The Analyst

Automated Monosegmented Flow Analyser. Determination of Glucose, Creatinine and Urea

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An automated monosegmented flow analyser containing a sampling valve and a reagent addition module and employing a laboratory-made photodiode array spectrophotometer as detection system is described. The instrument was controlled by a 386SX IBM compatible microcomputer through an IC8255 parallel port that communicates with the interface which controls the sampling valve and reagent addition module. The spectrophotometer was controlled by the same microcomputer through an RS232 serial standard interface. The software for the instrument was written in QuickBasic 4.5. Opto-switches were employed to detect the air bubbles limiting the monosegment, allowing precise sample localisation for reagent addition and signal reading. The main characteristics of the analyser are low reagent consumption and high sensitivity which is independent of the sample volume. The instrument was designed to determine glucose, creatinine or urea in blood plasma and serum without hardware modification. The results were compared against those obtained by the Clinical Hospital of UNICAMP using commercial analysers. Correlation coefficients among the methods were 0.997, 0.982 and 0.996 for glucose, creatinine and urea, respectively.

Keywords: *Monosegmented flow analysis; automated flow analyser; glucose; creatinine; urea*

Automatic analysers are being widely used nowadays owing to the demand for high turnover determinations, mainly in the clinical and environmental fields. These analysers give precise and accurate results with low consumption of both reagents and sample, allowing high laboratory productivity.

Analysers can be divided into three categories according to the sample processing method: robotic, batch (or discrete) and continuous analysers.[1,2] Robot based and batch automatic analysers need high precision mechanical parts and, therefore, are very difficult to maintain by small routine laboratories. On the other hand, continuous flow systems, such as flow injection (FI) and continuous flow analysis (CFA),[3] are simple and an automatic flow instrument can be easily implemented.

Automatic flow analysers employing the FI technique have recently been described.[4–10] The construction of this kind of analyser is relatively simple because samples are individually processed by these systems, that is, the software to control the instrument has to perform a number of sequential operations without any parallel processing because usually only one sample is processed in the manifold each time.

Monosegmented flow analysis (MSFA)[3] was proposed by Pasquini and de Oliveira[11] in 1985. In the MSFA system, the sample (previously mixed with reagents) is introduced into the analyser between two air bubbles. These bubbles minimise sample dispersion, allowing long residence times. The sampling frequency can be maintained as high as in FI systems with several samples simultaneously present in the reaction coil; therefore, there is no direct relationship between sample injection and sample detection. Two main approaches have been taken to add and mix reagents for the samples. The first, which was proposed in the original paper[11] and has since been frequently used,[12–15] employs differential pumping to mix reagents with the sample, before filling the sample loop. The second uses continuous addition of reagents through a confluence point after injection.[16–18] The first procedure does not allow methods based on sequential reactions to be adapted to MSFA systems such as the determination of urea by employing urease enzymatic hydrolysis followed by the Berthelot reaction for ammonium determination. The second, in addition to the high reagent consumption, destroys the integrity of the monosegment and is only feasible when the air bubbles are either removed before sample detection[16] or do not cause spurious signals in the detector, as when AAS is employed.[17,18] Air bubble removal has often been employed before the sample reaches the detector.[11–15,19] This operation eliminates spurious signals but increases sample dispersion. However, Facchin and Pasquini[20] have recently described monosegmented flow systems which perform liquid–liquid extractions, showing that it is possible to carry out the determination without removal of air bubbles.

This paper describes the construction of a microcomputer controlled automatic monosegmented flow analyser which has three main components: a sampling valve, a reagent addition module and a detection system with a photodiode array spectrophotometer.[21] Opto-switches were employed to detect the air bubbles limiting the monosegment, allowing sample localisation for reagent addition and for detection.

The analyser was applied to the determination of glucose, creatinine and urea in blood plasma and serum by employing the well established GOD–PAP, Jaffé reaction and urease–Berthelot methods, respectively. The manifold was designed to allow the determination of each of these analytes with only minor changes. These three analytes were chosen because they are often required in clinical tests; for example, they represent about 40% of the whole demand for analyses at the Clinical Hospital of UNICAMP.

Experimental

Fig. 1 shows a simplified diagram of the analyser. The instrument was controlled by a 386SX IBM compatible microcomputer (25 MHz, 2 Mbytes RAM) through an IC 8255 parallel port[22] that communicates with an interface which uses an address decoder similar to one described elsewhere.[23] The interface controls the peristaltic pump on–off state and the sampling valve. Sample localisation and sampling valve position were followed by employing opto-switches that generate TTL signals which can be accessed by the microcomputer as described previously.[24] A laboratory-made diode array spectrophotometer[21] was used as a detector and was controlled by the microcomputer through an RS232 serial interface.

Automatic Sampling Valve

The sampling valve was constructed by employing a proportional injector[25] whose sliding central bar was connected to a

stepper motor (24 V, 1 A, 7.5° per step). The sampling and injection positions of the valve were determined by using two PCST 2103 opto-switches (optos S and I in Fig. 1). The microcomputer sends a TTL pulse that enables an electronic circuit to switch the valve from sampling to injection position. A third opto-switch (opto R in Fig. 1) was used to generate another TTL pulse that is necessary to return the valve to its initial position. This last pulse is produced when the first bubble of the monosegment passes through opto-switch R, which was placed at a distance from the sampling valve equivalent to the size of the monosegment. Sampling valve commutation was found to occur in about 400 ms.

Automatic Reagent Addition Module

This device was constructed by inserting one (or more) hypodermic syringe needles in a PTFE tube (1.6 mm id), which was fixed with polyester resin, as shown in Fig. 2(*a*). This needle was placed between two opto-switches and each needle was connected to a three-way solenoid valve (12 V, 80 mA), as shown in Fig. 2(*b*). The opto-switches can locate the air bubbles and, therefore, the monosegment containing the sample for reagent addition. The first opto-switch was placed before the needle and the second one air bubble away from the needle. When the first air bubble reaches the second sensor, the solenoid valve is turned on and reagent is delivered into the sample monosegment. The valve is turned off when the second air bubble reaches the first opto-switch. The electronic circuit necessary to perform this operation is shown in Fig. 3

The analyser was constructed with two modules that can add up to three and up to two reagents, respectively (modules 1 and 2 in Fig. 1). The addition of the reagent can be selected and enabled/disabled by software.

Detection System

The detection system was constructed with a flow cell, an opto-switch and the diode array spectrophotometer.[21] An opto-switch (D) was placed after the flow cell as shown in Fig. 1, so that the central zone of the sample monosegment is inside the flow cell when its first bubble reaches the switch. At this moment, a logic signal is generated, triggering the microcomputer to perform the absorbance measurements.

Software for the Analyser

The software for instrument control, data acquisition and treatment was written in Microsoft QuickBasic 4.5. A simplified flow chart of the computer program is shown in Fig. 4. First, it allows start-up of the instrument, by filling the reaction coil with the carrier fluid and the tubing of the addition modules with reagents. The software requests from the operator the sample identification, the number of standards (3–7) and their respective concentrations (to construct calibration curves), the reagents that will be delivered (up to three in the first module and up to two in the second) and the wavelength at which the absorbance will be measured. The spectrophotometer is controlled as described elsewhere[21] and the intensity signals for three, five or nine diodes (covering about a 1.2, 1.9 and 3.5 nm wavelength range centred around the selected wavelength) are transferred to the microcomputer to obtain averaged absorption signals.

Before starting analysis, the microcomputer requests a reference spectral data set to perform absorbance calculations. The software asks for solutions (standards or samples) necessary to perform the determination. Data are processed in real time, results (as a report, showing the calibration curve and concentration of the samples) are shown on the microcomputer video and stored into a file named by the operator. A hard copy of the report can be obtained, if desired.

Reagents and Solutions

Analytical-reagent grade reagents and de-ionized water were used to prepare all solutions.

Chromium(VI) working standard solutions from 0.200 to 1.400 mg l^{-1} were prepared by dilution of a 1000 mg l^{-1} Cr^{VI} stock standard solution. A 0.25% m/v diphenylcarbazide (DPC) solution was prepared in 25% v/v acetic acid and 2.0 mol l^{-1} sulfuric acid solution was prepared by dilution of the concentrated acid.

A 0.01 mol l^{-1} PIPES buffer solution (pH 7.2) was prepared by 1 + 4 v/v dilution of the Merck (Darmstadt, Germany) solution (catalogue No. 14144). Merck reactive No. 14143 (GOD–PAP method) was diluted 1 + 40 with Merck solution No. 14144. β-D-Glucose standard solutions were prepared in the range 0.50–10.0 mg dl^{-1} in 0.01 mol l^{-1} PIPES buffer solution.

Creatinine standard solutions were prepared from 0.10 to 1.20 mg dl^{-1} in 0.1 mol l^{-1} hydrochloric acid and 4.0 mol l^{-1} sodium hydroxide and 5.5×10^{-2} mol l^{-1} picric acid solutions were prepared with de-ionized water.

Urea standard solutions were prepared from 0.50 to 5.00 mg dl^{-1}. A 0.10 mol l^{-1} phosphate buffer (pH 7.2) was prepared in 0.9% sodium chloride and 0.001% v/v Brij 30 solution. A 44 kU l^{-1} urease solution was prepared in water.

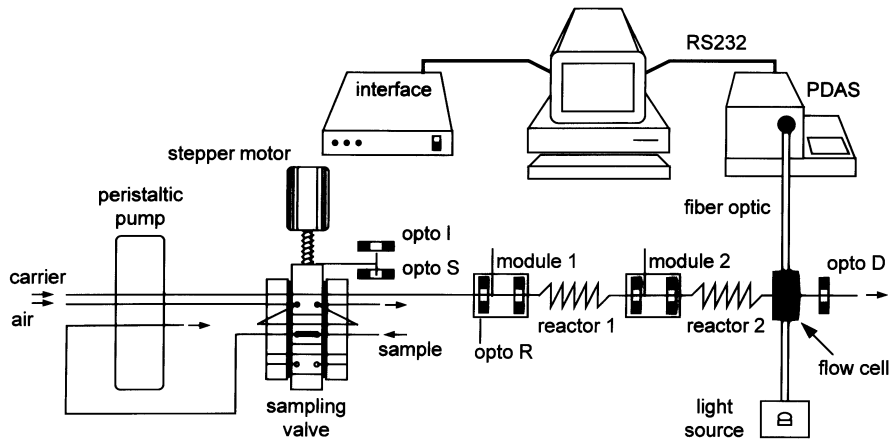

Fig. 1 Schematic diagram of the automated flow analyser. For details, see text.

Other solutions were 6.0% phenol plus 1.0% sodium nitroprusside and 1.0% sodium hypochlorite in 4.0 mol l^{-1} sodium hydroxide.

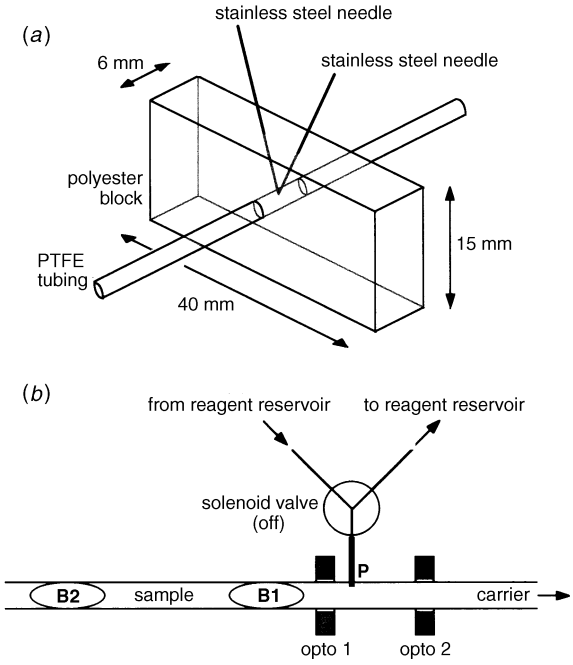

Fig. 2 (*a*) Reagent addition module and (*b*) addition point P (stainless-steel needle).

Procedures

Evaluation of the analyser

A manifold similar to that shown in Fig. 1 was employed, but with reactor 1 removed and a PTFE tubing coil of 1.5 m length and 1.6 mm id used as reactor 2. De-ionized water was used as the carrier at a flow rate of 2.0 ml min^{-1}. The first and the second air bubbles, limiting the monosegment, had volumes of 90 and 50 μl, respectively. These flow parameters allowed a residence time of 90 s for the samples, after the second module of reagent addition. The sample monosegment volume was 300 μl, except where specified otherwise.

Determination of glucose, creatinine and urea

The manifold shown in Fig. 1 was employed. Two glass reactors of 1.6 mm id were used; the carrier flow rate and bubble air volumes were kept as in the evaluation of the analyser, allowing residence times for samples of 2.0 and 6.5 min in the first and second reactors, respectively. The sample loop had a volume of 220 μl.

For glucose determination, blood plasma samples were manually diluted 1 + 45 v/v with 0.01 mol l^{-1} PIPES buffer solution. This buffer solution was also employed as the carrier and the reagent was delivered through the second reagent addition module at a flow rate of 0.16 ml min^{-1}. Absorbance measurements were carried out at 510 nm.

For creatinine determination, blood serum was deproteinized with 5% trichloroacetic acid solution (1 + 1 v/v). The supernatant was manually diluted 1 + 1 v/v with deionized water. Sodium hydroxide (0.16 ml min^{-1}) and picric acid (0.28 ml min^{-1}) were added to the sample through the first and

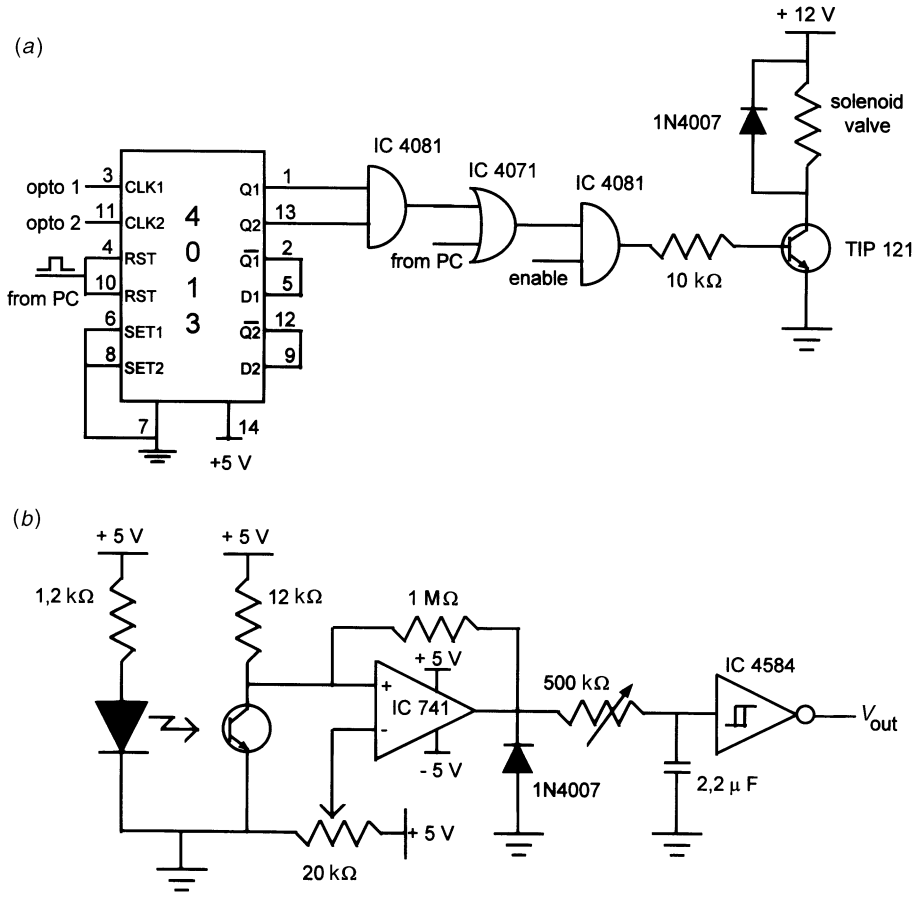

Fig. 3 Electronic circuit of the reagent addition module: (*a*) circuit to turn on and turn off the solenoid valve (enabled by the microcomputer) and (*b*) circuit to extract logic signal from the opto-switch.

second reagent addition modules, respectively. De-ionized water was used as the carrier. Absorbance measurements were made at 500 nm.

For urea determination, blood serum samples were deproteinized as in the determination of creatinine. The supernatant was diluted 1 + 45 v/v with phosphate buffer solution, which was also used as the carrier. Urease solution (0.16 ml min^{-1}) was added through the first module; phenol–sodium nitroprusside (0.16 ml min^{-1}) and sodium hypochlorite–sodium hydroxide (0.16 ml min^{-1}) solutions were both mixed with the sample through the second reagent addition module. Absorbance was measured at 620 nm.

Results and Discussion

Evaluation of the Analyser

MSFA analysers usually work with several samples being processed sequentially in the reaction coil, in order to allow for long residence times without decreasing the sample throughput. Therefore, one sample could be passing through the detector (and absorbance measurement must be performed) while some other tasks, such as switching of the sampling valve or addition of reagent, have also to be carried out. Thus, the hardware and software of the MSFA analyser was developed in order not to miss an absorbance measurement. The switching of the sampling valve from injection position to sampling position and the addition of reagents to the sample are performed automatically under hardware control enabled by the computer. The microcomputer has the task of sending a logic signal to perform sample injection and this action can be delayed for a few seconds if an absorbance measurement is being obtained for a sample present in the flow cell. Fig. 4 shows the flow diagram of the routine that allows the control of these tasks. In addition to releasing the microcomputer, the opto-switch, used to trigger the return of the sampling valve to its injection position, makes this event independent of the flow rate, which is an advantage when a method is being developed.

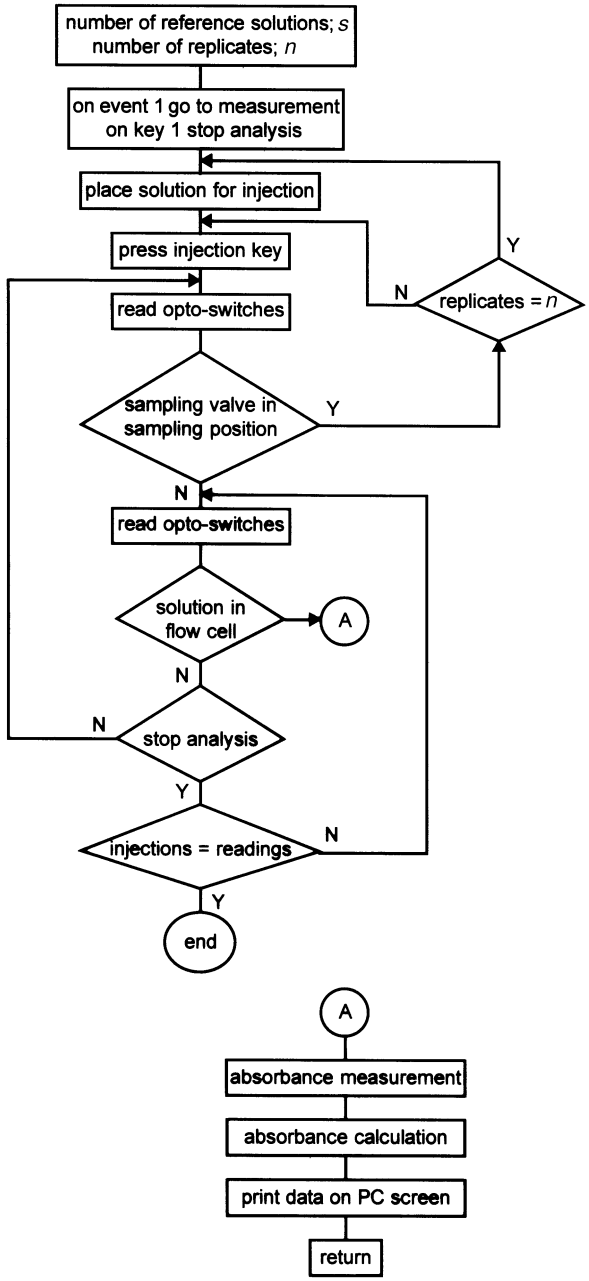

Fig. 4 Flow diagram of the software developed to control sample processing in the analyser (event 1 means solution in flow cell).

A disadvantage of an MSFA analyser, in general, is related to the admission and/or formation of small air bubbles in the reactor, because the air bubbles of the monosegment are used to drive reagent delivery through the addition modules and to control sample measurement. This problem was minimised by adjusting the opto-switch sensitivities with RC components of the circuit shown in Fig. 3(*b*). For a carrier flow rate of 2.0 ml min^{-1}, the opto-switch sensitivities were adjusted in order not to generate a logic level transition for air bubbles lower than 20 μl.

The Cr^{VI}–DPC reaction was used to evaluate the analyser performance, by adding 2.0 mol l^{-1} sulfuric acid and 0.25% DPC at flow rates of 0.07 and 0.15 ml min^{-1}, respectively, consecutively to 300 μl of sample through modules 1 and 2.The concentrations of the reagents and the flow rate ratios between reagents and carrier (sample) were determined according to the standard recommended method.[26] Absorbance measurements were performed at 540 nm, with a bandwidth of 3.5 nm (averaging signal intensities of nine diodes). Standard solutions of Cr^{VI} from 0.2 to 1.4 mg l^{-1} were injected in tripiclate at a sampling frequency of 60 h^{-1}. Absorbance values were obtained in the range 0.0611– 0.4069, with an average absolute standard deviation of 0.0017. The precision obtained in these absorbance measurements agrees with those obtained previously in the absence of reactions,[21] indicating that the analyser shows a very good performance. The injection of a blank solution (water, $A = 0.0016 \pm 0.0016$) after a 1.4 mg l^{-1} Cr^{VI} standard solution ($A = 0.4069 \pm 0.0021$) showed that there is no significant carry-over between samples. The calibration curve obtained with these data is $A = (0.0078 \pm 0.0028) + (0.2880 \pm 0.0031)C$ ($r = 0.9997$), where A is solution absorbance and C is the Cr^{VI} concentration, in mg l^{-1}. Considering that the analyser has a flow cell with only a 5 mm long pathlength, these results also agree with those obtained previously, with respect to sensitivity and linearity.[11]

The injection of 300 μl of a Cr^{VI} sample solution, as described, resulted in a consumption of 7.5 μl of sulfuric acid and 12 μl of the DPC solution. When 100 μl of sample were injected, these consumptions were lowered to 2.5 and 4.0 μl, respectively. Table 1 shows some parameters obtained under different conditions of analysis; the sensitivity is almost independent of the sample volume whereas the precision (determined by the standard deviation of ten replicates of a 1.00 mg l^{-1} Cr^{VI} solution) of the measurements decreases when the sample volume is decreased and at higher sampling frequencies. As can be seen, this automatic monosegmented flow analyser shows a good performance, allowing a sensitivity that is virtually independent of the sample volume and consuming less reagent than other instruments because the reagents are not delivered continuously but only into the monosegment. Fur-

Table 1 Dependence of the sensitivity and precision of the monosegmented flow analyser on sample volume and sample frequency

		Calibration curve			
V_{sample}/ µl	Frequency/ h^{-1}	Linear coefficient	Slope	r	$A \pm s^*$ (n = 10)
300	60	0.0027	0.2459	0.9996	0.2519 ± 0.0016
100	60	0.0020	0.2376	0.9999	0.2337 ± 0.0031
100	120	0.0029	0.2306	0.9996	0.2341 ± 0.0051

* Absorbance of a 1.00 mg l^{-1} Cr^{VI} reference solution ± standard deviation.

thermore, the reagent addition module makes possible the use of methods employing sequential reactions in MSFA, without disturbing the monosegment pattern.

Determination of Glucose, Creatine and Urea

Standard, manually processed, methods were adapted to the analyser in order to allow the determination of glucose, creatinine and urea with minor changes to the manifold. Therefore, the experimental parameters employed (mainly sample dilution and residence time) were not optimised for each analyte, but were aimed to suit the overall performance of the analyser. For example, although the glucose reagent was added through the second module, the first reactor (not necessary for this determination) was not removed from the manifold. However, this procedure did not alter the frequency of sample introduction but just increased both the delay necessary for the first sample to reach the detector and the sample dispersion to a minor extent. It is important to emphasise that in the urea determination, urease is added through the first module and, after this reaction has been processed, reagents for ammonium determination are delivered through the second reagent addition module. This operation is the main feature of the proposed analyser, *i.e.*, it became possible to perform sequential reactions without disturbing the monosegment and with reagents being added only to the sample.

Glass reactors were used in the manifold because the sample monosegment was not stable in PTFE reactors, mainly in the determination of creatinine. This probably occurs because blood proteins have a stronger affinity for PTFE. On the other hand, glass is wet by aqueous solutions and, therefore, when this material is employed, an increase in cross-contamination and a decrease in precision are observed. A manifold made with PTFE reactors allows insignificant cross-contamination and an RSD of 0.7% for six injections of a 1.00 mg dl^{-1} creatinine aqueous reference solution. A signal that is 2.5% of that obtained for any creatinine reference solution in the range 0.1–1.2 mg dl^{-1} was observed for the first blank introduced after the reference solution in a glass reactor. This characterises a cross-contamination that should be considered if the introduction of samples and/or reference solutions is not replicated. However, cross-contamination effects were minimised by injecting samples in triplicate and averaging the three signals obtained, because it only affects the first signal. Furthermore, as in real calibration and sample determinations the change in concentration is not so drastic, the cross-contamination is minimised. This is particularly true for the samples.

Table 2 shows the figures of merit for the methodologies adapted to the developed analyser. The results obtained with the analyser (MSFA) were plotted against those obtained by the Clinical Hospital (CH) of UNICAMP and the results for glucose, creatinine and urea were MSFA = 4.72 + 0.895CH (r = 0.997, n = 25), MSFA = 0.0785 + 1.155CH (r = 0.982, n = 29) and MSFA = 13.5 + 0.956CH (r = 0.996, n = 17), respectively. At the Clinical Hospital, the determinations were performed by automatic discrete analysers, *i.e.*, Merck–Vitalab Selectra (glucose) and Roche Cobas–Mira (creatinine and urea). Glucose and creatinine were also determined by the GOD–PAP and Jaffé methods, respectively. However, a kinetic procedure was employed in both determinations and the results were obtained from the difference between two absorbance measurements, made in a pre-defined time interval. Urea determination was based on the reaction of ammonium ion (produced by urease catalysed urea hydrolysis) with 2-oxoglutarate and NADH, in the presence of glutarate dehydrogenase, and the decrease in absorbance, due to the NADH consumed, was measured at 340 nm. Although a good correlation coefficient was always observed (r > 0.98) for the three analytes, the results do not agree completely and there are both constant and proportional systematic differences. The origin of these differences can be attributed to the different methodologies and/or instruments employed, as pointed out by Koch and Peters.[27] For example, in the creatinine determination some interferences (*e.g.*, from proteins) can be eliminated by employing a kinetic method, as in the procedure used in the Clinical Hospital. Differences such as those found in this work have often been reported for clinical methodologies[28–35] and seem to be tolerated from the clinical point of view. According to this point of view, these differences are not a serious drawback to the use of the proposed methodologies because the range of reference values for blood analyte concentrations is a function of the methodology and/or instrument employed for the determination.[27]

Conclusions

The automated analyser allows determinations to be performed with low reagent consumption. Furthermore, with the development of the reagent addition modules it is possible to adapt methods based on sequential reactions to MSFA without disturbing the monosegment, because the reagents are delivered only into the sample zone. Direct adaptation of the manual procedures can be made by setting appropriate flow rates for carrier fluid and reagents, which maintain the proportion of the manual procedures. The sensitivity of the analyser shows little dependence on the carrier flow rate if the reagent flow rate is kept proportional and the reaction reaches completion. Also, the sensitivity is almost independent of the sample volume, owing to low monosegment dispersion and proportional addition of reagents. Finally, the same manifold can be used to determine different analytes, although some straightforward dilution

Table 2 Figures of merit for glucose, creatinine and urea determination with the monosegmented flow analyser

Analyte	Averaged precision (RSD) (%)	Upper limit of linear range/ mg dl^{-1*}
Glucose	1.8	400
Creatinine	3.6	2.00
Urea	3.7	200

* Before sample dilution, as described under Experimental.

operations need to be performed before sample introduction into the system.

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