The Anagen System for Automated Fluorometric Immunoassay


We describe a new discrete microprocessor-controlled analyzer, the AN2000, which fully automates fluorometric immunoassays by using a magnetic separation of the solid phase and an alkaline phosphatase label. It can operate in random-access or batch mode with a dwell time typically <20 min. The analysis rate is 75 samples per hour and the system can hold refrigerated reagents for as many as 20 different analytes. The substrate and wash buffer are common to all analytes. The system can hold as many as 60 samples at once. The operator can select from the menu-driven operator interface any combination of the available analytes to be run for each sample, using either the touch screen or the keyboard. Results are calculated from a stored calibration curve that is stable for ≥1 month. The AN2000 is capable of automating most assay formats because the available timings, volumes, incubations, and wash cycles can be used in any combination.

Additional Keyphrases: discrete analysis • magnetizable solid phase • methyllumbelliferyl • enzyme label

The introduction of automation into clinical chemistry and hematology laboratories during the late 1950s marked the beginning of a new era of laboratory testing. The benefits of improved precision and accuracy plus increased rates of analysis, combined with a decrease in cost and a reduction in the skill and number of staff required to perform the work, resulted in more analyses being performed. Having seen the benefits of automation in these fields, researchers not surprisingly attempted to automate the more-difficult-to-perform immunoassays. Nevertheless, not until the late 1970s were attempts made to automate immunoassays. These early attempts had some success, but the methods of separation available, combined with the use of radioisotopes and long incubation times, made the systems unattractive for routine laboratory use (1). In an attempt to find better methods of separation, three of us introduced magnetizable solid-phase particles for this purpose in 1979 (2). Since then, these particles have been widely accepted for both automated and manual separations in immunoassay.

Principle

All assays reported here for the AN2000 discrete microprocessor-controlled analyzer (Anagen, Ltd., Hampshire, UK) use magnetic separation and an alkaline phosphatase (EC 3.1.3.1) label to achieve high sensitivity. The substrate used is 4-methylumbelliferyl phosphate (4-MUP), which is hydrolyzed to 4-methylumbelliferone (4-MU) and determined fluorometrically.1 The assays and the magnetizable particles have been developed together, and the instrument has been designed to provide optimum conditions for the assays.

The particles used are from a new generation of ceramic magnetizable particles that show no magnetic remanence. A newly developed coupling method (proprietary information) allows a wide range of molecules to be chemically bound to them. The particles are spherical, with a mean diameter of ~2 μm, and each assay cuvette contains ~12 × 10⁶ particles, which represents a surface area >2 cm². The particles have been engineered to provide rapid separation, typically within 2 s, with high retention of the solid phase on the magnets during aspiration of the unwanted liquid in the wash cycles. The particles do not clump with use and remain easily resuspendable, enabling exactly the same amount of solid phase to be taken in each aliquot during the life of the reagents.

The assays currently available are performed in either an immuno metric or a competitive format. The various forms of solid-phase immunoassays have been described by Butler (3). In the immunometric assay format, the sample and the solid phase are mixed and incubated for a fixed period (typically 7 min). The antigen to be determined in the specimen is bound by the antigen-specific polyclonal capture antibody, which is linked directly to the magnetizable solid phase. The conjugate, consisting of a solution of the developing monoclonal antibody, which is directed against the antigen and labeled with alkaline phosphatase, is then added and the contents of the cuvette are mixed and incubated for a fixed period (typically 7 min). The solid phase, carrying the immune complexes, is separated magnetically, and the supernatant liquid is aspirated to waste. Wash buffer is added to the solid phase, and the solid phase is resuspended. The magnetic separation is repeated and the wash buffer is aspirated to waste. After one more wash cycle, a buffered solution of the 4-MUP substrate is added to the solid-phase complex; the resulting mixture is incubated for a fixed period (typically 4 min). The alkaline phosphatase catalyzes the hydrolysis of 4-MUP to 4-MU, the latter being fluorescent when excited by light at 365 nm. At the end of the incubation period, the cuvette is transferred to the fluorometer and the fluorescent signal is measured at

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1Nonstandard abbreviations: 4-MUP, 4-methylumbelliferyl phosphate; 4-MU, 4-methylumbelliferone; SAW, separate and wash station; PMT, photomultiplier tube; and FU, fluorometer unit.

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450 nm. The selection of the filters is important because of the fluorescence of the substrate itself at similar wavelengths; this subject is covered in detail by Fiore et al. (4).

The competitive assay format uses the same analytical steps as the sandwich assay. However, the composition of the two reagents and the associated reaction mechanisms are different. The antigen to be determined in the specimen competes with the conjugate (alkaline phosphatase-labeled antigen) for the binding sites on the monoclonal antibody that is coupled to the magnetizable solid phase. After incubation, these immune complexes are separated and the analysis is completed as described above.

Materials and Methods

The Instrument

The AN2000 weighs 96 kg, measures 1181 × 631 × 562 mm (w × d × h), and can be operated at ambient temperatures between 18 and 28 °C with a maximum relative humidity of 85% (noncondensing). The complete system consists of the AN2000 itself, the reaction cuvettes, the reagent packs, the common reagents (wash buffer and substrate), the calibrator packs, and sample cups for small samples. Figure 1 illustrates the configuration of the AN2000 and its major subassemblies: the sample carousel, the load/unload station, the reagent carousel, the incubator/vortex-mixer, the gantry, the pipetting probes, the Cavro and pump module, the cuvette-transport mechanism (the "grabber"), the Separate And Wash station (SAW), the fluorometer, and the electronics pack.

Sample and reagent carousels. The sample and reagent carousels are concentric, the reagent carousel being the inner carousel, separated from the sample carousel by thermal insulation. The carousels are mounted on two separate concentric axles driven by different motors and can therefore move independently of each other. The carousel motors operate under the control of the computers, thus ensuring an optimal speed and path to whatever position is required.

The reagent carousel is cooled to 6 ± 2 °C. Cooling is achieved by using six sealed Peltier cooling elements (DuraTEC; Marlow Industries, Dallas, TX) rated at 69 W and fitted to the underside of the carousel housing. As ambient air passes over the cooled metal underside of the reagent carousel, the floor of the unit is designed so that any water condensing on the cooled metal is collected by a drain and periodically suctioned away to the system waste container.

The reagents are placed into their carousel in segment-shaped reagent packs made up of three containers. The segment is divided into two halves to form two of these containers; the third part is a cylindrical container mounted on the outer circumference formed by the wider end of the other two segments. The bottom axle of this container fits in a corresponding dry bearing in the base of the reagent pack frame. This cylindrical solid-phase container has an integral gear wheel molded into the lower part of its external circumference. This gear rotates the container to resuspend the solid material before aspiration. When a pack is present, it drives a small independent rubber wheel that allows the computer to check for the presence of a pack and to check that it can be rotated; it also provides a feedback signal to allow the computer to control the speed of rotation to ensure reliable resuspension of the solid phase before aspiration.

The sample carousel has 20 slots arranged around its circumference, each of which carries a sample tube rack.
that can carry as many as four individual sample tubes in the wells provided. The sample tubes can be loaded into a sample tube rack only when the rack is in the sample load/unload station. The sample tube rack can accept sample tubes with external diameters between 8 and 18 mm; thus, primary tubes can be used. The operator must inform the software initially what size tubes are being used; this information, stored as the default tube size, is required so that the sample probe can track the liquid meniscus while the sample is being aspirated, to minimize contamination of the outside of the probe with the sample. The sample load/unload station is covered with a lid, and sample tube racks can be transported between the load/unload station and the sample carousel only when this safety lid is closed. The station has four light-emitting diodes by which the computer can indicate to the operator which wells are available for insertion of sample tubes. Each sample tube well is monitored by an optical sensor, and the computer checks to see that the operator places the sample tube in one of the empty wells indicated by the sample tube position light.

After a sample tube rack has been filled and the lid closed, the sample tube rack is transferred to the sample carousel; another rack is presented for loading if required.

*Incubator/vortex-mixer.* Apart from the incubating/vortex-mixing function, this module also serves to store cuvettes. The cuvettes are used both as reaction vessels and as the measurement cells for the fluorometer. Constructed from a transparent acrylic material that does not absorb light or fluoresce at the wavelengths used in the fluorometer, the cuvette has a square lower section with four optical faces and an upper cylindrical section that carries four wings radiating from the cuvette's outer circumference.

The incubator can carry a maximum of 224 cuvettes, contained in four separate 56-place cuvette trays. These trays are disposable and are supplied filled with cuvettes. The cuvette trays sit on a ribbed temperature-controlled metal plate that keeps the temperature of the liquid contained in the cuvettes at 37 (±0.5) °C by means of a heater mat embedded in the base plate of the incubator. The temperature is controlled by a thermistor and a software servo loop. This base plate is mounted on a carriage that is driven on two rails so that the correct row of cuvettes can be positioned for processing. The carriage is also capable of mixing the contents of the cuvettes by a gyratory movement. The DC motor that powers this movement is accurately accelerated, decelerated, and positioned under control of the computers. This is necessary to avoid splashing liquid out of the cuvettes and to ensure that the rest position is always the same after shaking.

*Gantry.* The gantry runs along the back of a heated tunnel, allowing the movement of the sample and reagent probes between the carousels and the incubator and the movement of the grabber between the incubator, the SAW, and the fluorometer. The back plate of the gantry is heated and carries four fans to circulate the air within it, ensuring that no thermal gradient builds up across the gantry. The tubing carrying the wash buffer for the internal and external probe washes runs along the length of the gantry in good thermal contact with the back plate; this ensures that the wash buffer does not cool the probes during washing.

*Pipetting probes.* The sample probe and a separate specific reagent probe are mounted on a single carriage that can traverse the gantry along the length of the instrument. The carriage is propelled by a dc motor that carries a gear wheel meshed in a gear track running along the length of the back wall of the gantry.

The carriage has two other dc motors that can move the probes independently up and down by means of two screw drives. Each probe permanently carries a wash receptacle with it, thus allowing them to be washed as required even while the probes are traveling along the gantry. The external surfaces of the probes are washed by injecting wash buffer into one side of the wash receptacle and suctioning it away to waste from the other side by use of the system vacuum. The internal surfaces of the probes are washed by sending wash buffer, driven by the low-pressure air supply, through the probes. This liquid is also suctioned to waste by the same route as the external probe wash buffer. The wash buffer, which is supplied as a concentrate, is diluted with water and stored in the wash buffer bottle.

When a probe accesses a sample or a reagent for the first time, it uses its built-in capacitive liquid-level sensor to detect the level of the liquid, thus ensuring that the probe enters the liquid only to a depth of ~1 mm. Because the computer stores information about the level of the liquid, a probe revisiting the container can find the liquid level very quickly. After the liquid has been aspirated into the probe, it is drawn a further calculated distance up into the probe, thereby entering the part of the probe that is coiled around a heater block, where it is heated to 37 (±1) °C. The maximum temperature of this heater is controlled to 42 °C to prevent thermal degradation of samples or reagents.

*Cavro and pump module.* This module is situated at the rear of the instrument with an access door at the back of the right-hand side of the case. Each Cavro pump consists of a precision glass cylinder fitted with a stepper-motor-driven plastic piston. These stepper motors are controlled by dedicated microcontrollers that can be used to calibrate the cylinder and control the delivery volumes exactly by optimizing the rates of filling and expelling of the required volume. The system uses three Cavros: one for samples, one for specific reagents, and one for the substrate. There is no aspiration through the substrate probe; the substrate Cavro valve is connected by fixed tubing to the substrate bottle. The same enclosure also houses the vacuum pump and the pressure pump.

*Grabber.* The grabber arm is mounted on a carriage that can traverse the gantry between the above-mentioned modules. The horizontal and vertical motions of the grabber arm are implemented in exactly the same way as for the probes.
Details of the grabber arm are shown in the inset in Figure 1. The grabber has four spring-loaded fingers that position themselves in the four segments of the circumference of the upper portion of the cuvette formed by the four plastic wings that radiate from the cuvette’s outer circumference. These four fingers are held by a spring in the cuvette-gripping position. A cuvette can be released only by compressing the spring to open the fingers. To ensure that the cuvette is released from the grabber, a plunger moves vertically down through the middle of the four open fingers during a cuvette deposition. An optical sensor carried by the grabber mechanism detects whether or not the grabber is carrying a cuvette. Thus, the computer can always check that the grabber has correctly collected or deposited a cuvette.

Separation and wash station. The SAW consists of a small carousel, with circular cuvette holders placed at four equally spaced positions close to the carousel’s circumference. One of these positions is the load/unload position, two are the wash positions, and the fourth is the substrate addition position. The latter three positions are all equipped with two radially displaced sets of probes, one set of which aspirates liquid to waste, whereas the other set is used to add either wash buffer or substrate. The cuvettes are positioned either set of probes by rotating the carousel to the appropriate position. The circumference of each cuvette holder carries a plastic gear that meshes into a mating plastic gear on the internal face of the SAW housing. Thus, when the carousel rotates, the individual cuvette holders rotate at a faster speed than the carousel, in a manner resembling planetary motion about the sun. By repeatedly reversing the direction of travel of the SAW carousel, the resuspension of the solid phase in the cuvettes after magnetic separation is ensured. Apart from rotating to resuspend the solid phase or to select a set of probes as described above, the carousel cuvette positions can be indexed from one position to the next by rotating the carousel through 90°.

The cuvette position that is in line with the grabber arm is the load/unload position; this allows the grabber arm to transfer cuvettes into or out of the SAW. The next two positions (counterclockwise around the SAW carousel) are two identical wash stations. These two wash stations have powerful permanent magnets mounted close to the face of the cuvette to hold the magnetizable solid phase onto the wall of the cuvette before the liquid is separated. The solid phase is pulled to the side of the cuvette in ~2 s; after this, the aspiration probes are moved down by a pneumatic cylinder that is controlled by the software. In this position, the probes rest against the bottom of the cuvettes, which are held against the probes by a spring located under the cuvette holder. The vacuum system is switched on to aspirate the contents of the cuvettes to waste. This operation ensures that the amount of residual liquid left in the cuvette is small and that no solid phase is lost. The aspiration probes are then parked, after which the carousel is rotated so that the cuvettes are now under the dispense probes and wash buffer is added at the two wash positions. The base of the SAW contains an embedded heater mat that maintains a constant temperature within the SAW. The tubing carrying the wash buffer passes through a heat exchanger in this base to preheat the wash buffer to 37 °C before use.

The fourth position is the substrate addition position, which operates in a way similar to the two wash stations except that, after the aspiration step, a Cavro is used to add 300 µL of substrate through a specially heated probe that controls the substrate temperature at 37 (±0.2) °C. The use of this technique allows parallel processing of cuvettes during the SAW cycles.

Fluorometer. The fluorometer is a split-beam system with a low-pressure mercury lamp as the light source. The amount of light energy at the required wavelength of 365 nm is quite small, but this is increased by use of a phosphor reflector placed behind the lamp to convert the lower-wavelength emission to the required radiation at 365 nm. The light from the lamp can be shut off by activating the lamp shutter positioned on the lamp housing. The light from the lamp passes through a collimator lens, and the required wavelength is selected by the use of a band-pass filter. The emergent light passes through a lens system that focuses the light on the cuvette, which is accurately positioned in the fluorometer cuvette holder. Correct positioning is ensured by the four plastic wings on the cuvette, which mate into corresponding slits in the cuvette holder. To reduce the amount of stray light that enters the cuvette, the cuvette holder is covered by the cuvette shutter. This shutter can be opened to allow access for the grabber arm to insert or remove a cuvette from the fluorometer. Two optical detectors positively identify both the open and the closed position of the shutter to avoid any mechanical conflicts during a fault condition.

Fluorescent light energy is measured at right angles to the incident beam and passes through a secondary high-pass filter and then through a band-pass filter that, for use with 4-MU, has a transmission peak at 450 nm. The light passing through these filters falls on the sample photomultiplier tube (PMT), and the signal is ratioed with the signal from the reference PMT. The reference light for this PMT is obtained by splitting off a small portion of the primary beam with a beam splitter.

Before a measurement can be made, the magnetizable particles must be separated onto the two faces of the cuvette that are not in the optical path. The magnet draws the particles onto the sides of the cuvette at a point above the optical faces.

The fluorometer has its own electronics that are operated under the control of a dedicated microprocessor. The stability of the fluorometer is ensured by changing the PMT sensitivity by controlling the anode voltage through use of a software servo control loop. The reference light intensity for this control is obtained from a light-emitting diode that is driven by constant current source. The fluorometer block has embedded in it a heating mantle and a thermistor, which allow a soft-
Fig. 2. Computer configuration of the AN2000

ware servo loop to maintain the unit at 37 (±2) °C.

Electronics. The system electronics are contained in
the compartment at the back of the instrument. The
power supply is a switched-mode unit that allows the
system to be run from a wide range of supply voltages
and frequencies.

The computer consists of two Intel 80286 processor
boards in a master/slave combination that communicate
with one another over an RS232 link. The master
computer controls the VGA monitor with the touch
screen, the keyboard, the bar code reader, and the hard
and floppy disks. It also drives the external ports for the
RS232 link, the remote keyboard, and the printer. The
connections for these are on the bottom left-hand side of
the case below the floppy disk. The tasks performed by
the two computers are diagramed in Figure 2.

The electronics for the probe's liquid-level sensor is on
a small printed circuit board mounted on the probe
assembly itself. The rest of the electronics for the sys-
tem, except those for the fluorometer, are mounted in the
main electronics card cage.

Assay of Samples

To begin assaying samples, the operator must ensure
that adequate supplies of cuvettes, common reagents,
specific reagents, and waste-bottle capacity are avail-
able to allow completion of the tests to be run. The
software provides a guide to the operator performing
these functions as well as a resources report that allows
a check of the available resources at any time. However,
if the operator does not replace some resources and too
many tests are requested for the on-board resources, the
operator interface will inform the operator of any short-
ocnings and provide instructions for their replenish-
ment.

To load a sample, the operator selects the sample load
mode; a sample tube rack will be presented at the
load/unload station, and the operator will be requested
to enter the sample number. This can be either typed in
or read in with the bar code reader if the sample car-
ses a bar code label. The operator must then insert the
tube in the sample tube rack within 5 s to reduce the
Chance that a sample with another sample identification
number could be loaded by mistake. The user can then select
the required analytes to be assayed and any dilutions
that are necessary.

Analytes may be selected individually or as groups in
the form of set panels or intelligent panels. Set panels
are made up of a group of tests that are frequently run
together, e.g., a group of thyroid tests; when set panels
are selected, all the tests in the panel will be run.
Intelligent panels are similar except that the running of
one test can be made dependent on the result for another
test in the panel. For example, if, and only if, thyroxine
exceeds some set value will the thyrotropin assay be run. The analytes grouped in the panels are operator-
selectable, as are the decision values for the intelligent
panels. Similarly, the operator can define the conditions
for automatic dilutions. For example, if, and only if, the
concentration of human chorionic gonadotropin exceeds
the upper range of the method will the analysis be
repeated after a predefined dilution.

After the analytes are selected, the sample tube rack
will be loaded onto the sample carousel, and the oper-
tor can select either batch or random-access mode. If
batch mode is selected, the operator may change analyte
priority. If random-access mode is selected, the samples
may be run in load order, or the computer will select the
run order that gives the shortest time to last answer.
From this point on, the analyses proceed to completion
without further operator intervention. However, the
operator can add stats or additional samples at any time
during a run, up to the maximum sample capacity of the
instrument.

If the software detects that any operating condition
required for the system to continue to perform reliably
is not met, the operator will be informed, and the results
will carry a flag indicating the problem. If the problem
is serious, the system may stop aspirating samples but
will complete any tests already started, assuming that
this is possible.

Calibration and Calculations

There are two types of calibration. First, the fluoro-
meter is calibrated when the instrument is commissioned,
and at service intervals thereafter, by using 4-MU or
solid standards to ensure that one fluorometer unit (1
FU) corresponds to a 1 nmol/L solution of 4-MU in the
cuvette. This calibration is used to express the substrate
blank directly in 4-MU concentration units, these being
common to all analytes. All data used in the following
calculations are assumed to be corrected for substrate
blank.

Second, the system must be calibrated in terms of
analyte concentration for each method assay. The initial
calibration at Anagen is based on in-house reference
materials that have been calibrated against interna-
tionally accepted reference standards for each analyte.
This provides data that relate known concentrations of
each analyte to the instrument response (in FUs). An
iterative least-squares regression analysis is performed
to fit these data to the following model (5):

\[
R = A + BZ/(1 + Z)
\]

where \( R \) is the instrument response, \( Z = e^C + Dm_x \)
and \( x \) is the analyte concentration. The regression analysis
leads to values for the four fitting parameters \( A, B, C, \)

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Table 1. Total Assay Time on the AN2000

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Total assay time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human chorionic gonadotropin</td>
<td>16</td>
</tr>
<tr>
<td>Prolactin</td>
<td>16</td>
</tr>
<tr>
<td>Luteinizing hormone</td>
<td>16</td>
</tr>
<tr>
<td>Follicle-stimulating hormone</td>
<td>16</td>
</tr>
<tr>
<td>Thyrotropin</td>
<td>20</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>10</td>
</tr>
<tr>
<td>Triiodothyronine uptake</td>
<td>10</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>17</td>
</tr>
<tr>
<td>Free thyroxine</td>
<td>22</td>
</tr>
<tr>
<td>Estradiol</td>
<td>58</td>
</tr>
</tbody>
</table>

and D for determining analyte concentration as a function of FUs.

This process is repeated four times to establish four replicate curves. Provided the curves lie within an acceptable envelope, a mean curve is generated and the final values for A, B, C, and D are obtained. This mean curve is the absolute dose–response curve. From these data are derived the information encoded on the reagent pack bar code. This information is transferred, via the bar code reader or the keyboard, to the system software at the time the reagent pack is loaded.

It is necessary to recalibrate the reagent pack before use and at set intervals (typically 1 month) thereafter. This recalibration generates the working dose–response curve. Recalibration may be achieved by analyzing a calibrator pack for each analyte on the machine. Each calibrator pack contains four ready-to-use calibrator solutions in a single unit that can be inserted into a sample tube rack. To perform a recalibration, the operator needs only to scan the bar code label on the calibrator pack and load it into a sample tube rack, after which the recalibration proceeds automatically. When the recalibration is completed, the operator will be informed as to whether the calibration was acceptable or not.

Once the values for the four curve-fitting parameters A, B, C, and D are stored in the system, the computer calculates the concentrations (x) of an unknown sample from the instrument response (R) by using the following formula, derived from the above equations for R and Z:

\[ x = e^{-C_0D} \cdot \left( \frac{A-R}{R-(A+B)} \right)^{D-1} \]

Results and Discussion

Following is a very small sample of the large amount of data we have collected with the AN2000; additional data are to be published elsewhere. Table 1 shows the total assay times for the 10 analytes of our current thyroid and fertility panels. Table 2 shows data for the within-run precision obtained for 6 of the above 10 analytes. These data were obtained with the six second-generation analyzers we tested. All within-run CVs are calculated from ≥20 replicates.

Table 2 also shows data for the between-run precision for the same six analytes obtained by the same analyzers. All between-run CVs are calculated from 12 points, representing 12 individual analyses performed on 12 different days evenly distributed within 1 month. All results were calculated by using the same stored calibration curve without any intermediate recalibration of the instruments; therefore, Table 2 also indicates the stability of the standard curve over this period.

Figure 3 shows correlations of results for thyroxine and thyrotropin in comparison with results obtained by other commercially available methods.

![Fig. 3. Performance of AN2000 for (left) assays of thyroxine (nmol/L) compared with Boehringer Mannheim (Mannheim, FRG) Enzymun-Test and (right) assays of thyrotropin compared with Amerlite (Amersham, Bucks., UK) assay](image-url)
Modern developments in assay technology, particularly the ability to produce stable, high-activity noniso-topic labels and efficient separation technology, together with advances in hardware design and electronics, have made possible the development of this high-performance automated immunoassay system, the AN2000. We are confident that the system is now ready to be used for routine work and that it will bring to clinical laboratories of the 1990s the same benefits of improved assay performance, laboratory efficiency, and lower cost as were realized when automated chemistry systems were introduced in the 1960s.

We gratefully acknowledge that the software and hardware were developed and built by Allen Gregory and the project team at Wilj International Ltd. of Ashford, Kent, UK. We are also indebted to A. B. J. Nix of University College, Cardiff, UK, for assistance with developing the method of instrument calibration.

References