Anal Bioanal Chem (2007) 388:1109–1116
DOI 10.1007/s00216-007-1335-7

TECHNICAL NOTE

The dosage of small volumes for chromatographic quantifications using a drop-on-demand dispenser system

Matthias Englmann · Agnes Fekete · Istvan Gebefügi · Philippe Schmitt-Kopplin

Received: 28 February 2007 / Revised: 25 April 2007 / Accepted: 27 April 2007 / Published online: 5 June 2007

Abstract A commercially available piezo-driven drop-on-demand dispenser was tested for its suitability for the preparation of analytical calibration standards and in a standard addition approach prior to quantitative ultra performance liquid chromatography (UPLC) analysis of homoserines. The reproducibility of the drop-on-demand dosing system was tested and the verification of the droplet volume was performed by preparing a series of 1.0 mg/L caffeine standard solutions from a 1,000.0 mg/L stock solution and analysis of the concentrations obtained by UPLC. The reproducibility was better than 1% relative standard deviation from measurement to measurement and the highest was 1.6% from day to day. The results were compared with the conventional way of generating standard solutions (pipetting). A gravimetric method and a photography-based method for the determination of the average single droplet volume were compared and found to be in very good agreement. The system was employed for the quantification of *N*-decanoyl homoserine by standard addition in bacterial culture supernatants containing this analyte. The agreement with conventional quantification techniques was high. The paper shows the feasibility of the approach with advantages in low sample and solvent volume consumption and very good reproducibility and reliability combined with easy usage.

Keywords Piezo-driven drop-on-demand dispenser · Ultra performance liquid chromatography · Droplets · Dispenser · Calibration standards · *N*-Acyl homoserines

M. Englmann · A. Fekete · I. Gebefügi · P. Schmitt-Kopplin (✉)
Institute of Ecological Chemistry,
GSF - National Research Center for Environment and Health,
Ingolstädter Landstraße 1,
85764 Neuherberg, Germany
e-mail: schmitt-kopplin@gsf.de

Introduction

Deriving from inkjet technology, piezo-driven dosage system technology for the production of small single droplets in the nanoliter to picoliter range is slowly starting to conquer new fields of application in chemistry and life sciences. This technology is successfully used in biological applications, for example, in the production of oligonucleotide arrays [1] or in the handling of volumes that contain single or few bacterial cells [2]. Niles and Coassin [3] described a dispensing device for miniaturized assays. Different applications have been described, combining piezo dosage systems with sample enrichment systems prior to capillary electrophoresis [4] or with liquid chromatography–matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [5] for the spotting on the MALDI plates [5–8]. Especially the combination with levitation devices creates interesting new ways of partially wall-less handling of small volumes [4, 9–14].

All these applications benefit from the small volume to be handled, from the high accuracy of these devices and from the wide range of liquids that can be dosed. Also the possibility for automation is an interesting long-term perspective.

The control of physical properties (size, volume) of the droplets produced and the accessibility of these parameters are of key importance. Berggren et al. [15] found agreement within 20% by comparing a visualizing method and an approach based on a way to determine the average mass of single droplets by weighing the dispensed volume. Eberhardt and Neidhart [12] claim for their setup an accuracy of better than 1% and a precision of less than 1% relative standard deviation (RSD) for the determination of the droplet mass. Bruns et al. [2] found deviations "usually less than 1%," whereas Jacob et al. [13] reported deviations below 5%

with their instrument. Niles and Coassin [3] found root mean square deviations of less than 0.64%.

To our knowledge, no application has been shown to use these devices to generate precisely diluted standards for classic analytical purposes (highly reproducible and accurate dilution series, standard addition procedures in quantitative analysis). In this study, a simple, commercially available dosage system was tested for an application in chromatographic quantification.

Materials and methods

Chemicals and samples

Water was provided by a Milli-Q plus system (Millipore Corporation, Billerica, USA). All chemicals except where stated otherwise were from Merck (Darmstadt, Germany) and were at least of analytical grade. Caffeine and ammonium formiate were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. *N*-Decanoyl homoserine is not commercially available. To prepare the standard solution, to a 1,000 mg/L solution of *N*-docanoyl homoserine lactone in acetonitrile, 5% of 1 M NaOH (Sigma-Aldrich, Taufkirchen, Germany) in water was added to hydrolyze the lactone. After stirring, the suspension was dried under nitrogen and was ready for analysis.

As a real sample, a supernatant of a *Burkholderia cepacia*, strain LA-3, culture, grown for 12 h in nutrient broth medium was used. To increase the concentration of the analyte, the sample was reduced to 25% of the original volume by drying it under nitrogen and redissolving it in 0.05 M NaOH in water. After 15 min, 0.4% formic acid was added to an end volume of one fourth of the original sample volume. The solution was filtered through a 0.22-μm poly(tetrafluoroethylene) (PTFE) filter (VWR International, Darmstadt, Germany).

Dispenser

An MD-K-130 HV drop-on-demand system from Microdrop Technologies, Norderstedt, Germany, for vertical dosage was used in combination with an MD-E-201 controller. For monitoring, a model 7633 oscilloscope from Tektronix Holland (Heerenveen, The Netherlands) was used. The dispenser head was mounted on an *XYZ* micrometer stage and observations were made with a Zeiss Stemi 2000-C microscope (Carl Zeiss, Oberkochen, Germany).

The dispenser consists of a capillary with a 30-μm nozzle, a surrounding piezo actuator and a reservoir. The void volume in the dispenser was determined to be 91 μL (including the take-up tube). The reservoir can hold around 4 mL of fluid. The liquid is transported into the capillary only by capillary forces.

The time between two pulses, the duration of the pulse and the pulse voltage can be set. The system can be run in continuous mode as well as externally and manually block-triggered. The maximal block length is 999 pulses; the minimum is one pulse. A trigger pulse is shown in Fig. 1, which was captured while the dispenser was running. Oscillations along the pulse are due to resonances of the vibrating piezo crystal. The dispensed droplets can be visualized by a stroboscope diode, the delay of which can be set between 0 and around 600 μs.

The period, and the displaying of it, underlies constant oscillations in the sub-10-μs range, so the value has to be rounded to 10 μs. By comparison of the results obtained in continuous mode with those of the dispensing of a fixed number of shots, an additional average error of the displayed value of 1.7% was found and in all experiments, which were performed in continuous mode, was rectified.

A medium frequency of around 200 Hz was chosen for the experiments. At higher frequencies the system tends to become unstable, implying that reproducible droplet production is no longer possible (see also the results of Laurell et al. [14] with their instrument). Towards lower frequencies, the time for dosing higher numbers of droplets increases.

Stable conditions rely on a combination of all pulse parameters and these have to be found experimentally. Unstable conditions result in breakup of droplet production, angular trajectories and the appearance of satellite droplets, which are problematic when they have lower speed than and trajectories different from that of the main droplet. A satellite droplet with higher speed and the same trajectory as seen in Fig. 2 will unite with the main droplet within 100 μs. This effect is difficult to avoid and was found especially for the production of larger droplets.

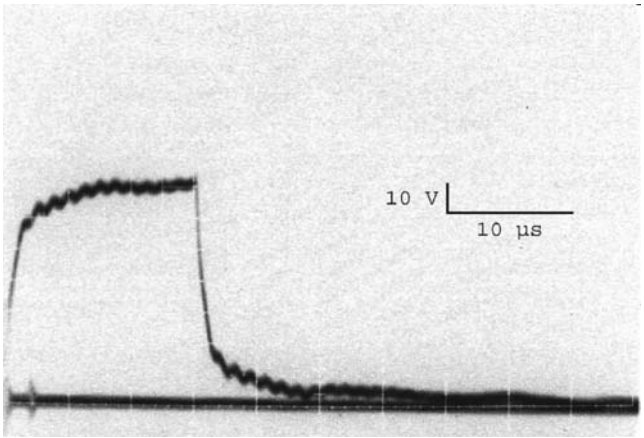

Fig. 1 Trigger pulse with a width of 15 μs and an amplitude of 70 V

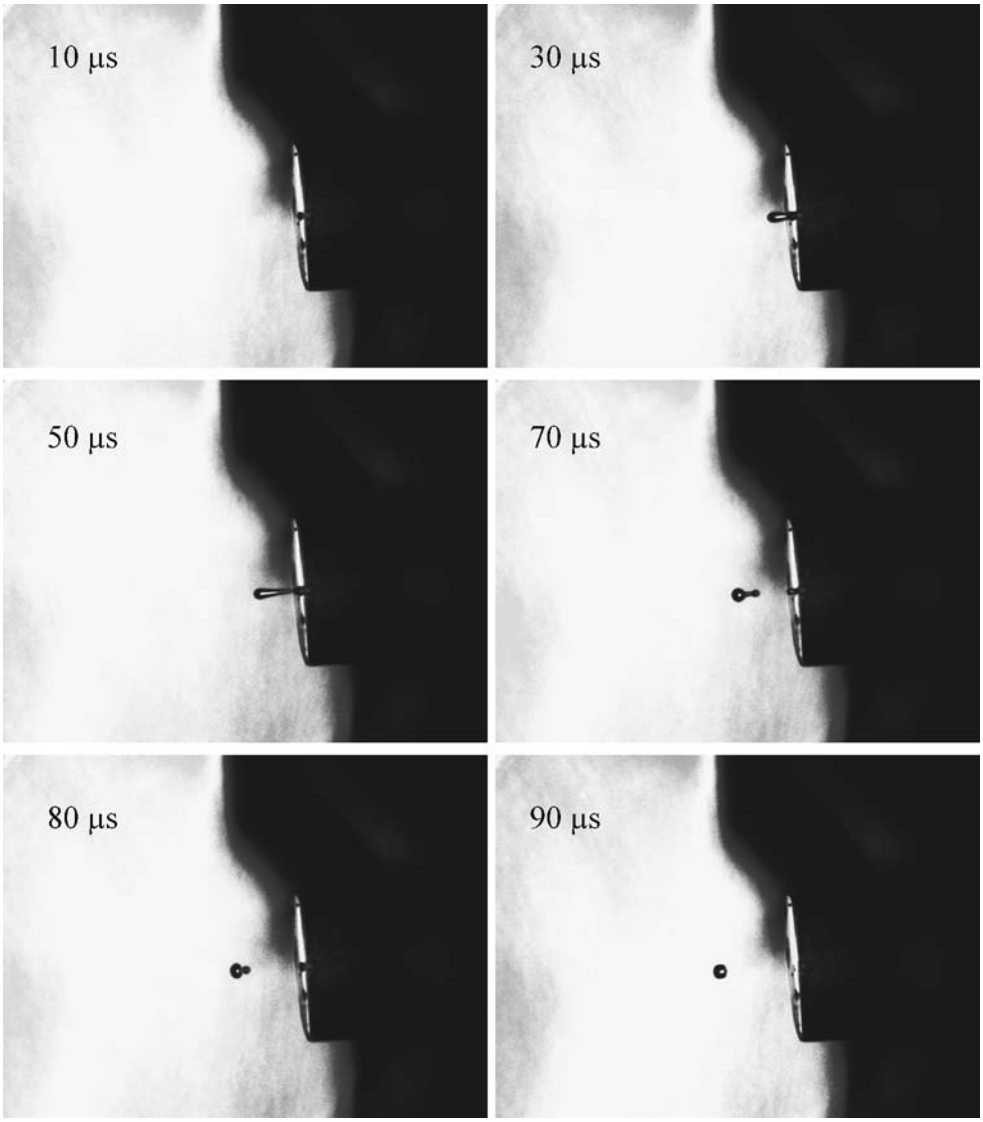

Fig. 2 Droplet production photographed with delay times of 10–90 µs of the stroboscope diode. The exposure time was 0.5 s and the period between two pulses 9 ms. This results in over 56 single pulses for every picture

The evaluation of the parameters for stable droplet production and calibration has to be made for every fluid, because viscosity and, especially for small droplet volumes, surface tension have a large impact on droplet formation [3, 16].

Droplet volume determination via photography

Photographs were taken with a Sony DSC-F717 camera (Sony Deutschland, Cologne, Germany) mounted on the microscope. At least nine shots were taken within each measurement with different delay times of the diode. For the processing, Adobe Photoshop 7 software (Adobe Systems, San Jose, USA) was used. The diameter of the droplet was determined in relation to the diameter of the nozzle, as done by Wu et al. [16]. In this case, the diameter determined is independent of the zoom factors of the microscope and the camera. The diameter was scaled twice at angles of 90° to each other and averaged. The period between two pulses was 5,050 µs, corresponding to about 200-Hz dispensing frequency.

Droplet volume determination by weighing the reservoir

In this method, the running system is stopped and the reservoir is taken off the system, encapsulated and weighed on a Sartorius CP 225 D microbalance (Sartorius, Göttingen, Germany). After at least 500,000 droplets had been dispensed, the reservoir was weighed again. The weighing procedure did not exceed 2 min to prevent losses due to evaporation. The mass divergence is equal to the dispensed mass, so the volume can be determined by dividing the mass, corrected by the density of the liquid, through the number of droplets dispensed. The period between two

pulses was as for the determination via photography, 5,050 μs, which corresponds to about 200-Hz dispensing frequency.

Droplet volume verification by ultra performance liquid chromatography analysis

Two setups for preparing 1 mg/L solutions from a 1,000 mg/L stock solution were compared.

- One preparation, a typical method of laboratory-scale sample preparation, was done on a 200-mL scale, using 200-mL graduated glass flasks (EM, In, class A from Hirschmann Laborgeräte, Eberstadt, Germany) and dosing 200 μL of the stock solution with a 200-μL pipette of the type "reference" (Eppendorf, Hamburg, Germany).
- In the alternative setup, 1 μL of the stock solution was dispensed directly into the ultra performance liquid chromatography (UPLC) vials (12 mm×32 mm, screw-neck glass vials, Waters), and 999 μL of water was added using a 1,000-μL Eppendorf reference pipette.

The weighing method described above was used to determine the droplet volume for pulse parameters of 22.5-μs pulse length, an amplitude of 80 V and a repetition rate of 5,050 μs. The droplet volume determined (79.41 pL) for the caffeine solution resulted in 12,590 droplets being dispensed in 20 blocks of each 630 pulses.

All the experiments were performed under normal laboratory conditions, which means that the laboratory environment was subject to normal shifts in temperature and humidity.

UPLC analysis was performed using a Waters Acquity system (Waters Corporation, Milford, USA) equipped with a 2996 photodiode-array detector and a 2.1 mm×100 mm C_{18} Acquity column from Waters with 1.7-μm particle size. The column was thermostated at 60 °C, and the sample system was maintained at 15 °C. The system was run with 2:8 (v/v) acetonitrile/water in isocratic mode. The flow rate was set to 0.8 mL/min, and 20 μL of sample was injected via full-loop injection. This resulted in a chromatographic runtime of 0.6 min and a total runtime of about 1.5 min per injection. Detection was performed at 273 nm, and peak areas were calculated using Waters Empower software. Each injection was repeated five times and the mean value was taken into account.

The standard addition approach

Preparation of the standard and samples

The homoserine was dissolved in the same volume of water as the original lactone solution had been. After filtration (0.22-μm PTFE filter; VWR International, Darmstadt, Germany), an aliquot of the solution was dissolved 1:99 in water and the concentration of the serine was determined by the UPLC method described in the next section. The concentrations determined were 747 mg/L for the solution used for the standard addition to the spiked samples and 832 mg/L for the solution used for the analysis of the real sample.

For the development of the standard addition method, spiked nutrient broth medium was used. The freshly made medium was spiked with the *N*-decanoyl homoserine standard to get a final concentration of 10 mg/L.

UPLC analysis

The method for the analysis of *N*-acyl homoserines is described in detail in Englmann et al. [17]. For the preparation of the external standards, the 1,000 mg/L stock solution of *N*-decanoyl homoserine lactone in acetonitrile was diluted in a mixture of one part acetonitrile and two parts 0.07 M NaOH (v/v). After 15 min, 0.4% formic acid was added. This standard solution was diluted in acetonitrile/water (1:2 v/v) to lower concentrations for the external calibration curve. A five-point calibration from 1.5 to 24 mg/L was performed every day.

The same UPLC system and column were used as for the droplet volume verification. In this case, the autosampler was thermostated at 27 °C, and the column oven at 60 °C. A 20-μL aliquot of sample was injected by full-loop injection. The detection was performed at 195.4 nm and 20 Hz.

The initial composition of solvent was 3:7 (v/v) acetonitrile/10 mM ammonium formiate buffer, adjusted to pH 4.25 with formic acid. A gradient profile of 0.2 min of the initial composition followed by a gradient of 0.5 min to 100% acetonitrile was performed at a flow rate of 0.9 mL/min.

Standard addition

The concentration in the sample was raised by a certain amount of standard dosed by the dispenser and the resulting difference in the peak areas was extrapolated to determine the concentration in the original sample.

The calibration of the dispenser was performed with the same parameters as used for the verification of the droplet volume. The standard solution was dispensed directly into the vials. The dispensed volume was dried under nitrogen, before 200 μL of spiked medium or real sample was added to it. For the development of the method, 0, 100, 500, 1,000, 5,000 and 10,000 droplets were dispensed in three parallel experiments. For the analysis of the real sample, 0, 500, 1,000 and 5,000 droplets were dispensed. Every sample was injected three times.

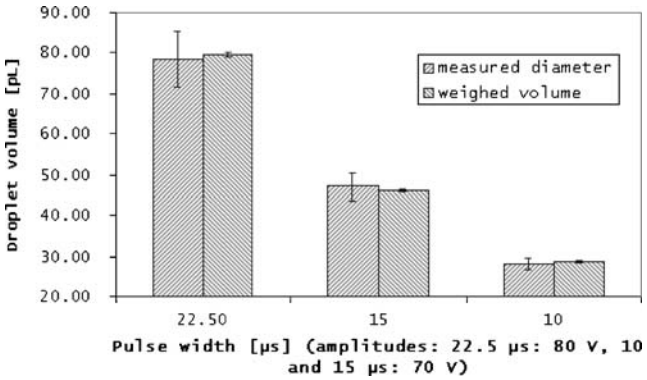

Fig. 3 Comparison of the results for the determination of the volumes of one droplet obtained by measuring diameters on a photograph and by weighing the dispensed volume with standard deviations with the usage of three different pulse parameters

Results and discussion

Comparison of the results obtained by measuring and weighing

First the water droplet volume produced by the dispenser was determined with three different settings by an imaging and by a gravimetric approach. Water was used as the test medium because most biological solvents are aqueous.

The accuracy of a dispenser-based dilution of an analytical standard, prepared on a 1-mL scale, was compared with that of a classic approach using Eppendorf™ pipettes and conventional glassware. The latter was performed on 200-mL scale. The validation was made using UPLC analysis following the dosage of a defined volume of a caffeine stock solution. Caffeine was chosen because of its high solubility in water and its convenient use in UPLC analysis.

Figure 3 shows the results of the calculation of the droplet volumes obtained by photographic and gravimetric methods with three different settings. Both results are in very good agreement for all experiments and are suitable for the determination of the droplet volume (no systematic underestimations or overestimations). The RSDs for the photographic method are relatively high and decrease with decreasing droplet size: 8.7% for the biggest droplet, 7.5% for the medium-sized droplet and 5.4% for the smallest droplet. The RSDs obtained by applying the weighing method are much smaller: 0.73% for the largest droplet, 0.67% for the medium-sized droplet and 1.07% for the smallest droplet. This reflects the high accuracy of this simple method, based on gravimetry.

Droplet size strongly depends on the pulse parameters: the shorter the pulse duration, the smaller is the resulting droplet. With water, a highly reproducible droplet volume of down to around at least 30 pL is shown to be easily achievable.

Reproducibility measurements

The results of the UPLC measurements of the 1 mg/L caffeine solutions obtained in the two different ways (pipette and dispenser) are displayed in Fig. 4. The average RSD for the measurement-to-measurement reproducibility in the classically diluted samples is 0.26% and in the dispensed samples 0.54%. The day-to-day reproducibility is 0.48% RSD for the classic dilution and 1.59% RSD for the dispenser-based dilution. It has to be kept in mind that the pipettes were calibrated every day, and the dispenser only once. The total mean deviation between the two setups is 1.46%. The accuracy of the UPLC method was independently determined to be 0.11% RSD from measurement to measurement (N=5).

On all days, an underestimation of the caffeine concentration seemed to occur for the dispensed samples. Only the measurements on the first day are an exception. This may be caused by the uncertainty in the determination of the droplet volume. These variations are in a very narrow band and by taking into account the errors of the different steps of preparation, it is more reasonable not to overinterpret these variations. An accuracy of around 1.5% RSD was also observed in preliminary experiments (not presented here) and this seems to be just the limit of the accuracy of the dispenser for day-to-day observations.

Overall, it can be found, that within 1 day, RSDs of below 1% are to be expected, while for day-to-day observations deviations should be around 1.5%.

Standard addition

A method was developed for the quantification of *N*-decanoyl D/L-homoserine in bacterial culture supernatants in order to demonstrate this novel modus of standard

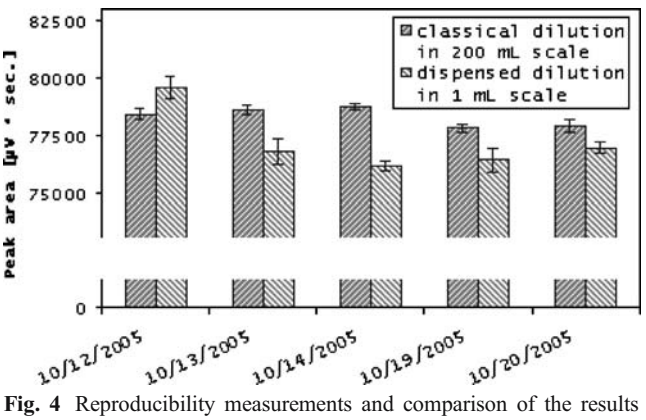

Fig. 4 Reproducibility measurements and comparison of the results obtained by classic pipette preparation in 200-mL volume (Eppendorf™ pipette) and by dosing with the dispenser directly into the 1-mL analysis vials

addition. This analyte is a degradation product of *N*-decanoyl D/L-homoserine lactone, which plays an important role in the cell-to-cell communication in Gram-negative bacteria [18]. The intention was to establish a fast and easy-to-use screening method for the detection of this homoserine lactone in bacterial cultures of *Burkholderia cepacia.* The ratio of lactone and corresponding serine changes with time [19]. If the lactone is hydrolyzed to serine, the amount of serine determined represents a sum parameter of both molecules. Because of considerable matrix effects in microbial extracts, identification and quantification via external calibration curves is frequently inferior and unreliable in comparison with precise addition of standards. Because of limited sample amounts, the quantification has to be performed with small sample volumes.

The external calibration and the calibration curve resulting from the additions to the spiked medium are compared in Fig. 5. The slopes of the two curves differ by 4%, which may be partly due to a slight extension beyond the linear range at 24 mg/L in the external calibration curve.

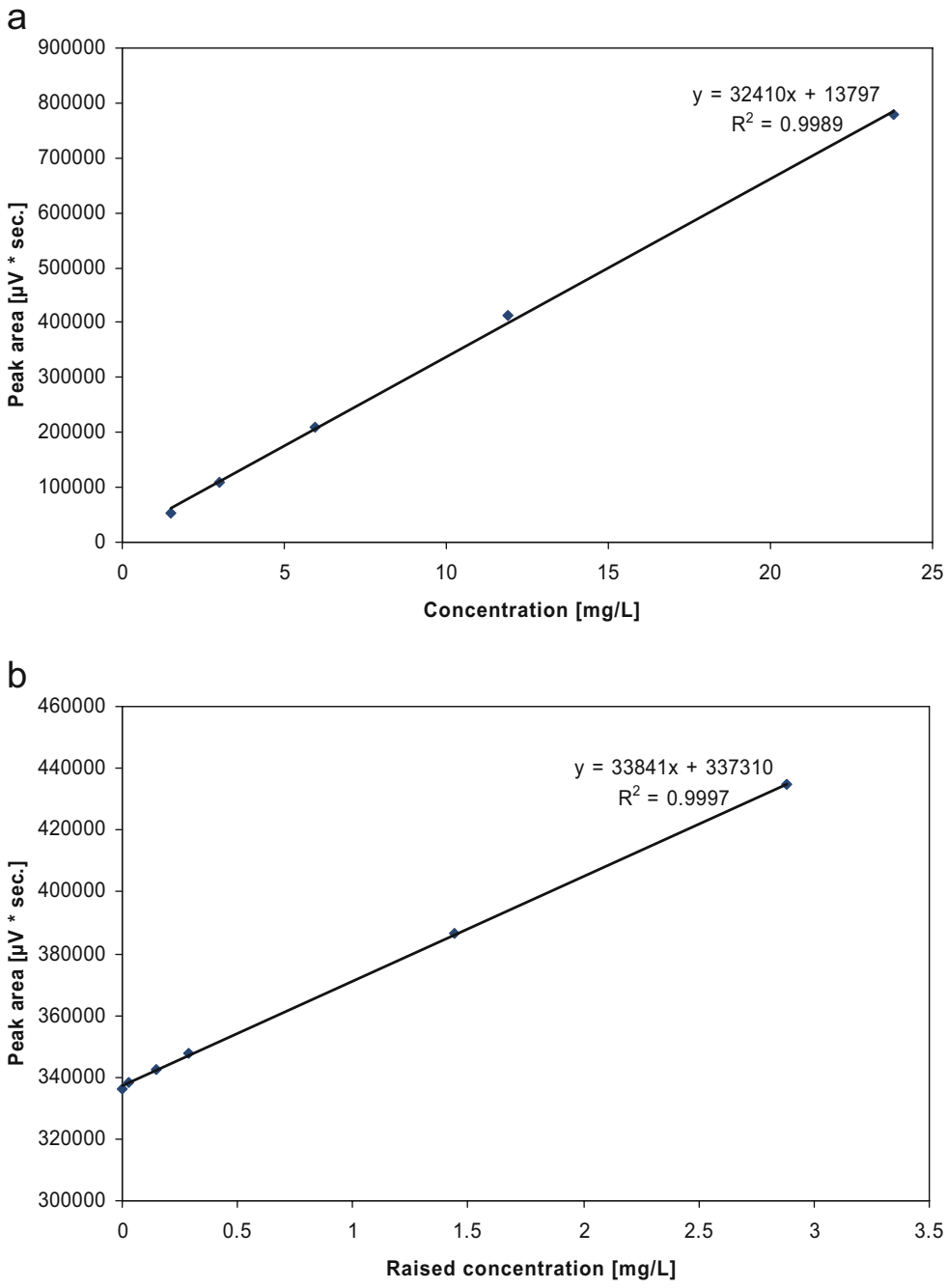

Fig. 5 Calibration curves for **a** the external calibration and **b** the concentration resulting from the standard addition to the spiked samples

Table 1 Results of the real sample measurements

	Raised concentration due to addition (mg/L)	Measured concentration (mg/L)	Concentration determined in original sample (mg/L)	Deviation from external calibration results (%)
External calibration		2.23	0.56	
500 droplets addition	0.16	2.48	0.62	11
1,000 droplets addition	0.32	2.17	0.54	3
5,000 droplets addition	1.59	2.24	0.56	0.4

The raised concentration means the concentration difference due to the standard addition. The concentration determined in the original sample compensates for the fourfold preconcentration of the sample

The calibration curve of the additions shows excellent linearity, which demonstrates again the highly reproducible and correct dosage. The complexity of the matrix does not influence this accuracy.

The results of the real sample measurements are shown in Table 1 and Fig. 6. Three settings are to be compared with the results obtained by external calibration. The deviation within the 500 droplet standard addition set is relatively high. This may be due to a too small number of droplets, so small deviations in the droplet volume result in a relatively large deviation when the amount of standard added is too small in relation to the amount in the sample. With addition of1,000 droplets, the deviation is much smaller and the agreement with the approach involving the addition of 5,000 droplets is very high. This shows that this method is applicable for real samples, producing reliable results.

One huge advantage, not directly obvious from the results presented herein, is the convenience and the speed of this method: the calibration of the dispenser may be time-consuming but once it has been calibrated, different volumes can be dosed just by pushing a button. The approach is also automatizable for preparation of many highly reproducible standards in large-scale experiments. This may increase the operator-to-operator reproducibility and prevents errors resulting from misusage of alternative dosage systems. The smallness of the volumes allows application directly in the vial used for the subsequent analysis without dilution of the sample in the case of the standard addition method.

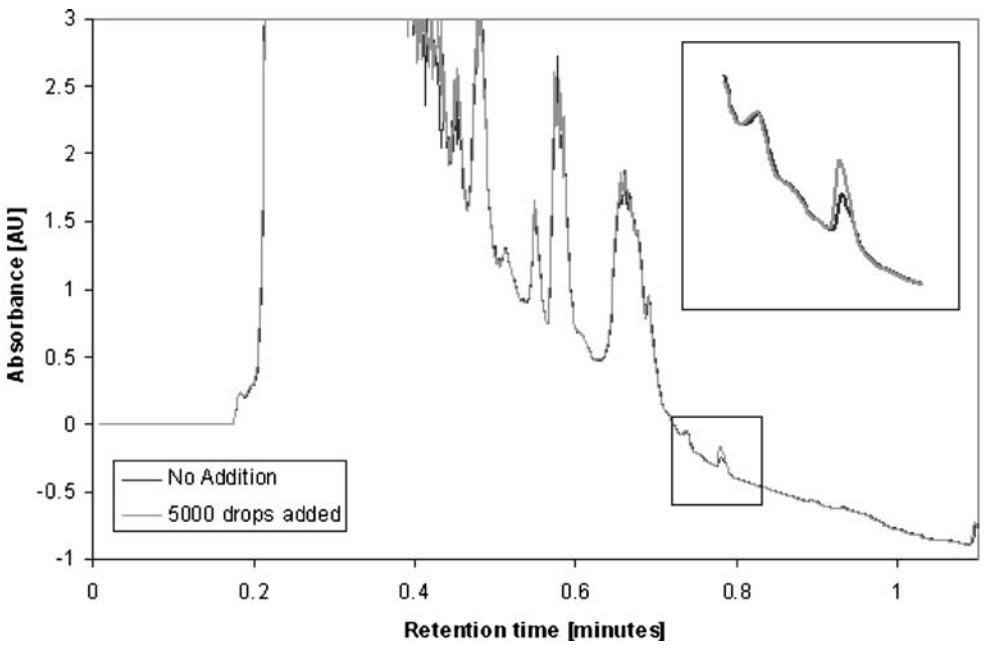

Fig. 6 Chromatogram of the original *Burkholderia cepacia* culture supernatant and the same sample with standard addition. The *N*-decanoyl homoserine peak is enlarged

Conclusion

With low RSDs, namely, 1.5% from day to day and below 1% from measurement to measurement, the dispenser presented herein is a reliable and easy-to-handle device for dosing volumes even in the microliter range and below with high precision to prepare small liquid samples of an exact analyte concentration (ideal for calibration solutions in routine analysis in large-scale analytical laboratories) prior to quantitative chromatography. The accuracy and the reproducibility are comparable to those for the classic method of sample preparation.

The strength of the dosing approach using single droplets lies in the constant accuracy over a wide volume range (over a wide number of created droplets), because the droplet volume is constant over time. The reproducibility of the system was shown to be very high and the key approach, the determination of the average volume of one single droplet, can be made with the same high accuracy in a very simple way (only a microbalance is needed). Additionally this leads to much lower consumptions of sample and solvents compared with conventional methods (in this work, a 1-mL-scale dilution showed results comparable to that of a conventional 200-mL-scale dilution).

It was shown that the system is applicable for quantifications via standard addition as an example. It also shows the potential for a wide range of different applications, wherever volumes in the low volume range have to be handled in a highly accurate and reproducible way, a key demand for further downscaling processes.

Acknowledgements A. Hartmann (AMP/GSF) is thanked for the bacterial culture sample used in this study. A. Krainz, (RCC, Itingen, Switzerland) is thanked for giving us the impulse to test the dispenser technology for routine throughput work.

References

1. Stimpson DI, Cooley PW, Knepper SM, Wallace DB (1998) Biotechniques 25:886–890
2. Bruns A, Hoffelner H, Overmann J (2003) FEMS Microbiol Ecol 45:161–171
3. Niles WD, Coassin PJ (2005) Assay Drug Dev Technol 3:189–202
4. Petersson M, Nilsson J, Wallman L, Laurell T, Johansson J, Nilsson S (1998) J Chromatogr B 714:39–46
5. Onnerfjord P, Nilsson J, Wallman L, Laurell T, Marko-Varga G (1998) Anal Chem 70:4755–4760
6. Allmaier G (1997) Rapid Commun Mass Spectrom 11:1567–1569
7. Ekstrom S, Ericsson D, Onnerfjord P, Bengtsson M, Nilsson J, Marko-Varga G, Laurell T (2001) Anal Chem 73:214–219
8. Luginbuhl P, Indermuhle PF, Gretillat MA, Willemin F, de Rooij NF, Gerber D, Gervasio G, Vuilleumier JL, Twerenbold D, Duggelin M, Mathys D, Guggenheim R (2000) Sens Actuators B 63:167–177
9. Santesson S, Nilsson S (2004) Anal Bioanal Chem 378:1704–1709
10. Bogan MJ, Agnes GR (2004) J Am Soc Mass Spectrom 15:486–495
11. Bogan MJ, Agnes GR (2002) Anal Chem 74:489–496
12. Eberhardt R, Neidhart B (1999) Fresenius J Anal Chem 365:475–479
13. Jacob P, Stockhaus A, Hergenroder R, Klockow D (2001) Fresenius J Anal Chem 371:726–733
14. Laurell T, Wallman L, Nilsson J (1999) J Micromech Microeng 9:369–376
15. Berggren WT, Westphall MS, Smith LM (2002) Anal Chem 74:3443–3448
16. Wu HC, Hwang WS, Lin HJ (2004) Mater Sci Eng A 373:268–278
17. Englmann M, Fekete A, Frommberger M, Li X, Gebefügi I, Fekete J, Schmitt-Kopplin P (2007) J Chromatogr A (in press)
18. Fuqua C, Greenberg EP (2002) Nat Rev Mol Cell Biol 3:685–695
19. Frommberger M, Hertkorn N, Englmann M, Jakoby S, Hartmann A, Kettrup A, Schmitt-Kopplin P (2005) Electrophoresis 26:1523–1532