Contributions of Walter A. Pons, Jr., to Development of Methodology for Mycotoxins

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ABSTRACT

Walter Pons began work on mycotoxins in 1963, well prepared by 20 years' experience in the Analytical Section of the SURDD, USDA. His first paper on aflatoxin methodology, presented at the fall meeting of the AOCS in 1964, was rated as one of the 3 best. In this paper, he introduced acetone as the extraction solvent for aflatoxins, moved away from the lengthy, exhaustive Soxhlet extraction to the far more rapid equilibrium shaker extraction and introduced lead acetate to precipitate major interfering impurities. He early saw the need for the calibration of reference aflatoxin standards and for their availability. He prepared and supplied more than 3,000 aflatoxin standards, used worldwide, as a public service of USDA before such standards were available commercially. Walter Pons pioneered in every aspect of development of analytical methodology for aflatoxins. He was instrumental in development of objective densitometric methods for determination of aflatoxins in diverse commodities, including milk, eggs and mixed feeds; in multimycotoxin methodology; in application of minicolumns for the rapid estimation of aflatoxins in various agricultural commodities; and, most recently, in application of high pressure liquid chromatography to the precise objective determination of aflatoxins. Many of Walter's contributions resulted from the generous gift of his time. He organized symposia, presented seminars, participated in workshops and responded freely and enthusiastically to numerous requests from analysts for assistance.

This symposium is in honor of Walter A. Pons, Jr., who died February 18, 1979, ending a career of more than 36 years in analytical chemistry at the Southern Regional Research Laboratory. The symposium was organized to allow his friends and associates to shown their respect and to recognize Walter's brilliant contributions to mycotoxin methodology. He was clearly the right person in the right place at the right time. In this discussion, it should be noted that his work dealt exclusively with physicochemical assay, not biological assay. Also, he was concerned primarily with aflatoxin, although his innovations, of course, could be applied to other mycotoxins.

Walter began his work on mycotoxins at the USDA Southern Regional Research Laboratory, now SRRC. He had worked in the Analytical Section of the SRRL for 21 years after undergraduate training at Loyola University and a Master's degree at Tulane University, both in New Orleans. While in the Analytical Section, Walter developed and applied analytical methods for the determination of important components of diverse agricultural commodities. He originated the first analytical system for determining the types and amounts of phosphorus compounds—total, inorganic, acid soluble, phosphatide and phytin phosphorus —in plant materials. He developed accurate, simple methods for determining "free," "bound," and "total" gossypol in cottonseed, cottonseed products and mixed feeds.

The methods he developed were selected as the official methods of the American Oil Chemists' Society and are still used throughout the world. In developing these methods, which he described in detail in his Wiley Award Address in 1976 (1), he introduced aqueous acetone as the extraction solvent, equilibrium extraction on a shaker, and lead acetate as a precipitating reagent to remove impurities selectively. These procedures and the knowledge he acquired concerning the behavior of plant materials in a wide variety of situations made him the obvious choice to initiate our work on aflatoxin methodology when we first recognized, in 1963, that we had an aflatoxin problem with peanuts and cottonseed in the US. These were the 2 major oilseeds studied at the SRRL.

His first paper on aflatoxin, entitled "Determination of Aflatoxin in Cottonseed Products," was presented at the fall meeting of the American Oil Chemists' Society in 1964 (2) and won honorable mention as one of the 3 best papers presented at that meeting. In that paper, he introduced aqueous acetone as the extraction solvent for aflatoxin, replaced Soxhlet extraction with the far more rapid half-hour equilibrium extraction on a shaker, and introduced lead acetate to remove major interfering impurities.

This method, after a collaborative study (3) that he conducted, was adopted as the Official Method by the AOAC (4) and by the American Oil Chemists' Society as Tentative Method Aa 8-71T for determination of aflatoxins in cottonseed products. He served the AOAC as Associate Referee for Mycotoxins in Cottonseed Products from 1965-79. Later, he modified the procedure for determination of aflatoxins to make it applicable to other agricultural products. Aqueous acetone extraction of aflatoxins and lead acetate precipitation for cleanup were successfully applied to determination of added aflatoxin to 20 agricultural commodities (5).

Aqueous acetone did not prove to be the ideal extraction solvent for use with mixed feeds, which present unique analytical problems because of the multiplicity of ingredients. Aqueous acetonitrile, which at first seemed to offer promise, also was unsuitable. On the basis of many extraction experiments, he developed a system that afforded a purified extract suitable for thin layer chromatography (TLC) (6). That system involved prewetting the feed sample with dilute acid, followed by extraction with methylene chloride (dichloromethane), concentration of the crude extract followed by dilution with aqueous acetone, precipitation with lead acetate solution, and further purification by column chromatography on acidic alumina and elution with methylene chloride/acetone (75:25). This procedure, the first method suitable for determination of aflatoxin in mixed feeds, was also found "applicable to all peanut and cottonseed products, to other oilseeds and oilseed meals, copra and coconut, grains and fish meals" (6).

During his work on gossypol, Walter developed a process for the preparation of high-purity gossypol. With the advent of official methods for the determination of gossypol, primary gossypol standards were needed. Recognizing this, he undertook the preparation and distribution of authentic primary standards for gossypol, as the more stable gossypol acetate. For many years, the Southern Regional Research Center has been the source of these standards that are used throughout the world.

Similarly, with the advent of official methods for determining aflatoxins, reference standards were again

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needed. Walter met this need, as he had with gossypol, by preparing and distributing high-purity analytical standards for aflatoxins B_1, B_2, G_1 and G_2, as judged by their ultraviolet (UV) and fluorescent characteristics (7). From the inception of the program in 1964 to its termination when aflatoxin standards became available commercially, he prepared and supplied more than 3,000 authentic standards to investigators in the U.S. and more than 100 other countries. This distribution greatly enhanced the accurate estimation of aflatoxins and reduced the confusion that then existed in reports by different laboratories, using the official methods, of the aflatoxin content of various materials. Preparation of such standards was not simple. Conflicting literature data made preparation of reliable standards difficult. Walter was a major contributor in cooperative research on the establishment of molar absorptivity values for aflatoxins and for justification for their use as criteria of purity of analytical standards (8). This research, together with his studies of the stability of the aflatoxins in various solvents (9) and under different conditions of storage (10) and of the techniques for handling them, provided suitable procedures for preparation of authentic standards. These procedures are now incorporated in the official methods of the AOAC for the preparation of standards (11).

Walter pioneered in establishing the principle that fluorodensitometry could be used routinely to improve markedly the precision and accuracy of aflatoxin determinations. His original research established the linear relationship between the emitted visible fluorescence with concentration of individual spots of aflatoxin B_1, B_2, G_1 and G_2 resolved on silica-gel-coated thin layer plates and excited to fluorescence by long-wave UV light (12). He established the solid-state fluorescence excitation and emission maxima of individual aflatoxins on silica-gel-coated plates and the relative fluorescence emission response of each aflatoxin, $B_2>G_2>B_1>G_1$ (13), and he proposed instrumental evaluation of aflatoxin resolution on TLC plates (14). His research demonstrated that aflatoxins can be measured objectively by fluorescence densitometry on thin layer plates with a precision of ±2-4% as contrasted to about ±20-25% by the customary subjective visual analysis (12). He further established the practicality of fluorodensitometric measurement of aflatoxins in 2 collaborative studies on naturally contaminated agricultural products (3,15).

The results established the precision and accuracy of his fluorodensitometric method of estimating aflatoxins and led to adoption of fluorodensitometry in officially approved measurement systems by both the AOAC and AOCS. In recommending the fluorodensitometry method for adoption as official, A.D. Campbell, then General Referee for Mycotoxins, in his verbal report delivered to the Association, spoke in glowing terms of the high scientific caliber of the collaborative work on the method, and the devotion and energy displayed by Pons in his capacity as Associate Referee. He also observed that the design and execution of the work for which Walter had assumed responsibility set a high standard for future collaborators. Walter Pons served the AOAC as Associate Referee for densitometry during 1969-75.

In response to a need for a rapid method to detect aflatoxin-contaminated cottonseed, Walter developed a rapid, simple, qualitative screening technique capable of detecting aflatoxins at a level as low as 10 parts per billion (ppb) (16). This method was based on small columns (4 mm id × 20 cm) adapted from the work of Holaday (17), who first used a 4 mm id column packed with silica gel to detect aflatoxin contamination in extracts of peanuts. Walter called this column a "millicolumn." The entire analysis requires only about 15 min. Because aflatoxins B_1, B_2, G_1 and G_2 are not separated from each other, precise quantitative estimates cannot be made, but it is possible to make quick qualitative estimates of total aflatoxins. Aflatoxin content below 10 ppb is easily distinguished from one in the 20-30 ppb range.

In September 1971, Walter was strongly urged by members of the cottonseed processing industry to demonstrate this new screening test to quality control chemists. They then used it extensively in sampling truckloads of ginned seed before storage at the various mills. Their analyses provided an opportunity for separate processing of contaminated lots and avoided contamination of storage piles of uncontaminated seed. The millicolumns used to screen the 1971 crop numbered in the thousands, and the method is still widely used in the cottonseed industry.

In modified form it is an official screening method of the AOAC for aflatoxin in corn (18). A screening method using the same type of columns (now designated minicolumns), was subsequently applied successfully to 24 agricultural commodities spiked with aflatoxins (19). The contamination level could be placed in one of 5 categories: <10, 10-20, 20-50, 50-100, and 100-200 ppb, based entirely on visual observation of the fluorescent aflatoxin band.

Another example of the diversity of Walter's analytical ability was his sensitive method for the determination of aflatoxin M_1 in milk (20). Aflatoxin M_1 may be found in extremely low levels (generally less than 1 ppb) in milk from dairy cows ingesting cottonseed containing aflatoxin B_1. Because the toxin is present in such small amounts, extracts of milk must be almost devoid of interefering substances before quantitation. The unique feature of Walter's method for M_1 was his novel cellulose-aqueous methanol partition column used for extract cleanup. Spiking experiments showed that no toxin was lost on the column. This finding was extremely important because spiking levels were below 0.5 ppb. After passage of the partially purified extracts of milk or milk products through this column, levels of M_1 as low as 0.05 μg/L could be detected. This method is now an official first action method (21).

The cellulose column was subsequently adopted in methodology for aflatoxin in eggs. Clean-up of extracts by passage through this column followed by 2-dimensional TLC permitted the detection of 0.02-0.04 ppb of aflatoxin in eggs. The egg method was published jointly with the U.S. Food and Drug Administration (22).

In 1977, Walter turned his attention to methodology for mycotoxins other than aflatoxins and undertook with Alva Cucullu the development of a multimycotoxin method for determination of 10 mycotoxins in peanuts. These included zearalenone, sterigmatocystin, ochratoxin A, penicillic acid, citrinin, and the 5 aflatoxins, B_1, B_2, G_1, G_2 and M_1. In the procedure that he devised but never published, the 10 mycotoxins were extracted together with methanol and 0.1 N HCl. Interfering substances were precipitated with zinc acetate and sodium chloride, and the filtrate was divided into 3 portions. Partition of 1 portion into methylene chloride permitted the quantitation of the 5 aflatoxins and penicillic acid by conventional TLC. A second portion was acidified to pH 2 before partition with methylene chloride. Citrinin was quantitated from this portion by TLC or by HPLC. After partition of the third portion of the primary extract into methylene chloride, the extracts was purified by column chromatography on a small florisil column. Zearalenone and sterigmatocystin were quantitated after elution with methylene chloride/methanol. Ochratoxin A was quantitated after elution with methylene chloride/formic acid. These 3 mycotoxins and citrinin were quantitated by HPLC on a C-18 reverse-phase Bondapak column with methanol/phosphoric acid as the

developing solvent. Spiking experiments on sound peanuts indicated 500 ppb as the lowest detectable level for citrinin and penicillic acid, and 50 ppb the lowest detectable level for the 5 aflatoxins. Although time ran out before he was completely satisfied with the procedure, it was used successfully for the determination of aflatoxins B_1, B_2, G_1, G_2 and M_1 in a survey of 59 samples of pickout peanuts from 6 states.

Walter's last published contributions to methodology for mycotoxins were in the application of high pressure liquid chromatography (HPLC) for separation of aflatoxins, first to cottonseed products and later to peanut products and corn.

Application of HPLC techniques that Walter found satisfactory for separation and quantitation of aflatoxins B_1, B_2, G_1 and G_2 in primary standard mixtures (23), when applied to semipurified extracts of naturally contaminated cottonseed products, showed that residual nonaflatoxin artifacts interfered with resolution of the aflatoxins. The HPLC procedure he then devised (24) incorporated aqueous acetone extraction, lead acetate precipitation, and methylene chloride partition techniques analogous to those specified in the AOAC rapid modification for cottonseed products (25). Twelve samples of naturally contaminated cottonseed products previously used in a collaborative study were analyzed by the rapid cottonseed method that used TLC for quantitation and by the new HPLC method (24). Walter stated that the HPLC data developed indicated "a high degree of precision in the resolution of aflatoxins, and ultraviolet absorbance quantitation, with coefficients of variation of 0.2-1.0 and 1-2%, respectively," and that "this strongly suggested HPLC as significantly more precise than TLC for estimating aflatoxin contamination in agricultural products." He stated that "since HPLC is a closed system, it should be reproducible in different laboratories, as contrasted to TLC, where both environmental conditions and type of silica gel coatings lead to significant errors" (24).

Walter next turned his attention to HPLC determination of aflatoxin in peanut products. This paper was presented at the 91st annual meeting of the AOAC held in Washington, DC, Oct. 17-20, 1977, and was the last paper he presented to the AOAC. In this paper, he proposed "a precise and sensitive high pressure liquid chromatographic method for determination of aflatoxins B_1, B_2, G_1 and G_2 in all types of peanut products" (26). Although this paper on HPLC determination of aflatoxins in peanut products was the last paper Walter presented to the AOAC, it was not his last paper submitted to *JAOAC*. In a paper submitted after his death, Walter proposed an HPLC method for determination of aflatoxins in corn (27). In this method, he introduced a new, apparently superior, extraction solvent, methanol/10% NaCl (4:1). He reported that in 5 of 6 samples containing aflatoxins B_1, B_2, G_1 and G_2, this solvent system extracted more aflatoxin than did the official CB solvent, chloroform/water. In samples containing only B_1 and B_2, the 2 extraction solvents were virtually equivalent. The aflatoxins were measured by UV fluorescence from a silica-gel-packed flow cell that was originally introduced by Panalaks and Scott (28). Agreement was good between HPLC and TLC procedures with the same extraction solvents.

Walter's final research contribution was a collaborative study he conducted on his proposed method for aflatoxin in cottonseed that included a comparison of HPLC and TLC as the determinative step. The HPLC detection system was the same as for toxins extracted from peanuts or corn. After his retirement, he tabulated the analytical results in anticipation of publication. At the suggestion of Leonard Stoloff, these results were analyzed statistically, evaluated, and presented at the 93rd meeting of the AOAC in October 1979, 8 months after Walter's death. This collaborative study is Walter's last publication (29).

The collaborators in this study, as well as the many other analysts with whom Walter had a warm professional relationship, attest to his unparalleled ability and concern for others involved in analytical methodology for mycotoxins. Research on aflatoxins was organized and those conducting it were instructed and guided as a result of Walter's personal involvement. He arranged symposia on aflatoxins and organized workshops in which analysts from government, academia and industry shared their expertise or acquired new skills in aflatoxin methodology. He traveled extensively to give seminars on such methodology. At SRRC, he was always willing to interrupt his work and respond to requests for help or advice on the phone, by mail, or face-to-face on any of the many problems so frequently encountered in the analysis of agricultural products. Those who knew him only through his accomplishments and technical knowledge miss his skillful guidance and expertise; those who knew him personally deeply miss a friend.

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