JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2012) **9**, 1892–1897
doi:10.1098/rsif.2011.0809
Published online 15 February 2012

Using digital photography to implement the McFarland method

L. Lahuerta Zamora* and M. T. Pérez-Gracia

Departamento de Química, Bioquímica y Biología Molecular. Facultad de Ciencias de la Salud, Universidad CEU Cardenal Herrera, 46113 Moncada, Spain

The McFarland method allows the concentration of bacterial cells in a liquid medium to be determined by either of two instrumental techniques: turbidimetry or nephelometry. The microbes act by absorbing and scattering incident light, so the absorbance (turbidimetry) or light intensity (nephelometry) measured is directly proportional to their concentration in the medium. In this work, we developed a new analytical imaging method for determining the concentration of bacterial cells in liquid media. Digital images of a series of McFarland standards are used to assign turbidity-based colour values with the aid of dedicated software. Such values are proportional to bacterial concentrations, which allow a calibration curve to be readily constructed. This paper assesses the calibration reproducibility of an intra-laboratory study and compares the turbidimetric and nephelometric results with those provided by the proposed method, which is relatively simple and affordable; in fact, it can be implemented with a digital camera and the public domain software ImageJ.

Keywords: colorimetric imaging analysis; digital photography; McFarland method; charge-coupled device; ImageJ

1. INTRODUCTION

Microbial concentrations can be determined in various ways, including direct counting, plate counting and measurement of light scattering by bacterial cells in a liquid medium. Although these methods are all non-destructive, the last has the advantage that it is much more expeditious than the other two. Light passing through a microbial suspension is partly absorbed by the microbes and subsequently re-emitted in all directions, as a result, the suspension has a milky appearance under visible light [1]. Measuring the amount of light absorbed (turbidimetry) or scattered (nephelometry) under appropriate conditions allows one to estimate the amount of biomass present in the suspension.

McFarland [2] devised a nephelometer for measuring suspended bacteria based on standards optically mimicking bacterial suspensions and obtained by chemical precipitation. Thus, mixing appropriate amounts of sulphuric acid (H_2SO_4) and barium chloride ($BaCl_2$) produced known amounts of a fine barium sulphate ($BaSO_4$) precipitate with the same light-scattering capacity as a suspension of bacterial cells. Although visual comparison is indeed possible, obtaining precise results entails comparing microbial suspensions and McFarland standards via turbidimetric or nephelometric measurements made at wavelengths over the range of 420–660 nm. Some dedicated commercial instruments have even been designed to provide measurements in 'McFarland units' [3].

*Author for correspondence (lahuerta@uch.ceu.es).

This technique has the advantage that the standards are chemical and thus require no incubation, and also that (if visual) no instrument other than one's eyesight is required for comparison. However, it can be directly applied only to Gram-negative bacteria such as *Escherichia coli* because others differ in volume and mass—and hence in their ability to scatter light.

Although McFarland standards currently consist of suspensions of latex or titanium dioxide particles [4,5], which are more stable and longer lived than the former precipitates, barium sulphate remains in use for this purpose—in fact, its suspensions have been shown to remain stable for nearly 20 years if stored in tightly sealed tubes at room temperature in the dark [6].

In microbiology, McFarland standards continue to be used as reference suspensions for comparison with bacterial suspensions in liquid media for purposes such as obtaining antibiograms [7] and biochemical testing [8].

The increasing technical improvement and affordability of digital photography hardware and software [9] have promoted their use in quantitative chemical analyses. Today's digital cameras capture images by a light-sensitive chip called 'charge-coupled device' (CCD), which plays the same role as photographic film in conventional (chemical) photography. Each cell in a CCD acts as a light-sensitive individual element providing an electrical response to light that can be digitized to build an optical image. Because CCD pixels respond to light intensity—but not to colour—reproducing the three primary colours the human eye can perceive [10] (red, green and blue) requires using a 'colour' CCD. Colour digital images are the additive combination of the three colours, which most of the sensors used in digital

Received 22 November 2011
Accepted 20 January 2012

cameras obtain by superimposing a mosaic of red, green and blue filters (*viz.* a Bayer mask) over the pixel array in order to interpolate colour-related information for each individual pixel.

A CCD consisting of eight-bit pixels can respond to $2^8 = 256$ levels of grey ranging from 0 (black) to 255 (white). This allows each pixel in the red, green or blue channel in a CCD-captured image to be assigned a numerical value from 0 to 255, which can be subsequently used for analytical calibration. As a result, a digital camera can be used as an analytical sensor because each captured image provides a vast amount of information [9].

Commercial CCD cameras have been available for 30 years, and they have been well appreciated and widely used for analytical purposes. Analytical imaging (CCD) methods are increasingly being used in Raman spectroscopy [11], chemiluminescence spectroscopy [12] or electron transmission microscopy [13] at research laboratories. This has promoted the development of dedicated commercial equipment where analyses are implemented on inductively coupled plasma-atomic emission spectroscopy (ICP-AES) [14], mass spectrometers [15] or fast-scan systems [16]. In biological research, CCDs have been used to capture digital images for UV-fluorescing substances with a variety of purposes including documentation and quantitative analysis in, for example, electrophoretic separations of nucleic acids and proteins—even in *in vivo* tests in the latter case [17]. In the biotechnology field, digital image analysis techniques have been even used for the *in situ* characterization of multiphase dispersions [18] and for the monitoring of activated sludge processes [19]. CCDs have also been used to obtain digital images with a view to quantifying chemical species following separation by thin-layer chromatography (TLC) or high-performance thin-layer chromatography (HPTLC) [20–23]. However, in spite of their extensive use, the CCDs intended for the analytical work are quite expensive.

On the other hand, nowadays the very low-cost mass-produced digital cameras (based on CCD technology) for the home consumer market have not yet been widely appreciated and have scarcely been used for analytical purposes.

These low-cost devices have been employed in various fields, including forensic science [24,25], telemedicine and laboratory analyses. In fact, digital cameras have become an effective, inexpensive alternative to commercially available equipment (scanners) for qualitative and quantitative thin layer chromatographic analysis [26]. Quantitative imaging analysis with a digital camera and the software ImageJ recently proved an effective, affordable choice for fluorimetric measurements in teaching laboratories [27]. This software–hardware combination has also enabled the quantitation of haemoglobin and melanin with a view to assess erythema and pigmentation in human skin [10].

Digital images obtained with very inexpensive CCD cameras known as 'webcams' have been used to assess colour changes during acid–base titrations and found to provide results on a par (*viz.* no significant differences at the 95% confidence level) with those of spectrophotometric monitoring [28]. Also, a computer monitor has been successfully used as a light source and, a webcam as detector, for purposes such as distinguishing wine samples [29].

Even built-in cameras in mobile phones have been used in telemedicine to capture and transfer biotesting results obtained by paper-based microfluidic devices to quantify glucose and proteins [30].

The method reported in this paper, which is similar to one used in previous work and very recently proposed by one of the authors to quantify analytes absorbing visible light in aqueous solutions [31,32], uses digital images of a series of McFarland standards in combination with appropriate software (ImageJ) to assign a numerical value to each colour hue (turbidity). Such a value is directly proportional to the concentration of the McFarland standard concerned and can thus be used for calibration. The proposed method performs on par with the classical turbidimetric and nephelometric methods for this purpose, but has the advantage that it uses much more accessible and inexpensive hardware (a low-cost mass-produced digital camera for the home consumer market) and software (ImageJ, which is public domain software).

2. MATERIAL AND METHODS

2.1. Material

All reagents used were analytically pure unless stated otherwise. Solutions were prepared from water purified by reverse osmosis, de-ionized to 18 MΩ cm with a Sybron/Barnstead Nanopure II water purification system furnished with a fibre filter of 0.2 μm pore size. H_2SO_4 and anhydrous $BaCl_2$ were obtained from Panreac, both in analytical reagent grade.

Once prepared, the McFarland standards (3 ml of each one) were transferred to the wells of an Iwaki 3820-024N polystyrene microplate (their holder for photographing; figure 2) with the aid of an Accumax VA-900 micropipette.

All photographs were taken with a Nikon Coolpix E995 digital camera and processed with the public domain software ImageJ (windows version) developed by the National Institutes for Health and available for free download at http://rsbweb.nih.gov/ij.

Lighting was provided by two parallel fluorescent strips (Philips Master TL-D 36 W/840) located 1.5 m over the microplate (figure 1). The diffusing screen was made with white paper filter from ALBET (LabScience), 60 g m^{-2} (in reams) 420 × 520 mm (code RM2504252). The microplate was placed on a piece of black cardboard (NE 30K A4, 180 g) from Hermanos Cebrián, Spain. Absorbance measurements were made with a Spectronic Genesis 20 UV–vis spectrophotometer, and fluorescence measurements with a Perkin Elmer LS50 luminescence spectrometer equipped with the software FL WinLab. The McFarland value for each standard was obtained by a BD CrystalSpec dedicated nephelometer.

2.2. Procedure

All experimental work was performed in five sessions (A–E), involving the operations described below. Solutions containing 1 per cent (w/v) $BaCl_2$

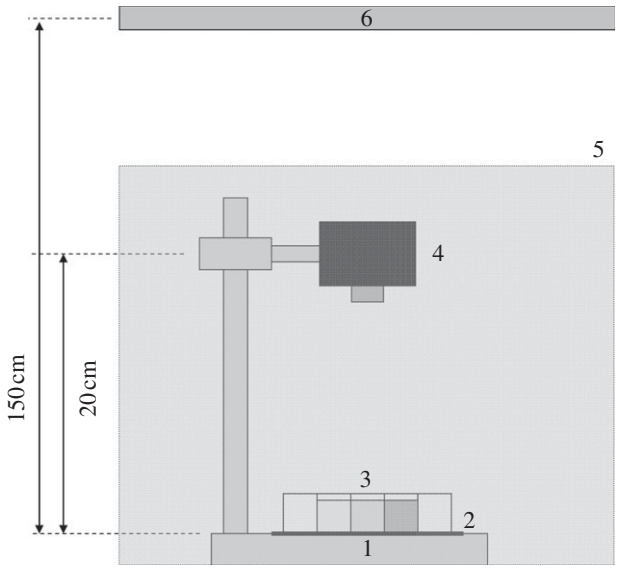

Figure 1. Schematic of the system for obtaining images. (1) Static support for microplate and camera, (2) black cardboard, (3) microplate containing the McFarland standards, (4) camera, (5) diffusing screen and (6) fluorescent strips.

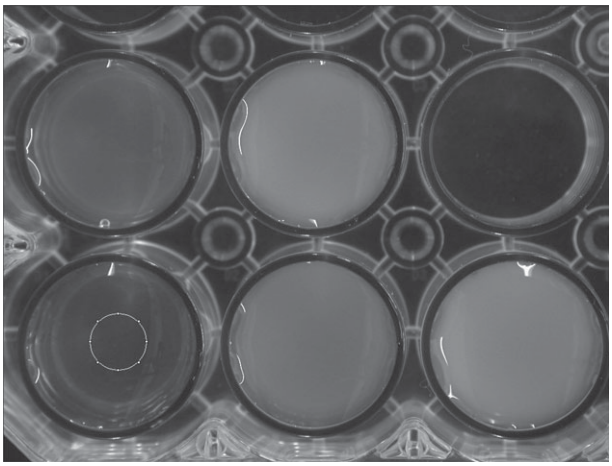

Figure 2. Image of a microplate holding McFarland standards and the circular area used to measure the average grey level by ImageJ. (Channel, eight-bit, 2048 × 1536 pixels.)

(equivalent to 0.04802 mol l^{-1}) and 1 per cent (v/v) H_2SO_4 (equivalent to 0.18010 mol l^{-1}) were used to prepare a series of McFarland standards and their composition and equivalence in colony forming units (CFU) per millilitre of microbial suspension, which are shown in table 1. The standards were prepared in disposable, thread-cap test tubes.

Next, the optimum photographic conditions were established (figure 1). The supports for McFarland standards were polystyrene microplates on account of their advantageous geometry and transparency. This facilitated the simultaneous capturing of an image of all calibration standards under identical lighting conditions. A volume of 3 ml of each standard was measured with the micropipette and transferred to a plate well. Once an aliquot of all standards was transferred to the plate, then an image was captured and processed with the software ImageJ.

Table 1. Composition and equivalences of the standards in the McFarland series.

McFarland standard no.	1.0% anhydrous $BaCl_2$ (ml)	1% H_2SO_4 (ml)	approximate bacterial density ($\times 10^8$) (CFU ml^{-1})
0.5	0.05	9.95	1.5
1	0.1	9.9	3.0
2	0.2	9.8	6.0
3	0.3	9.7	9.0
4	0.4	9.6	12.0

The camera was operated as follows: because illuminating with the camera strobe light (flash) would have caused reflections on solution surfaces, all lighting was provided by fluorescent strips. This makes the procedure easy to implement at virtually any laboratory. The effect of potential reflections of the strips on the solutions was avoided by placing a diffusing screen made with a sheet of white paper filter over and around the camera and plate. This setup provided soft lighting; in addition, using the largest aperture (i.e. the smallest F-number) on the camera lens minimized exposure times, which were set as recommended by the camera in order to avoid too dark (underexposed) or too light (overexposed) images. The camera was placed 20 cm from the plate, on a static support to ensure reproducible framing and shooting under identical conditions: F/5 as lens aperture and 1/2 s as exposure time. The plate was placed on a piece of black cardboard to enhance the milky turbidity of the McFarland standards.

Images were processed with the software ImageJ. Operation A > B > C means select command B on menu A and then select subcommand C in command B. First, the original colour image (in jpg format, 24 bit, 2048 × 1536 pixels) was split into three according to its RGB (red, green, blue) values (Image > Colour > RGB split), each split image being in jpg format, eight-bit and 2048 × 1536 pixels. The image for the green channel, which exhibited better linearity than those for the other two, was used to determine the 'grey level' for each standard; this was taken to be the average for a uniform circular area that was selected with the software's drawing tool (mean of 23 000 pixels) in each plate well (figure 2). The influence of the size of the mentioned circular area was studied by testing five different sizes: namely (in pixel) 230 000 (maximum allowed by the well size), 100 000, 50 000, 25 000 and 12 500. A linear calibration curve was obtained for each tested size. Only the obtained with the maximum tested area showed a slope smaller (0.2728) than the others, among which there were no significant differences (mean slope and s.d.: 0.3188 ± 0.0014; CV = 0.4%). This difference could be attributed to the reflections produced by the illumination near the internal edge of each well, which can be easily observed in figure 2. Finally, a circular area containing 23 000 pixels was selected as the optimum, because it avoids the inclusion of any reflection feature in the measured area.

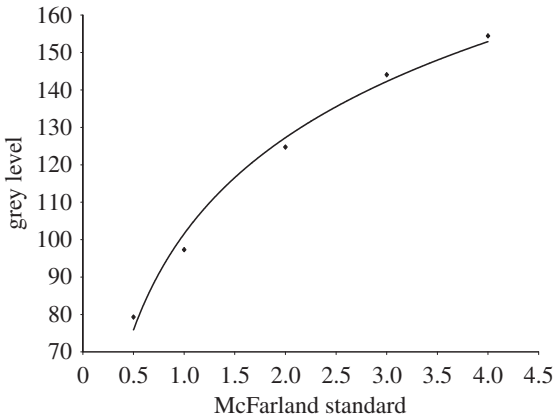

Figure 3. Calibration curve directly obtained with the proposed method.

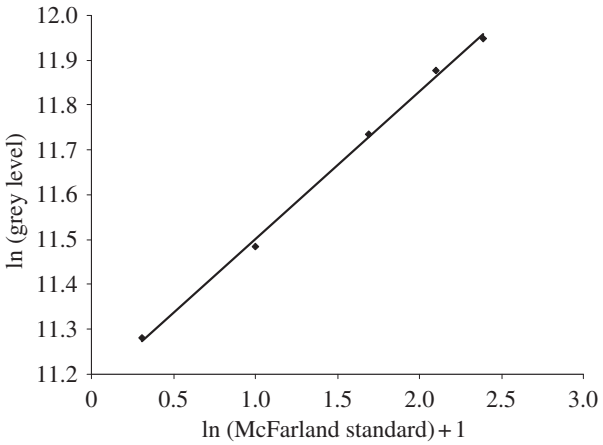

Figure 4. Linear transformation of the calibration curve of figure 3 via a log–log plot.

Table 2. Results obtained with the four instrumental techniques compared.

	slope of the calibration curve			
working session	nephelometer	spectrophotometer	spectrofluorimeter	ImageJ
A	1.492	0.1263	168.1	0.3200
B	1.539	0.1317	160.4	0.3304
C	1.378	0.1474	128.2	0.3289
D	1.525	0.1424	137.4	0.3107
E	1.521	0.1384	140.3	0.3450
s.d.	0.07	0.008	17	0.013
average slope	1.49	0.137	147	0.327
CV (%)	4.7	5.8	11.6	4.0

Then, each McFarland standard was used to make absorbance (625 nm) and fluorescence measurements ($\lambda_{ex} = 625$, $\lambda_{em} = 625$ nm, both slits being set at their minimum value and signal attenuation at 1%) during each working session.

Finally, each solution was assigned a McFarland value by the BD CrystalSpec nephelometer.

3. RESULTS AND DISCUSSION

By way of representative example, this section presents and discusses the results of working session C.

As stated in §2.2, the first step in the determinations involved capturing colour images of the McFarland standard series. The images were then processed with the software ImageJ for splitting into the three primary channels, and the green one (G) was stored for subsequent processing as it resulted in more linear calibration curves than the red (R) and blue channel (B). Each standard was used to measure the average grey level in a circular area spanning 23 000 pixels (figure 2). As can be seen from figure 3, plotting the results exposed a logarithmic trend in them.

In order to accurately compare the calibration curves with the turbidimetric (spectrophotometric) and nephelometric (spectrofluorimetric) results, which exhibited a linear trend, the logarithmic calibration curve was easily converted into a straight line (figure 4) by a log–log plot. Negative x-values were avoided by adding one unit (+1) to all values. Then, the absorbance of each McFarland standard was measured and a calibration curve run from both these results, and those of the spectrofluorimetric and nephelometric measurements of the standards. These operations were all conducted in each working session.

Table 2 shows the slopes of the calibration curves obtained with the four instrumental techniques used in each session. Following application of Dixon's Q criterion for rejection of outliers—none was in fact detected—averages and their corresponding standard deviations were calculated and rounded off to the required decimal place by the usual criteria, and the coefficients of variation between slopes of the calibration curves for each technique were obtained (table 2).

4. CONCLUSIONS

On the basis of the overall results, the proposed method provides results comparable with those of the conventional turbidimetric and nephelometric methods, with correlation coefficients between calibrations exceeding 0.99 in all cases. This imaging method possesses a high repeatability (CV = 4.0% with $n = 5$) exceeding even that of the nephelometric method used by the dedicated instrument for measuring McFarland standards (CV = 4.7%, $n = 5$).

The proposed method is operationally simple and inexpensive: in fact, all it requires is a digital camera

and a computer as measuring instruments, and both are more flexible, accessible and affordable than the conventional spectrophotometers, spectrofluorimeters or dedicated nephelometer typically used for this purpose. In addition, images can be readily processed with the public domain software ImageJ, developed by the National Institutes of Health and freely available on the Internet, which is very user-friendly.

In a scenario dominated by increasingly sophisticated and expensive commercial instruments, the proposed method provides an interesting alternative inasmuch as it enables quantitative determinations in teaching laboratories and modest facilities in developing countries, where economic resources for purchasing and maintaining measuring equipment are typically scant.

The authors thank the students Mª Isabel Cano Esteban and María Solana Altabella, for their assistance during the experimentation.

REFERENCES

1 Koch, A. L. 1994 Growth measurement. In *Methods for general and molecular bacteriology* (eds P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg), pp. 248–277. Washington, DC: American Society for Microbiology.

2 McFarland, J. 1907 The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J. Am. Med. Assoc.* **49**, 1176–1178.

3 BD CrystalSpec. Nephelometer user's guide. See http://www.bd.com/ds/technicalCenter/inserts/8809791JAA(201010).pdf (accessed May 2011).

4 Roessler, W. G. & Brewer, C. R. 1967 Permanent turbidity standards. *Appl. Microbiol.* **15**, 1114–1121.

5 Pugh, T. L. & Heller, W. 1957 Density of polystyrene and polyvinyl toluene latex particles. *J. Colloid Sci.* **12**, 173–180. (doi:10.1016/0095-8522(57)90004-1)

6 Washington II, J. A., Warren, E. & Karlson, A. G. 1972 Stability of barium sulfate turbidity standards. *Appl. Microbiol.* **24**, 1013.

7 Performance standards for antimicrobial susceptibility testing. 2009 Nineteenth informational supplement. CLSI document no. M100-S19.

8 Murray, P. R., Baron, E. J., Jorgensen, J. H., Landry, M. L. & Pfaller, M. A. 2007 *Manual of clinical microbiology*, 9th edn. Washington, DC: American Society for Microbiology (ASM) Press.

9 Byrne, L., Barker, J., Pennarun-Thomas, G. & Diamond, D. 2000 Digital imaging as a detector for generic analytical measurements. *Trend. Anal. Chem.* **19**, 517–522. (doi:10.1016/S0165-9936(00)00019-4)

10 Yamamoto, T., Takiwaki, H., Arase, S. & Ohshima, H. 2008 Derivation and clinical application of special imaging by means of digital cameras and Image J freeware for quantification of erythema and pigmentation. *Skin Res. Technol.* **14**, 26–34. (doi:10.1111/j.1600-0846.2007.00256.x)

11 Schlücker, S., Schaeberle, M. D., Huffman, S. W. & Levin, I. W. 2003 Raman microspectroscopy: a comparison of point, line, and wide-field imaging methodologies. *Anal. Chem.* **75**, 4312–4318. (doi:10.1021/ac034169h)

12 Goldmann, T., Zyzik, A., Loeschke, S., Lindsay, W. & Vollmer, E. 2001 Cost-effective gel documentation using a web-cam. *J. Biochem. Biophys. Methods* **50**, 91–95. (doi:10.1016/S0165-022X(01)00174-9)

13 Fan, G. Y. & Ellisman, M. H. 2000 Digital imaging in transmission electron microscopy. *J. Microsc.* **200**, 1–13. (doi:10.1046/j.1365-2818.2000.00737.x)

14 Hanley, Q. S., Earle, C. W., Pennebaker, F. M., Madden, S. P. & Denton, M. B. 1996 Charge-transfer devices in analytical instrumentation. *Anal. Chem.* **68**, A661–A667. (doi:10.1021/ac9621229)

15 Koppenaal, D. W., Barinaga, C. J., Denton, M. B., Sperline, R. P., Hieftje, G. M., Schilling, G. D., Andrade, F. J. & Barnes IV, J. H. 2005 MS detectors. *Anal. Chem.* **77**, 418A–427A. (doi:10.1021/ac053495p)

16 Merk, S., Lietz, A., Kroner, M., Valler, M. & Heilker, R. 2004 Time-resolved fluorescence measurements using microlens array and area imaging devices. *Comb. Chem. High Throughput Screen* **7**, 45–54.

17 Chakravarti, B., Loie, M., Ratanaprayul, W., Raval, A., Gallagher, S. & Chakravarti, D. N. 2008 A highly uniform UV transillumination imaging system for quantitative analysis of nucleic acids and proteins. *Proteomics* **8**, 1789–1797. (doi:10.1002/pmic.200700891)

18 Galindo, E., Larralde-Corona, C. P., Brito, T., Córdova-Aguilar, M. S., Taboada, B., Vega-Alvarado, L. & Corkidi, G. 2005 Development of advanced image analysis techniques for the in situ characterization of multiphase dispersions occurring in bioreactors. *J. Biotechnol.* **116**, 261–270. (doi:10.1016/j.jbiotec.2004.10.018)

19 Mesquita, D. P., Dias, O., Amaral, A. L. & Ferreira, E. C. 2009 Monitoring of activated sludge settling ability through image analysis: validation on full-scale wastewater treatment plants. *Bioprocess Biosyst. Eng.* **32**, 361–367. (doi:10.1007/s00449-008-0255-z)

20 Lancaster, M. D., Goodall, M., Bergström, E. T., McCrossen, S. & Myers, P. 2005 Quantitative measurements on wetted thin layer chromatography plates using a charge coupled device camera. *J. Chromatogr. A* **1090**, 165–171. (doi:10.1016/j.chroma.2005.06.068)

21 Lancaster, M. D., Goodall, M., Bergström, E. T., McCrossen, S. & Myers, P. 2006 Real-time image acquisition for absorbance detection and quantification in thin-layer chromatography. *Anal. Chem.* **78**, 905–911. (doi:10.1021/ac051390g)

22 Hayakawa, T. & Hirai, M. 2003 An assay of ganglioside using fluorescence image analysis on a thin-layer chromatography plate. *Anal. Chem.* **75**, 6728–6731. (doi:10.1021/ac0346095)

23 Prosek, M. & Vovk, I. 1997 Reproducibility of densitometric and image analysing quantitative evaluation of thin-layer chromatograms. *J. Chromatogr. A* **779**, 329–336. (doi:10.1016/S0021-9673(97)00442-1)

24 Wagner, J. W. & Miskelly, G. M. 2003 Background correction in forensic photography I. Photography of blood under conditions of non-uniform illumination or variable substrate color. Theoretical aspects and proof of concept. *J. Forensic Sci.* **48**, 593–603. (doi:10.1007/s11284-006-0035-7)

25 Wagner, J. W. & Miskelly, G. M. 2003 Background correction in forensic photography II. Photography of blood under conditions of non-uniform illumination or variable substrate color. Practical aspects and limitations. *J. Forensic Sci.* **48**, 604–613. (doi:10.1007/s10531-010-9817-x)

26 Hess, A. V. I. 2007 Digitally enhanced thin-layer chromatography: an inexpensive, new technique for qualitative and quantitative analysis. *J. Chem. Ed.* **84**, 842. (doi:10.1021/ed084p842)

27 Cumberbatch, T. & Hanley, Q. S. 2007 Quantitative imaging in the laboratory: fast kinetics and fluorescence

quenching. *J. Chem. Ed.* **84**, 1319–1322. (doi:10.1021/ed084p1319)

28 Gaiao, E. N., Martins, V. L., Lyra, W. S., Almeida, L. F., Silva, E. C. & Araujo, M. C. U. 2006 Digital image-based titrations. *Anal. Chim. Acta* **570**, 283–290. (doi:10.1016/j.aca.2006.04.048)

29 Alimelli, A., Filippini, D., Paolesse, R., Moretti, S., Ciolfi, G., D'Amico, A., Lundstrom, I. & Di Natale, C. 2007 Direct quantitative evaluation of complex substances using computer screen photo-assisted technology: the case of red wine. *Anal. Chim. Acta* **597**, 103–112. (doi:10.1016/j.aca.2007.06.033)

30 Martinez, A. W., Phillips, S. T., Carrilho, E., Thomas, S. W., Sindi, H. & Whitesides, G. M. 2008 Simple telemedicine for developing regions: camera phones and paper-based microfluidic devices for real-time, off-site diagnosis. *Anal. Chem.* **80**, 3699–3707. (doi:10.1021/ac800112r)

31 Lahuerta Zamora, L., Alemán López, P., Antón Fos, G. M., Martín Algarra, R., Mellado Romero, A. M. & Martínez Calatayud, J. 2011 Quantitative colorimetric-imaging analysis of nickel in iron meteorites. *Talanta* **83**, 1575–1579. (doi:10.1016/j.talanta.2010.11.058)

32 Lahuerta Zamora, L., Mellado Romero, A. M. & Martínez Calatayud, J. 2011 Quantitative colorimetric analysis of some inorganic salts using digital photography. *Anal. Lett.* **44**, 1674–1682. (doi:10.1080/00032719.2010.520394)