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Metabolic-sensing characteristics of absorption-photometry for mammalian cell cultures in biopharmaceutical processes

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Background: In mammalian cell culture processes, metabolite concentrations have to be routinely monitored to ensure the consistency of both process operation and product quality. The absorption photometric-based technology (APBT) has been introduced recently as a new option for characterizing metabolic profiles of the cell-culture processes. In order to understand the measurement technology, the benchmarking study was conducted under various conditions, including known standard samples and untreated cell-culture samples. **Results:** The standard samples, with known concentrations of the metabolites, were analyzed using APBT, and its repeatability, as well as its accuracy was compared with the other two common technologies used in the industry: membrane-based technology, and supplementary HPLC. The three technologies were further validated using the supernatant collected from batch cell-cultures from two different Chinese hamster ovary cell lines with extended culture duration, covering a broad range of experimental conditions. A statistical analysis was conducted to evaluate the different technologies, and it revealed that the APBT exhibited the best accuracy, while both APBT and membrane-based technology provided good reproducibility. On the other hand, HPLC was highly susceptible to instability when under sub-optimal conditions, especially to the untreated cell culture samples with high complexity and interference. At the same time, the investigation of correlations among the different technologies indicated that APBT was highly compatible with other two technologies. **Conclusion:** Both good accuracy and high precision of the metabolite analysis achievable with APBT suggest that it might be another viable option for analyzing the metabolites in mammalian cell cultures.

Mammalian cells, such as Chinese Hamster Ovary (CHO) cells, are the most frequently used host cells in therapeutic protein production due to the ease of their maintenance and their established safety profile of the protein products for human use [1,2]. However, despite the enormous efforts made for improving the efficiency of therapeutic protein production from mammalian cell cultures, the processes easily suffer from poorly understood instability and perturbations which often result in inconsistent performance and product quality [3,4]. Therefore, in mammalian cell culture, several parameters are closely monitored on a regular basis in order to properly track progression of the cells' growth and their protein production with consistent process operation. Especially in the context of process analytical technology and Quality by Design [5–7] which have recently been initiated by the US FDA, this can lead to improved understanding of the underlying process by characterizing the critical process parameters and their influence on the overall cell culture performance as well as final product quality.

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Key Terms

Membrane-based technology: Utilizing the immobilized enzymatic membranes with amperometric electrodes to quantify the metabolites of interests.

Absorption photometric-based technology: Utilizing enzymatic reactions with reagents and subsequent photometric assays to quantify the metabolites of interests.

Among the several critical process parameters in a typical mammalian cell culture, the metabolite concentrations, and their evolution during the culture process, are the most fundamental and paramount of them as the current metabolic status of mammalian cells can be directly inferred from these measurements, relating them to the cell growth characteristics as well as therapeutic protein production [8–10]. Here, the essential metabolites, which are usually monitored throughout the culture, generally include glucose and glutamine (substrates), glutamate, lactate and ammonium (by-products or waste). To measure those parameters, several platforms and methodologies have been proposed, including off-line, at-line and on-line techniques, such as membrane-based technology (MBT) [11], HPLC [12–14], or on-line near-infrared/Raman/fluorescence spectroscopies [15–17]. Among them, one of the most widely used techniques is the multi-analyzer system based on MBT due to its simplicity and convenience [11]; commercially available systems, such as BioProfile® (Nova Biomedical, MA, USA) or YSI Biochemistry Analyzer (YSI Life Science, OH, USA) are widely adopted in the biotechnology industry. In practice, they use membrane-based biosensors or electrodes together with flow injection analysis techniques for the sample delivery, providing an efficient way to analyze the metabolite's level during the cell culture in both off-line and at-line applications. However, one of the main drawbacks of this technology is the relatively high consumptions of materials (e.g., buffer solutions and standards), short lifetime of the membrane sensor units, and changes in the quality of the enzymatic membrane over time, thus necessitating frequent recalibrations and high maintenance costs [18,19].

On the other hand, a technique based on absorption photometric-based technology (APBT) has recently been introduced in the market as a way to characterize the metabolites by using the alternative principle of a photometric assay in an automated fashion (Cedex Bio; Roche Diagnostics, USA) [19]. In APBT, the samples are mixed with a set of reagents for the enzymatic reactions, and their absorption levels are measured by a photometric measurement unit to quantify the metabolite concentrations, which differentiate it from other enzymatic biosensor systems, such as MBT. Under normal conditions, calibration with the standards is only performed when the reagents are replaced or changed, thus providing good reproducibility and requiring lower maintenance and materials demands compared with MBT. Therefore, in mammalian cell culture, the corresponding APBT platform can be a good alternative in-process monitoring tool for the metabolites, potentially replacing, or complementing, the current multi-analyzer systems based on MBT or the more laborious HPLC techniques. However, to really take advantage of the full benefits of this technology in a diverse range of cell culture processes, a systematic evaluation of its accuracy, repeatability and comparability to other techniques must to be performed in advance, and it has to be compatible with the currently utilized technologies.

Based on the above considerations, in this study, a benchmarking study was conducted for the APBT, and its repeatability as well as reliability was compared with the other most common techniques, MBT and HPLC, under various conditions. To accomplish this, the standard samples, with known concentrations, and the cell culture samples obtained from two different CHO cell lines were comprehensively analyzed by the corresponding technologies (i.e., APBT, MBT and HPLC), and their accuracy, consistency and similarity were examined by employing statistical analysis tools. Note that, here, the HPLC analysis was only used for the supplementary comparison between the different technologies since our in-house protocol for the HPLC analysis of the metabolites was only suboptimal with low sensitivity and high interferences. At the same time, other more advanced techniques, such as LC-MS or NMR [20,21] were not considered in this comparative study, due to their laborious, expensive and time-consuming procedures, which make them unsuitable for the routine monitoring of the major metabolites in the corresponding biotechnology industry. The results obtained in this study clearly revealed that the accuracy and precision of the APBT was comparable, or superior, to MBT, suggesting that it might be used as another viable option for analyzing the metabolites in mammalian cell cultures with the reduced maintenance and material costs as well as improved accuracy.

Materials & methods

» Instrumentation

To assess the performance of APBT, a Cedex Bio (Roche Diagnostics, Indianapolis, IN, USA) was utilized in this study as a model platform. Here, glucose, glutamine, glutamate, lactate, ammonia and (extracellular release of) lactate dehydrogenase for given samples were analyzed using the automated enzymatic photometric assays by following the user instruction manual provided from the manufacturer (Roche Diagnostics). In addition, sodium and potassium were measured separately using an integrated

Table 1. Measuring range of glucose, lactate, glutamine and glutamate in each of the technologies employed here.

	Glucose (g/l)	Lactate (g/l)	Glutamine (mmol/l)
Absorption photometric-based technology	0.02–7.5 (0.02–75†)	0.018–1.4 (0.018–14†)	0.4–10.3 (0.4–102.6†)
Membrane-based technology	0.2–15.0	0.2–5.0	0.2–6.0
HPLC	-‡	-‡	-‡

†Values represent the measuring range of absorption photometric-based technology when the automatic dilution capability of the instrument was taken into account.
‡The measuring range of HPLC is dependent on the instrumental setting and conditions.

ion selective electrode module equipped in the instrument. All of the reagents and control standards for calibration were stored at 4°C in the refrigerator prior to using them, and the calibrations were performed regularly whenever the corresponding reagents were replaced. At the same time, when the measured concentration values were outside of the calibration range, automatic dilutions with subsequent repeated measurements were performed for the corresponding samples.

For MBT, a Bioprofile 400® (Nova Biomedical, Waltham, MA, USA) was employed. Here, sodium, potassium, pH, carbon dioxide and ammonium were analyzed with potentiometric electrodes, while oxygen, glucose, lactate, glutamine and glutamate were measured by amperometric electrodes equipped in the instrument [22]. During the analysis, the reagent pack (Nova Biomedical, USA) was regularly replaced every 2 weeks, following the user instruction manual provided by the manufacturer, and the automatic calibrations were performed with default settings. The concentrations were internally calculated by the instrument and recorded on thermal paper.

The HPLC analysis of the selected metabolites was conducted using a Waters® 2695 Separations Module (Waters Corporation, Milford, MA, USA) equipped with a Waters 2414 Refractive Index detector and an Aminex® HPX-87C 300 × 7.8 mm column (Bio-Rad Laboratories, CA, USA) thermally set at 60°C. The column was packed with a polystyrene divinylbenzene matrix and a 5 mM calcium nitrate solution at pH 5.5 was utilized as the eluent. Prior to the HPLC analysis, the calibration standards: glucose, glutamate, lactate and glutamine, were prepared in a specified range and analyzed together with the samples.

Note that in this study, only the concentrations of glucose, glutamate, lactate and glutamine were further analyzed and compared among different technologies despite the availability of other metabolite measurements, since only those four metabolites were overlapped across all the technologies, and in fact, they are of major interests in typical mammalian cell culture. Table 1 summarizes the measuring range of these four metabolites in each of the technologies, with exception of HPLC since its measuring range depends on instrumental and analysis conditions.

Standard samples with known concentrations

The standard samples with known metabolite concentrations for glucose, lactate, glutamine and glutamate were provided by Roche Diagnostics, and were used to check the accuracy and precision of the APBT as well as the MBT and HPLC throughout this study. For glucose and lactate, the standard samples were prepared by adding a known amount of each component into a mixture with two different concentration levels (i.e., low and high), while the independent standard samples were utilized for glutamine and glutamate with two different concentration levels (i.e., low and high). Table 2 summarizes the conditions of each standard sample with their specified concentration ranges. All of the standard samples were stored at 4°C in a refrigerator prior to the experiments and were analyzed in duplicate. After measuring the standard samples by each of the technologies (i.e., APBT, MBT and HPLC), the recovery (%) was calculated for each of the metabolites using the following equation.

$$\text{Recovery} = \frac{\text{Concentration measured by instrument}}{\text{Known actual concentration}} \times 100\,(\%)$$

Equation 1

Furthermore, the same standard samples were analyzed on different days (10 consecutive days) using an identical procedure in order to track the stability of the accuracy and precision for each of the technologies.

Table 2. Sample conditions for the glucose, lactate, glutamine and glutamate standards at high and low levels.

Standard sample condition	Glucose (g/l)	Lactate (g/l)	Glutamine (mmol/l)	Glutamate (mmol/l)
Low	0.879	0.149	1.204	2.861
High	2.270	0.308	8.364	7.963

Mammalian cell-culture samples

In order to validate the APBT and compare it with the other techniques under more realistic situations, the cell culture samples obtained from CHO cell cultures were analyzed with each of the technologies. Here, all of the experiments were designed to investigate the consistency and machine variability of each technology, as well as the similarities among the different technologies under various conditions. Therefore, two different cell lines, CHO-DG44 and FreeStyle™ CHO-S, having different media formulations, were employed as model systems to simulate a broad range of metabolite concentrations and compositions as well as possible interferences from other components in the corresponding media. At the same time, two different vessel types (i.e., shaking flask vs glass bioreactor vessel) were employed for each of the cell lines in order to eliminate any bias towards a particular culture vessel or cover the different level of salt concentrations originating from the supply of gases and bases in the bioreactor operations.

Both cell lines were previously adapted to suspension culture and chemically defined media prior to the experiment. All of the media components were purchased from Invitrogen (Life Technology, NY, USA), unless otherwise stated. The composition of the chemically defined media for CHO-DG44 consisted of CD OptiCHO™ Medium (55%), CHO CD EfficientFeed™ A (20%), CHO CD EfficientFeed™ B (20%), 200 mM L-glutamine (4%) and HT Supplement (1%). For FreeStyle CHO-S cells, FreeStyle™ CHO Expression Medium supplemented with L -glutamine (4%) was used throughout this study. Both of the cell lines were cultivated in 500 ml shake flasks with a working volume of 200 ml (three flasks for each cell line) and a target seeding cell density of 0.5×10^6 cells/ml in a carbon dioxide incubator maintained at 130 rpm, 8% CO_2 and 37°C, in a humidified atmosphere. In the shaking flask, each cell line was grown independently to avoid possible cross contamination, and they were cultivated with an extended culture duration until the cells were in the death phase, covering a broad range of experimental conditions and metabolite concentrations. At the same time, one additional batch for each of the cell lines was grown in a 3 l glass vessel (working volume of 2 l) equipped with a marine impeller under the bioreactor operating conditions. Here, an Applikon ADI 1010 Bio Controller and an ADI 1025 unit (Applikon Biotechnology, CA, USA) were utilized to maintain agitation (130 rpm), temperature (37°C), pH (6.95) and dissolved oxygen (40%) in the bioreactor at a constant level using 0.5 M sodium carbonate, carbon dioxide and oxygen gases.

In order to evaluate the different technologies for measuring the metabolites in the corresponding CHO cell cultures, samples were collected from the flasks and bioreactor once a day, and analyzed immediately using the Cedex Bio (i.e., APBT) and Bioprofile 400 (i.e., MBT) in triplicate. For the HPLC analysis, all of the collected samples were centrifuged at 1000 rpm for 2 min in order to remove any particulate matter and cell debris from the supernatant. Then, the samples were stored in a freezer at -20°C and analyzed by HPLC in triplicate on the same day after thawing and centrifuging them again at 10,000 rpm for 10 min.

Statistical analysis & evaluations

In this study, statistical analyses were performed in order to evaluate the metabolite concentrations measured by three different technologies: APBT, MBT and HPLC. For this, an analysis of variance (ANOVA) was utilized in order to determine whether there are the statistically significant differences within the triplicate measurements of each technology or among the different technologies. Here, the null hypothesis was that the mean values of the measurements for each group are equal. Therefore, whenever the probability value (p-value) based on the F-test was less than 0.05, the null hypothesis was rejected for the given ANOVA test, and the follow-up pair-wise multiple comparison test was performed using the Tukey–Kramer honestly significant difference test [23].

In addition to ANOVA analysis, in this study, the residual standard deviation (RSD) was utilized to further determine the accuracy of the measurements. RSD was calculated from the measured values of the metabolite concentrations by dividing the residuals by the standard deviation, thus allowing the comparison of different groups of measurements even though the scales of each group are significantly different from each other. Note that all of the statistical analyses and mathematical computations employed here were conducted using JMP® software (SAS, NC, USA).

Results & discussion

» Standard sample analysis

First, high and low standard samples with known metabolic concentrations were used to investigate the measurement accuracy of each technology. The same standard samples were analyzed on 10 consecutive days using identical procedures, and their accuracy, as well as stability, were assessed from these measurements. Figure 1 shows the recovery for the four metabolites measured from the low and high standard samples by using the three different technologies: APBT, MBT and HPLC. In this figure, one can see that the measurements of APBT are in excellent agreement with the known concentrations of the standard samples (i.e., recovery close to 100%) for all four metabolites

investigated here, while MBT and HPLC suffered from low recovery for some of the metabolites. For example, MBT could not provide good accuracy for lactate, although the other three metabolites had acceptable recovery. Possible reasons for the poor accuracy of MBT observed for the lactate might be due to the relatively low concentrations of the lactate in both the low and high standard samples (0.149 and 0.308 g/l), which were below, or very close to, the lowest level of the measuring range (i.e., 0.2–5.0 g/l) for the lactate sensor in the Bioprofile 400 system [101]. In fact, it is worth noting that similar trends were also observed for the cell culture samples with low concentration of lactate, further confirming the above explanation.

On the other hand, the accuracy of HPLC was not satisfactory for the majority of metabolites, except for lactate, as shown in Figure 1. The reason for the poor recovery for these metabolites is not clear at this time, but, in general, the observed chromatograms obtained from the standard samples suffered from low resolution and overlapping peaks, indicating that the in-house routine and analysis conditions employed in this study might not be optimal for analyzing those metabolites under the given sample conditions. As a result, in this study, only the APBT could exhibit reliable precision, as well as good recovery, for the standard samples investigated here, and the observed recovery remained stable within the acceptable range (i.e., 99 ± 4%), regardless of the types of metabolites and the level of their concentrations. However, to further generalize these findings, a broader range of the metabolite concentrations in the standard samples should be investigated in more comprehensive ways, as it turned out that the lactate concentrations employed here were not favorable for the given MBT platform. Note that, although the lactate and other metabolites (i.e., glucose) had lower concentrations in these standard samples compared with the typical level encountered in mammalian cell culture, it can be easily expected that under the conditions with moderate to high concentration of the metabolites within the measuring range of each instrument, each technology will perform reasonably well, as illustrated in other studies [19,24]. The same trends could be also observed for APBT and MBT in Figure 1, in that the glucose and lactate showed better recovery for the high standard samples. Therefore, the results obtained in this study indicate that, under the conditions close to limit of detection, which might be met during the initial (e.g., for lactate) or late-growth phase (e.g., for glucose) of the cell culture, APBT might provide best accuracy compared with other technologies.

In order to further illustrate the differences between the three technologies, the RSD was calculated from the above measurements and shown in Table 3 for both the low and high standards. The mean values and standard deviations for each of the technologies were also presented together for a comprehensive comparison between them. In Table 3, it can easily be recognized that the RSD value for the APBT was the lowest among of the three technologies for all cases, again demonstrating the supe-

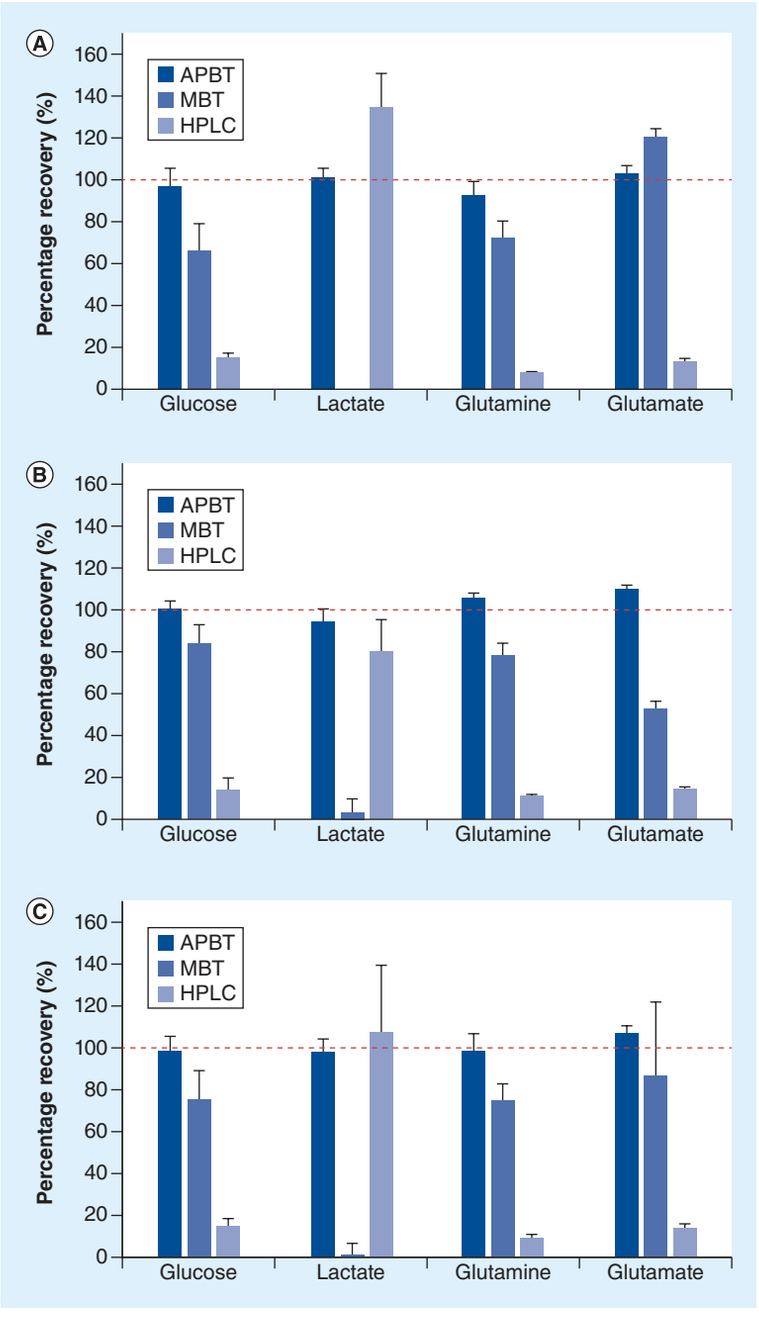

Figure 1. Recovery rate of absorption photometric-based technology, membrane-based technology and HPLC. (A) Low standard samples. **(B)** High standard samples. **(C)** Average of low and high standard samples. A horizontal dashed line with recovery rate of 100% allows a clearer comparison. APBT: Absorption photometric-based technology; MBT: Membrane-based technology.

Table 3. The calculated residual standard deviation values for glucose, glutamine, lactate and glutamate for each technology for low and high standard solutions.

Component		APBT	MBT	HPLC	Standard sample
Low standard solution					
Glucose	σ	0.075	0.116	0.016	
	μ	0.854	0.604	0.132	0.879
	RSD	8.8	19.14	11.97	
Lactate	σ	0.006	0	0.024	
	μ	0.153	0	0.201	0.149
	RSD	4.08	-	12.1	
Glutamine	σ	0.073	0.109	0.01	
	μ	1.11	0.857	0.095	1.204
	RSD	6.62	12.72	10.06	
Glutamate	σ	0.085	0.133	0.057	
	μ	2.952	3.424	0.383	2.861
	RSD	2.9	3.88	14.86	
High standard solution					
Glucose	σ	0.107	0.231	0.127	
	μ	2.296	1.936	0.307	2.270
	RSD	4.67	11.94	41.51	
Lactate	σ	0.017	0.02	0.05	
	μ	0.289	0.006	0.245	0.308
	RSD	5.98	346.4	20.26	
Glutamine	σ	0.182	0.549	0.034	
	μ	8.779	6.391	0.874	8.364
	RSD	2.08	8.59	3.85	
Glutamate	σ	0.094	0.402	0.061	
	μ	8.723	4.053	1.107	7.963
	RSD	1.08	9.91	5.51	

APBT has smaller RSD values than HPLC and MBT. The standard mean concentration is most comparable with APBT.
σ: Standard deviation; μ: Mean value; APBT: Absorption photometric-based technology; MBT: Membrane-based technology; RSD: Residual standard deviation.

Table 4. Analysis of variance between the mean measurements from the different technologies and the standard samples with known concentrations.

Component	Standard vs APBT	Standard vs MBT	Standard vs HPLC
Low standard solution			
Glucose	0.9999	<0.0001†	<0.0001†
Lactate	0.4608	<0.0001†	0.0001†
Glutamine	0.0126†	<0.0001†	<0.0001†
Glutamate	<0.0001†	<0.0001†	<0.0001†
High standard solution			
Glucose	0.7822	<0.0001†	<0.0001†
Lactate	0.9851	<0.0001†	<0.0001†
Glutamine	0.0144†	<0.0001†	<0.0001†
Glutamate	0.0718	<0.0001†	<0.0001†

The p-values associated with low and high standard levels were presented for the four metabolites. APBT was the only technology that showed some comparability the standard concentrations.
†Any value less that 0.05 indicates an observed significant difference.
APBT: Absorption photometric-based technology; MBT: Membrane-based technology.

riority of the corresponding technology. At the same time, the standard deviations of the APBT calculated for the same standard samples over 10 days show that they were either the smallest, or close to the smallest one, among the three different technologies, thus exhibiting relatively good stability (or consistency) over 10 days. A further evaluation of the different technologies was made by using ANOVA for a pair of the technologies and standard samples as shown in Table 4. It clearly reveals that the measurements taken by MBT and HPLC were significantly different from the known concentrations of the metabolites in all of the standard samples, which was in line with the previous results. On the other hand, in APBT, only glutamine and glutamate had p-values close to or less than 0.05, indicating that these measurements might not be accurate. However, its p-values were still much higher than those of MBT and HPLC, illustrating that APBT had better performance for these metabolites when compared with the others. In addition, the concentrations of the glucose and lactate measured by the APBT were statistically the same with the known values of the standard samples, demonstrating the performance of the APBT as the best for precisely quantifying these metabolites, at least within the range of concentrations investigated here. In the meantime, the MBT had moderate accuracy for the glucose, glutamine and glutamate, but showed poor performance when the lactate concentration is relatively low. HPLC had the lowest accuracy among the three technologies, but needs further investigation with more optimized routines. Therefore, throughout this study, the results obtained from HPLC were only used as supplementary measurements to check the reliability of other two techniques, APBT and MBT. Note that with other instrumental setting with use of

other chromatography column and conditions, different results might be produced.

» Benchmarking with cell culture samples

The metabolites in the supernatants of CHO cell cultures were analyzed by the three different technologies: APBT, MBT and HPLC, in order to further evaluate them with the cell culture samples. Therefore, two different cell lines were cultivated within two different types of culture vessels (three shaking flasks vs one bioreactor vessel), producing a total of eight (2 × 4) batches of CHO cell culture. From these batches, the supernatants were drawn on a daily basis and analyzed by each of the three technologies in triplicate. Note that, since, in this case, the absolute values of the reference concentrations for the different metabolites were not available, only the precision of each technology (or consistency) and the similarity among the different technologies were assessed by using statistical tools. In addition, all the HPLC analyses were conducted with the samples stored at -20°C by thawing them, and a preliminary study with ANOVA indicated that there were no statistical differences between the HPLC results before and after the storage at -20°C. However, due to the poor accuracy and precision of HPLC as illustrated with the standard samples, those results were only used for the supplementary comparisons among different technologies.

Figure 2 shows the average glucose profiles during the culture with two different cell lines. Here, the average, and error bars were calculated from four different batches of each cell line and for each technology. In both cell lines, the glucose was taken up continuously by the cells and completely depleted prior to the harvest, covering broad concentration ranges. At the same time, the three technologies showed comparable trends of decreasing patterns accordingly, although the HPLC exhibited relatively wide variations among the different batches in the CHO-S cell line compared with the other two technologies. The glutamine profiles for the CHO-S cell line are also presented in Figure 3, showing comparable trends among the different technologies with wide variations in the results of the HPLC again. For the other metabolites, the lactate shows quite different trends of the accumulation in the supernatants, while the glutamate exhibited less variation, all of which were expected trends in typical CHO cell culture (data not shown) [9,25].

With these metabolite measurements taken by the APBT, MBT and HPLC for the same cell culture samples, an ANOVA analysis was performed first, to compare the similarity among different technologies. Table 5 illustrates the results obtained from the ANOVA comparing the APBT with other two technologies for the different cell lines. Here, one could see that the glucose showed good agreement among the different technologies, illustrating that all of the analytic platforms investigated here provided comparable measurements for the glucose. For the lactate, all of the comparisons between ABPT and other two technologies indicated that they were statistically the same, but the comparison between the APBT and HPLC produced p-values very close to 0.05, thus necessitating more evaluations on this. In the meantime, an ANOVA for the glutamine showed that the measurements of APBT and MBT were statistically

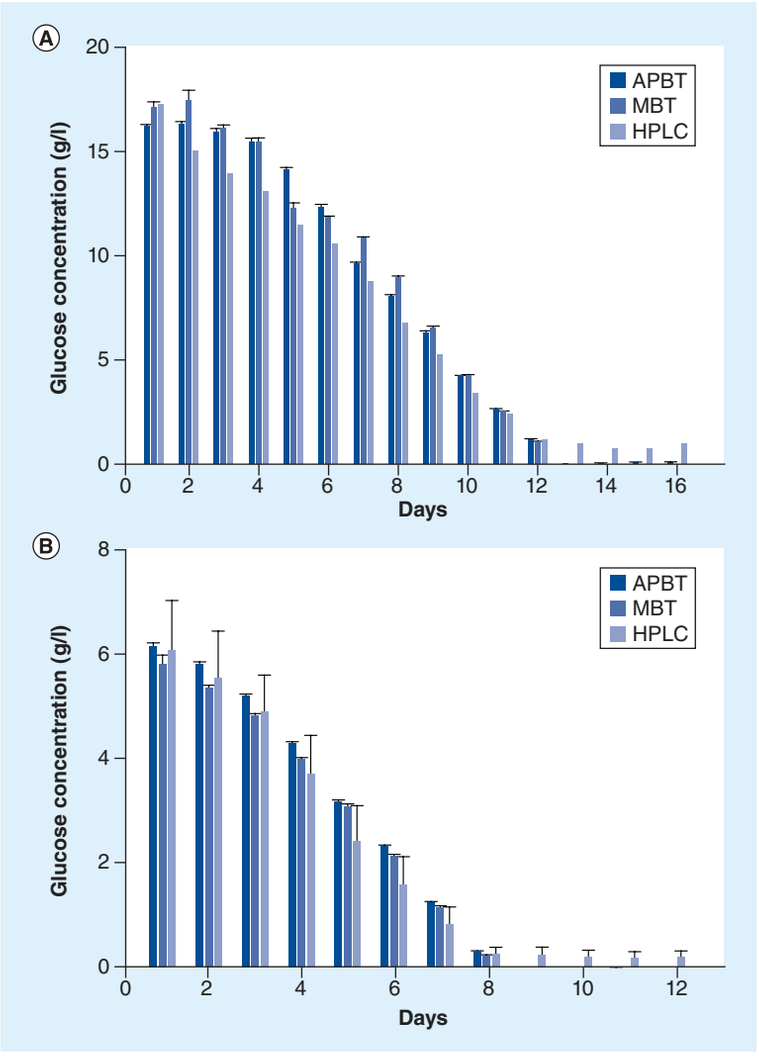

Figure 2. Average glucose concentrations measured by APBT, MBT and HPLC for cell culture samples. (A) Cell culture samples with DG44 Chinese hamster ovary (CHO) cells and **(B)** with FreeStyle™ CHO-S cells. There is comparability between the measured values from all three technologies. The included error bars signify three standard deviations from the mean and are significantly different for HPLC measurements with FreeStyle™ CHO-S cells. Note that there were insufficient data to determine the error bars for HPLC in case of CHO-DG44.
APBT: Absorption photometric-based technology; MBT: Membrane-based technology.

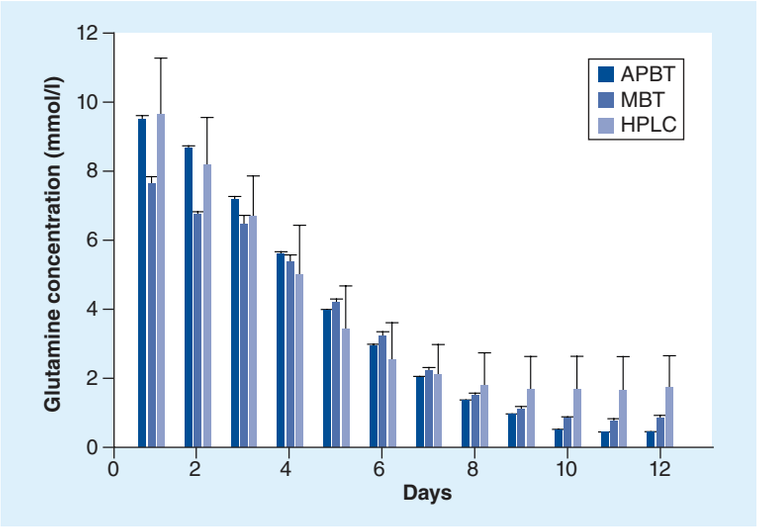

Figure 3. Average glutamine concentrations measured by APBT, MBT and HPLC for cell culture samples with FreeStyle™ Chinese hamster ovary-S cells. There is comparability between the measured values but the error bars are significantly different for each technology.
APBT: Absorption photometric-based technology; MBT: Membrane-based technology.

in the same range, while the glutamine measurements of APBT and HPLC were statistically different from each other. On the other hand, the greatest differences between APBT and the other two could be seen for the cases of glutamate (i.e., all the p-values were less than or close to 0.05 for two different cell lines), revealing that there were fundamental discrepancies among the different technologies for this metabolite. A further investigation revealed that the MBT could not provide reliable measurements of glutamate when its concentration was relatively high and close enough to the upper limit of its measuring range (i.e., 0.2 – 6.0 mmol/l; Table 1), while the HPLC could not successfully quantify the glutamate for the given samples possibly due to the high interferences with glutamine, as will be more illustrated in the next section.

Based on the above results, a further comparison was made using the ANOVA to investigate the similarity among the different technologies on different days of the cultivations as the metabolites' levels changed. Therefore, the metabolites' measurements for each of the cell lines were grouped according to the cultivation days, and multiple sets of ANOVA were conducted repeatedly for each of the metabolites (data not shown). In these results, each cell line had noticeably different p-values overall, which might reflect the effects of the different media compositions and interferences. For the CHO-DG44 cell line, the day-to-day analyses based on the ANOVA indicated that there were significant differences for all of the metabolites investigated here, when the cells are in the initial stages with highest concentrations of glucose, glutamine and glutamate as well as with lowest concentration of lactate (near zero). However, as the cultures progressed, the differences between the technologies were reduced, and in some cases no significant difference was observed as the metabolite concentrations fall within the acceptable measuring ranges due to the subsequent uptake or secretion by the cells. During the late stages of cultivation, glucose, glutamine and lactate concentrations approached near zero by depletion, and significant differences are again seen among the three technologies. On the other hand, for the CHO-S cell line, the same analyses with ANOVA revealed that only the lactate exhibited statistically significant differences among the different technologies in the initial stage of cell culture when its concentration was close to zero. During the exponential and stationary phases, significant differences were only observed when the cultivation vessel was a small-scale bioreactor. At the late stages of the cell-culture, glucose and glutamine approached near zero again by depletion, and significant differences were subsequently observed among the different technologies. These results clearly illustrated that the three technologies were relatively comparable when the metabolite concentrations were within the acceptable ranges, thus producing the greatest discrepancies among the different technologies when the metabolites were in the extreme levels, such as those at the beginning or late periods of cell cultures.

Furthermore, pair-wise comparison for glucose indicated that during the early and late stages of CHO-DG44 cells, there were significant difference between HPLC and MBT, while for the CHO-S cells, the significant difference resided between HPLC and APBT. Given the facts that the HPLC was significantly different from the other technologies for both two cell lines, it might be considered that the measurements taken

Table 5. Probability factors between the technologies for each of the metabolites.

Cell Line	Technology	Glucose	Lactate	Glutamine	Glutamate
DG44	APBT and HPLC	0.4577	0.0704	0.0048†	0.0076†
	APBT and MBT	0.8923	0.1328	0.5689	<0.0001†
CHO-S	APBT and HPLC	0.5402	0.0661	0.6338	<0.0001†
	APBT and MBT	0.6113	0.1353	0.5921	<0.0001†

†Any value less that 0.05 indicates an observed significant difference.
APBT: Absorption photometric-based technology; CHO: Chinese hamster ovary; MBT: Membrane-based technology.

by the HPLC were not accurate, which is also in line with the previous results obtained for the standard samples with known concentrations. At the same time, the pair-wise comparisons of lactate for the different technologies indicated that MBT had significantly different measurements when compared with both HPLC and APBT in both of the cell lines: CHO-DG44 and CHO-S. A further, investigation revealed that the MBT had poor accuracy when the lactate concentration was relatively low, below 0.5 g/l, as will be seen in the next section, thus explaining the observed discrepancies. In the cases of glutamine and glutamate, the APBT showed significant differences when compared with the other two technologies in both of the cell lines. However, when considering that only the APBT could exhibit good accuracy for the glutamine and glutamate in the standard samples, the metabolite concentrations measured by the MBT and HPLC for these ones might not be reliable, thus illustrating the better performance of APBT compared with other two technologies for analyzing the metabolites from the given cell culture samples.

» Machine variability

The machine variability was determined from an ANOVA using the triplicate measurements taken for the same cell culture samples. Here, the machine variability was defined as the consistency (or precision) of the metabolite measurements when the same samples were measured in a replicated manner using identical instruments and analysis procedures. Table 6 shows the probability factors for each of the three technologies for both cell lines and four different types of metabolites, illustrating how much different the triplicate measurements were for each technology. As can be seen in Table 6, APBT and MBT performed well, with values statistically the same among the triplicate measurements for all four metabolites. In HPLC, the p-values were not very high when compared with MBT and APBT, and were even lower than 0.05, especially for glutamine, indicating poor consistency and precision of the measurements for the given cell culture samples. Typically, HPLC is used to analyze purified samples [3,4], but in this study, the samples were unpurified supernatant samples having high interferences. As a result, the resultant chromatograms in the given HPLC analysis suffered greatly from low resolution in that the glutamate peaks were overlapped with broad peaks of the glucose, leading to poor precision and inaccurate quantification of the glutamate, which illustrated the typical shortcomings of HPLC when the analysis conditions are sub-optimal. Both the APBT and MBT had good consistency among the replicate measurements, and were much easier to use without any extensive optimization, signifying its' great potential as an at-line metabolite monitoring tool in mammalian cell culture.

» Compatibility of APBT with the other technologies: MBT & HPLC

Finally, the compatibility of APBT with the other two technologies, MBT and HPLC, was assessed by investigating the correlations between the metabolite measurements taken by the different technologies, since the absolute values of the quantified values might be different from each other due to bias or different instrument conditions. During this step, a special focus was given to the potential of the APBT in complementing, or replacing, the existing technologies, MBT and HPLC, by examining whether there are any significant linear relationships between the measurements taken by those technologies.

Figure 4 shows the relationship between the metabolite measurements taken by APBT and the other two technologies for all batches, including two different cell lines, and different types of vessels. Here, one could clearly see that some of the metabolites, such as glucose and lactate, measured by the different technologies are well aligned with a linear line, illustrating that they were providing, essentially, similar information despite the differences in their absolute values as was illustrated in the previous sections. In this figure, the correlation coefficients were also presented together, and it should be worth noting that all of them were statistically significant (i.e., p-value <0.01), except for the glutamate in a pair of APBT and HPLC. Glucose, especially, shows an excellent match between the APBT and each of the MBT and HPLC, indicating that all of the technologies were highly compatible with each other for glucose under the linear relationships established here with the different cell lines and vessel types. Lactate and glutamine also exhibited moderate correlations between the measurements taken by APBT and the other two

Table 6. Analysis of variance results between the triplicate measurements of the same daily sample for each metabolite with each technology.

Cell line	Technology	Glucose	Lactate	Glutamine	Glutamate
CHO-DG44	APBT	0.9998	0.9995	0.9991	0.9538
	MBT	0.9993	0.9823	0.9932	0.9703
	HPLC	0.6120	0.2817	0.9209	<0.0001†
CHO-S	APBT	0.9982	0.9737	0.9995	0.8940
	MBT	0.9912	0.9992	0.9973	0.8244
	HPLC	0.5612	0.7375	0.0059†	<0.0001†

The probability values for CHO DG44 and FreeStyle™ CHO-S show that the majority of the results indicate no observed significant differences between replicate samples. HPLC analysis of glutamate and glutamine was poor due to very poor resolution between metabolite peaks.
†Value less than 0.05 indicate an observed significant difference.
APBT: Absorption photometric-based technology; CHO: Chinese hamster ovary; MBT: Membrane-based technology.

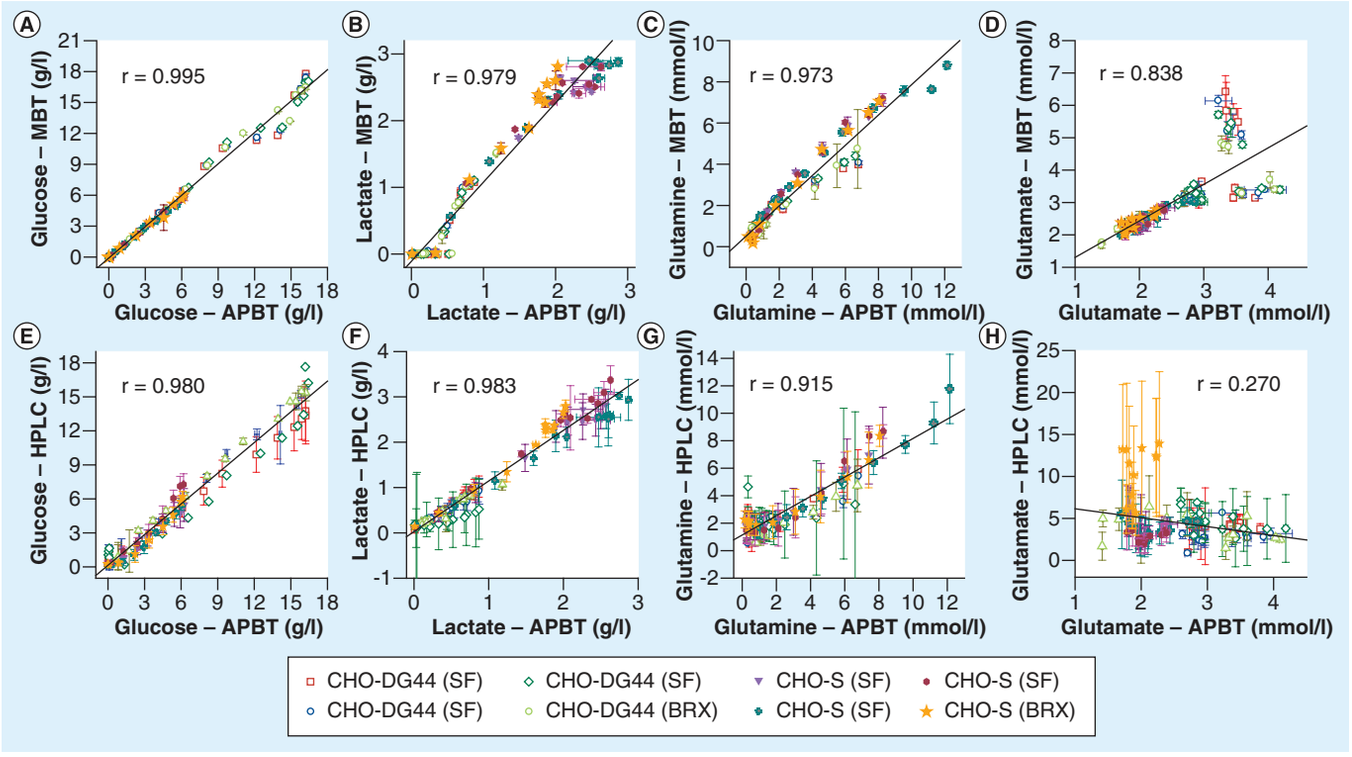

Figure 4. Correlation of the different metabolites measured by absorption photometric-based technology and the other two technologies: membrane-based technology and HPLC. (A) Glucose measured by APBT and MBT, **(B)** lactate measured by APBT and MBT, **(C)** glutamine measured by APBT and MBT, **(D)** glutamate measured by APBT and MBT, **(E)** glucose measured by APBT and HPLC, **(F)** lactate measured by APBT and HPLC, **(G)** glutamine measured by APBT and HPLC, **(H)** glutamate measured by APBT and HPLC. All of the cell culture samples obtained from different batches with two CHO cell lines were included and differentiated by distinct symbols and colors. At the same time, a linear regression line is presented together by simply regressing the two sets of metabolite concentrations measured by a pair of technologies.
APBT: Absorption photometric-based technology; CHO: Chinese hamster ovary; MBT: Membrane-based technology.

technologies, although the metabolite measurements of HPLC were highly variable with poor consistency among the triplicate measurements. Furthermore, evaluation of the lactate concentrations measured by the MBT indicated that it could not provide sufficient accuracy when the concentrations were below 0.5 g/l or above 2.0 g/l as can be seen in Figure 4B. In contrast, the glutamate did not show any clear linear relationship between the APBT and HPLC (Figure 4H), while the comparisons of APBT and MBT revealed that there was a good linear relationship between them (p-value <0.001) for concentrations below 3 mmol/l. The poor correlation between the APBT and HPLC for the glutamate could be easily explained by the low accuracy and precision of the HPLC when analyzing glutamate, as illustrated previously. Therefore, these results clearly support the use of APBT in conjunction with the other existing technologies, and its' good accuracy and precision, compared with other two technologies, further encourage the wide spread of this technology as a reliable at-line monitoring tool for analyzing the metabolites in mammalian cell culture.

Future perspective

In this study, the accuracy and precision of the different metabolite analyzers, APBT, MBT and HPLC-based ones, were tested with the standard samples with known concentrations and the cell culture samples from two different cell lines, for the purpose of benchmarking the newly introduced APBT technology in measuring the different metabolites of glucose, lactate, glutamine and glutamate. The standard sample analysis illustrated that APBT was the most accurate technology among the three, in that it had a good recovery rate, close to 100%, for both high-level and low-level standards. A further validation of APBT with the other two technologies using cell culture samples obtained from two different cell lines indicated that the APBT and MBT exhibited acceptable precision for the given culture samples, but the similarity of their metabolite quantifications were generally dependent on the range of concentration, media compositions and cell line differences. On the other hand, HPLC suffered from poor accuracy and precision for some of the metabolites, especially for the glutamate in the cell culture

samples due to poor resolution, indicating that further optimization would be needed for this assay compared with other two technologies which do not require any involvement from the end user in setting the automated analysis of the metabolites. Given the fact that the APBT exhibited both high accuracy and precision, with low variability, for the replicate samples, the use of APBT demonstrates great potential for complementing, or replacing, the existing technology for monitoring the metabolites in off-line or at-line manners with reduced consumption of materials and less calibration demands. Furthermore, the correlation study among the different technologies revealed that there is high compatibility between the measurements taken from APBT and the other two technologies, except for glutamate, thus encouraging the accelerated adoption of this technology in existing cell culture processes. Accordingly, the development of novel technologies to analyze the metabolites in cell culture processes will further complement the current efforts of implementing the on-line analyzers (e.g., near-infrared or Raman spectroscopy) in the biopharmaceutical industry.

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Executive summary

Background

- A technique based on absorption photometric-based technology (APBT) to measure the multiple metabolites in mammalian cell culture was newly introduced as an alternative to the existing techniques, such as membrane-based technology (MBT) and HPLC, thus requiring a systematic evaluation of it under the various experimental conditions.

Materials & methods

- To assess the performance of APBT, three different technologies, APBT, MBT and HPLC were implemented with the corresponding instrumentations.
- Standard samples with known metabolite concentrations of glucose, lactate, glutamine and glutamate and cell culture samples from two different Chinese hamster ovary cell lines were analyzed with three technologies for the systematic comparisons of them.

Results & discussion

- APBT exhibited highest accuracy of metabolite quantification for the standard samples among three different technologies.
- APBT and MBT provided acceptable precision for the real cell culture samples, but the similarity of metabolite quantifications among different technologies were generally dependent on the range of concentration, media compositions and cell line differences.
- A correlation study among the different technologies revealed that there is high compatibility between the measurements taken from APBT and the other two technologies.

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