup>14</sup>	1194	59
2035-C <sup>14</sup>	1249	61
2035-C <sup>14</sup>	1263	62
2035-C <sup>14</sup>	1055	52
None	66	

1426 20.7

1196 17.3

# 3

"Microdetermination of Phosphorus" by P. S. Chen, Jr., T. Y. Toribara, and Huber Warner, University of Rochester; **1956, 28, 1756–1758.**

Philip Chen was only 22 years old when he completed his University of Rochester Ph.D. thesis on canine renal excretion of calcium. Before his final oral exam, as he waited for the graduate committee to complete

their review of his work, Chen busied himself with perusing the publications in the university's medical school library.

Among the papers Chen stumbled across was a microanalytical study of brain tissue constituents, by Oliver H. Lowry et al. (*J. Biol. Chem.* **1954, 207, 1–17**). It wasn't long before Chen realized that an ascorbic acid method, similar to one outlined in Lowry's paper, might be more effective for the determination of phosphorus than the Fiske–Subbarow method that Chen had relied on for a portion of his thesis work.

Chen set to work confirming his hypothesis under the guidance of his mentor Taft Toribara, a prolific and animated University of Rochester analytical chemist. Huber Warner, a Williams College freshman and summer employee in Toribara's laboratory, was assigned the task of comparing Chen's approach with the Fiske–Subbarow method.

As Warner helped demonstrate that summer, Chen's technique is about 8 times more sensitive than the Fiske–Subbarow test. In addition, the data are linear with respect to phosphorous concentration and sufficiently constant to reduce the need for frequent calibration with blanks and standards.

Chen attributes the current popularity of the article to applications that were not readily apparent in 1956. "Most people using the method today," Chen explains, "are probably involved in studies of DNA and RNA, and phosphorus is very important in those kinds of research."

Toribara is now an emeritus professor at Rochester, but he remains an active researcher. Chen left research in 1967 to pursue an administrative career at the National Institutes of Health (NIH), where he is currently a senior advisor on intramural research. As for the intrepid undergraduate Huber Warner—he and Chen occasionally run across each other in the halls of NIH, where Warner is an associate director of the National Institute on Aging.

# 4

"Theory of Stationary Electrode Polarography" by Richard S. Nicholson and Irving Shain, University of Wisconsin–Madison; **1964, 36, 706–723.**

In the early 1960s, the technique of stationary electrode polarography—what is now called cyclic voltammetry—seemed destined to be just an intellectual curiosity. "Everyone thought it was a great technique, but no one thought it would ever become use-

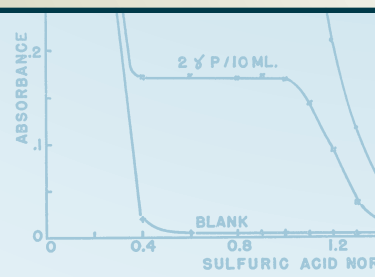
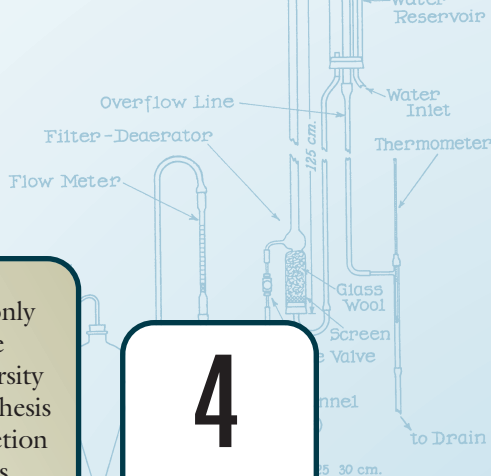
ful because the theory was so complex," recalls Irving Shain. The problem was that the theory was represented by a series of differential equations that no one could solve as a set of general equations. "The first efforts to solve these equations were done with a desk calculator and would take months. However, the results were only good for one concentration, one scan rate, and one set of experimental parameters. For every set of experimental parameters, you had to do it over again. It was useless."

That was the state of affairs when Richard Nicholson joined Shain's group as a graduate student in 1960. Shain gave him the seemingly impossible problem of generalizing the theory for a reversible electron transfer. It turned out to be an inspired assignment. "[Math] was easier for me than chemistry," admits Nicholson.

The breakthrough came in a paper in the Russian literature by A. Y. and Y. P. Gokhshtein. "They had solved a diffusion problem by first converting the equation into a single integral equation and then solved it numerically," says Nicholson. "When you do that, you can pull all the parameters out in front, like the scan rate, concentration, diffusion coefficient, and the only thing left to calculate is a dimensionless parameter," says Shain.

Nicholson began working through the math for various experimental cases and testing the results with known examples using an old mechanical adding machine. However, Nicholson's finger was getting sore from punching the keys. Fortunately, Shain had access to one of the first computers, an IBM 704. They could use it evenings and through the night when it was otherwise idle.

"We went into a kind of production mode. I learned FORTRAN on my own, and we started running these problems on this huge vacuum tube machine," recalls Nicholson. "It would take two hours to make each run, and we would do four or



P added $\gamma$	Phosphate Found in Presence of			
	10 mg. gelatin $\gamma$	50 mg. gelatin $\gamma$	10 mg. blood albumin $\gamma$	10 mg. hemoglobin $\gamma$
0.0	1.0	0.5	7.8	7.4
56.4	57.0, 55.0	54.4, 53.3	51.0, 54.4	55.7, 55.7
84.6	83.8, 82.5	83.3, 79.8	78.8, 79.4	82.8, 82.8

Table VI. Inorganic Phosphorus in Alfalfa

Sample <sup>a</sup>	A.O.A.C. <sup>b, c</sup>		Berenblum-Chain <sup>b, c</sup>		Modified <sup>b, c</sup>	
	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$

"Smoothing and Differentiation of Data by Simplified Least Squares Procedures" by Abraham Savitzky and Marcel J. E. Golay, Perkin-Elmer Corp.; **1964**, *36*, 1627–1639.

five each night," says Shain. "We did that night after night for several months, until we got all these parameters calculated."

During the night, they talked about their work, checked their results because the vacuum tubes constantly went bad, drank soft drinks, and tried to sleep. "It was really a drag to go home, take a shower, and then give a lecture," adds Shain. The intense work affected Shain and Nicholson personally. "We were no longer graduate student and faculty member," says Shain. "We became real pals, real buddies."

The finished work was gathered up in a single paper, which sailed through peer review. Allen Bard even sent Shain a copy of his review, which simply stated: "This is a remarkably good paper. Don't change any of it." However, Lawrence Hallett, then the editor of *Analytical Chemistry*, was not happy about publishing such a long article and wanted it broken up into several shorter papers. Shain went to Washington, DC, and convinced Hallett to keep it together, because the first section on separating the parameters for the reversible case was then used as the reference for all the other kinetic cases. It was a key decision that probably helped the paper's popularity.

Nicholson went on to a faculty position at Michigan State University, and, later, he served as a program officer at the National Science Foundation. Today, he is the executive director of the American Association for the Advancement of Science. Shain became the chancellor of the University of Wisconsin–Madison and later, vice president and chief of research at the Olin Corp. He is now vice president of the university research park in Madison, which boasts 60 companies and around 2100 employees.

So who found the crucial paper in the Russian literature? Appropriately, Shain and Nicholson credit each other.

It can be argued that the dawn of the computer-controlled analytical instrument can be traced to this article. It actually includes two examples written in FORTRAN code.

In 1989, *Analytical Chemistry* (pp 921 A–923 A) published a reminiscence by Abraham Savitzky about the work. Marcel Golay had just died that year, at the age of 86. (Savitzky passed away last year.) Here are some excerpts from that remembrance.

"In late 1959 or early 1960, John Atwood, then director of research for the Perkin-Elmer Instrument Division, Norman Adams, Marcel Golay, and I discussed the analysis and interpretation of IR spectra untouched by human hands. Our first problem was the recognition of spectral characteristics, such as peaks, valleys, and shoulders. It appeared that these features could be determined from changes in sign in the first and second derivatives.

"We asked Golay to look into a simple and fast method for calculating the derivatives. [In 1960] he provided me with sets of first- and second-derivative convoluting integers for parabolas. We used those on synthetic spectra to prove our ability to find spectral features. This method worked well enough for spectra without noise but worked poorly when we added synthetic noise.

"By early 1962 we had efficient routines for first and second derivatives, but a notebook entry for March 1962 reads: 'I need a general purpose convolution program' and defines its characteristics.

"At about this time, my reading and work prompted me to look more closely at smoothing as well as derivatives. In April 1962, I wrote to Golay: 'Extend the application of the methods you suggested for determination of the first and second derivatives into other areas. The class of methods which should be discussed is characterized by a convolution of the experimental data with a set of integers, followed by normalization. The importance of these methods is that, for the smoothing functions and for the derivative functions, the computer program is essentially the same. Thus, a single general purpose program, which provides for entry of the set of convolution integers and the normalizing factor, can handle a very broad class of problems.

"What I would like to have is a cookbook procedure for generating the set of integers for any desired function and for derivatives of any degree, from zero on up. The useful thing for the paper would be [to] have a simple set of rules for obtaining the integers and normalizing function, plus a simple, elegant proof in the appendix."

"The paper was submitted to *Analytical Chemistry* in February 1964. In thinking about why the technique has been so widely used, I've come to the following conclusions. First, it solves a common problem—the reduction of random noise by the well-recognized least-squares technique. Second, the method was spelled out in detail in the paper, including tables and a sample computer subroutine. Third, the mathematical

basis for the technique, although explicitly and rigorously stated in the article, was separated from a completely non-mathematical explanation and justification. Finally, the technique itself is simple and easy to use, and it works."

"Determination of Inorganic Phosphate" by James B. Martin and D. M. Doty, Purdue University Agricultural Experiment Station; **1949, 21, 965–967.**

Biochemistry Department. Despite the passage of 51 years, Martin clearly remembers the method paper he wrote with his adviser, the late Dilbert M. Doty of Purdue University. Still, he was surprised to learn that the article is a top-ranked *Analytical Chemistry* paper.

Martin's popular paper was also among his first publications; the Martin and Doty method was part of his 1949 doctoral thesis. At that time, Martin was primarily studying the availability of phosphorus in alfalfa and other cereals. "I think the subject matter has generally dropped from the literature," says Martin, "since we normally get plenty of phosphate compounds from other sources." Martin's procedure, however, rapidly captured the attention of other researchers in need of phosphorus determinations. The technique was an improvement over a prior determination described by Berenblum and Chain, with the added benefits of simplified color development, a shorter extraction procedure, and the elimination of protein interferences.

For the past quarter century, Martin has turned his attention to another lifelong passion: gladiolus cultivation. In fact, since his retirement from Proctor & Gamble in 1977, he has spent nearly as many years toiling in his garden as he worked in the laboratory as a research chemist. *Lady Lucille*, a variety Martin cultivated and subsequently named after his wife of 59 years, is now one of the top show gladioli in the country. The purple *Jennifer K.* and the unusual gray-colored *Becky's Beauty* gladioli are among hybrids Martin christened for his granddaughters.

Ever since his childhood days in rural Oregon, James Martin has been a farmer at heart. It's an inclination that sustained his lifelong interest in horticulture and led Martin to pursue a doctorate at Purdue's Agricultural

"Protein Determination for Large Numbers of Samples" by Gail Lorenz Miller, Quartermaster Research and Engineering Center (now called U.S. Army Natick Research, Development, and Engineering Center); **1959, 31, 964.** "Use of Dintrosalicylic Acid Reagent for Determination of Reducing Sugar" by Gail Lorenz Miller; **1959, 31, 426–428.**

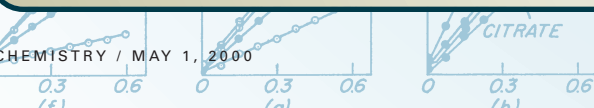
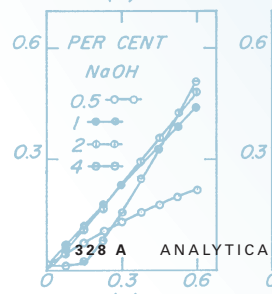
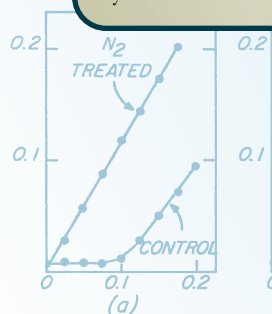
Gail Lorenz Miller is the only author with two solo papers in this top 10 list, and together, his papers are a testament to the endurance of simple, reliable techniques. Both papers were expansions on methods that were already old in 1959. Yet 41 years later, both papers are still being cited.

The protein determination paper was a variation of the colorimetric total protein assay by Oliver H. Lowry et al. (*J. Biol. Chem.* **1951, 193, 265–275**), which relied on the reaction of a copper solution and the Folin phenol reagent. Miller modified the method so that most of the color development would not occur until the final mixing step, thus making it possible to analyze many samples at once. Similarly, he adapted James B. Sumner's 1921 colorimetric assay for estimating the amount of glucose in a sample (*J. Biol. Chem.* **1921, 47, 5–9**). In the original method, some of the sugar was lost to oxidation. Miller determined that the loss was caused by the presence of Rochelle salt, which was needed for color stability, and he compensated either by adding known amounts of glucose or by adding Rochelle salt after the color had developed.

Both papers were published while Miller worked at the Quartermaster Research and Engineering Center in Natick, but he spent only a fraction of his

varied career there. He began with a Ph.D. in biochemistry at George Washington University in 1937, and after many years in industrial and academic research, he taught microbiology at the University of Michigan and Jefferson Medical College. He was still publishing papers in 1968, when he headed the Tumor Immunology Unit at Philadelphia's Lankenau Hospital, and, over the years, his research interests ranged from the mechanisms of cancer to an assay for the chemical responsible for sexual attraction in cockroaches.

Now retired, Miller lives in Pennsylvania. Meanwhile, researchers still use both of his methods. Nam Sun Wang of the University of Maryland, for example, included the glucose assay in a protocol for cellulose degradation. Wang concedes that there are newer techniques for glucose measurement, and some of them are more selective. But he says that Miller's method is still valuable because it is inexpensive and quick. "One of the ideas that our students have is that new methods are automatically better," he adds. "That's not true. The old methods are still good. They have stood the test of time."



"Measurement of Low Energy Beta-Emitters in Aqueous Solution by Liquid Scintillation Counting of Emulsions" by Michael S. Patterson and Ronald C. Greene, Veterans Administration Hospital and Duke University School of Medicine; **1965**, 37, 854–857.

At first, Michael Patterson seems an unlikely author of one of the top 10 articles in *Analytical Chemistry*. He was an undergraduate when his paper, co-authored by Ronald C. Greene, was published. He was also a history major. He later earned a Ph.D. and became a professor at California State University–Northridge, specializing in the colonial era of the United States.

But Patterson was known for having a wide range of expertise and interests, according to Charles Macune, chair of Northridge's history department. So perhaps it's not too surprising that, as an undergraduate at Duke University, Patterson would seize an opportunity to conduct research in biochemistry. He worked with Greene at the Duke-affiliated Veterans Administration Hospital, where Greene is still an active researcher despite his retirement.

The 1965 paper grew from a very simple problem: Greene and Patterson wanted to perform liquid scintillation counting (LSC), which measures the activity of radionuclides based

on the rate of photon emission, on biological samples. Usually the liquid in LSC is a solvent for the sample; this ensures the efficient transfer of energy from the  $\beta$ -particle emitted by the sample to the solution. In the mid-1960s, the solvents with the best energy-transfer properties were toluene and xylene, which are insoluble in water. This posed a problem for the analysis of biological samples, which must take place in water. "People [were trying] to figure out solvents with good energy-transfer properties that could accommodate water," Greene explains. "And it turns out that's really an impossible thing to do."

Greene and Patterson realized that there was an alternative—an emulsion, in which toluene (the organic phase) would be suspended in water (the aqueous phase). "You could have a  $\beta$ -particle generated on a little water droplet, and if it immediately gets into a toluene phase, that's fine," Greene explains. "[LSC] still works." In theory, this approach would accommodate biological samples in their proper environment, handle large volumes of water, and could be used with inorganic salts and other components that are insoluble in the organic phase.

The researchers wrote to detergent companies asking for emulsifiers, but the ones they received either absorbed or emitted light, Greene recalls.

So he and Patterson tried Triton X-100, which happened to be on the shelf. "It worked like a charm," Greene says.

It was the kind of discovery that "anybody who'd stopped to think" would have come up with, Greene says. But no one else did. Indeed, it turned out that nothing was better suited to this purpose than Triton X-100 and similar alkylphenol surfactants, and the researchers' paper continued to be cited until LSC was replaced by nonradioactive methods. Greene published one other paper on the subject, showing that xylene was better than toluene. Then he went on to study the binding of regulatory proteins to DNA and other subjects.

Patterson also moved on, but the paper served him well. "One of the things that appealed to [our department] was his publication in chemistry," says Macune. It was early evidence of Patterson's versatility. Later, as a professor of American history, Patterson would also speak fluent French, teach computer science, and run a small computer business. In the 1970s, he earned Northridge's distinguished teaching award, Macune says, "And that's about the sweetest prize there is, because it's given by your students and colleagues." Sadly, in 1993, Patterson had a stroke caused by a brain tumor, and it brought his rich career and life to an abrupt end.

*James Riordon and Elizabeth Zubritsky are assistant editors, and Alan Newman is managing editor with Analytical Chemistry.*