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Analytical Methods Section

Automated microbiological assay for quantitation of niacin performed in culture microplates read by digital image processing

Marianne Sølve, Helle Eriksen & Carl-Henrik Brogren*

Institute of Food Chemistry and Nutrition, National Food Agency of Denmark, DK-2860 Søborg, Denmark

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An automated microbiological assay for the quantitation of niacin is described. The assay, involving the use of *Lactobacillus plantarum*, was performed in culture microplates. The performance of the assay in both 48-well and 96-well culture microplates was investigated. Pipetting was carried out by a computer-controlled laboratory robot. Growth response was read by video digital-image processing (VDIP) and by ELISA reader. The effect of shaking the microplates prior to reading was also investigated.

The use of a niacin-depleted culture as inoculum shows lower blanks and an increased growth rate at low concentrations of niacin. The incubation time is reduced from 48 to 24 h by using the culture of *L. plantarum* directly after thawing of the cryopreserved working culture.

The 48-well plate assay showed better performance than the assay performed in 96-well culture plates. The coefficient of variation (CV) was improved in the 48-well plate assay when compared with the conventional microbiological assay. The relative intra-assay variation ranged from 3·7 to 8·5% in the 48-well plate assay and from 16·7 to 25·8% in the 96-well plate assay for samples of foods and feeds. The recovery of added standard in the 48-well and in the 96-well culture-plate assay was 101% ± 3% ($n = 6$), and 105% ± 12% ($n = 6$), respectively. The results of the 48-well plate assay correlated well with the results obtained by the conventional microbiological assay ($r = 0{\cdot}985$, $n = 40$). The detection limit for the 48-well assay was found to be 0·5 ng niacin per well. Avoiding shaking the 48-well plates prior to reading of growth response increased the precision significantly.

The technique used in this assay reduces reagent costs and time spent on pipetting and evaluating results when compared with the conventional microbiological assay performed in 5-ml tubes. The microplate assay is more compact and easier to perform. Digital image processing is introduced as a measurement of turbidity.

INTRODUCTION

Vitamin analysis of foodstuffs is necessary to control the quality of products during manufacture as well as that of the final product, to examine the effect of new technologies, to ensure compliance with legislation, and to provide data for food tables and nutritional studies. Reliable analytical techniques for vitamin measurements are therefore still needed in food-science studies as well as in the food industry.

Official methods for quantitation of niacin in foods are colorimetric methods and a microbiological assay employing *Lactobacillus plantarum* as test organism (AOAC, 1990). The microbiological assay is more sensitive and applicable to a wider variety of materials without requiring extensive sample preparation and is therefore preferable and still widely used for routine purposes (Favell, 1990).

Microbiological assays for the quantitation of vitamins are based on the fact that a given microorganism can reproduce and grow only in the presence of a specific vitamin. An extract of the test sample is mixed with a medium and inoculated with the microorganism. The growth response proportional to the vitamin content in the sample is measured and subsequently related to a standard curve (AOAC, 1990). The conventional microbiological assays for the quantitation of vitamins are costly, laborious, and time-consuming. The need

* To whom correspondence should be addressed

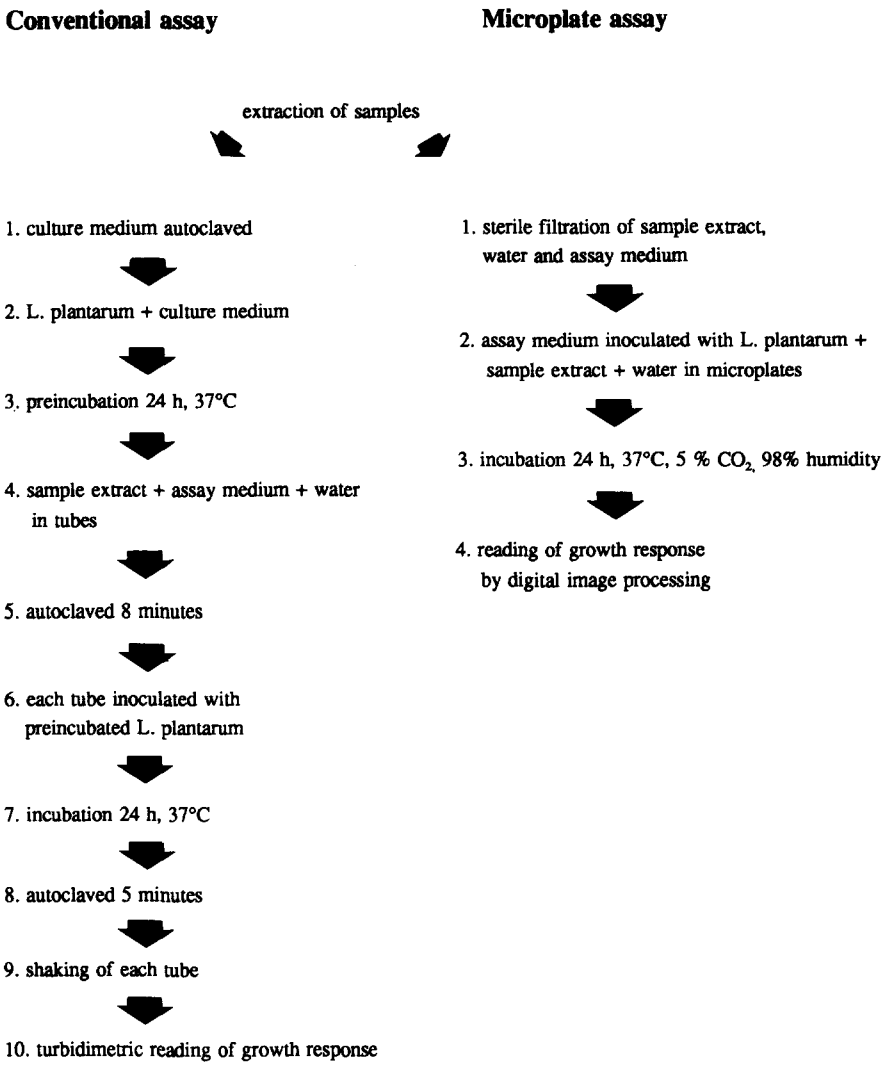

Fig. 1. Flow diagram for the conventional assay and the microplate assay.

for monitoring the nutritional quality of food and the effect of technological processes on foodstuffs requires rapid vitamin assays enabling a large number of samples to be analysed (Kirk, 1977).

In order to meet these needs, several microbiological assays performed in microplates have been developed. The use of a cryoprotected folate-depleted culture of *L. casei* in a microbiological tube assay of folates in blood was first described by Grossowicz *et al.* (1981). Their procedure was later simplified by Wilson and Horne (1982). Newman and Tsai (1986) have described a procedure for a microbiological assay of folates in sera performed in 96-well plates read with an automatic plate reader. The organism used in this assay was a folate-depleted culture of *L. casei*. This method was subsequently simplified by Horne and Patterson (1988) by using the cryoprotected folate-depleted culture directly after thawing the culture. Eguchi *et al.* (1990) have described microbiological assays of biotin, nicotine amide, and pantothenic acid in sera. The addition of CO_2 to the incubation chamber was found to affect the growth response of *L. plantarum*, and 5% of CO_2 gave the best result. A microbiological assay for vitamin B_{12} in sera involving the use of a colistin-resistant strain of *L. leichmanii* performed in 96-well plates has been described by Kelleher and O'Broin (1991). The use of a chloramphenicol-resistant organism of *L. casei* was later applied in the 96-well plate assay for folates in sera and red cells in order to avoid aseptic precautions (O'Broin & Kelleher, 1992). Recently, Eguchi *et al.* (1992) described a microbiological assay of vitamin B_{12} in blood performed in 96-well plates incubated under anaerobic conditions. Olkowski and Goonerate (1992) presented a microbiological 96-well microplate assay of thiamin in biological material. These microplate assays are all for use in routine clinical diagnosis.

In this paper, an automated microbiological assay for the quantitation of niacin in foods and feeds performed in 48-well and 96-well plates is described.

A niacin-depleted cryopreserved culture of *L. plantarum* is used for the inoculation of the assay medium directly after thawing the working culture. Plates are read by computerised VDIP and by ELISA reader (Brogren *et al.*, 1992). The VDIP system is based on digitalisation of a grey-level image of the microplate processed by a video camera. The digitised image is transferred to data-handling software, resulting in a graph of the standard curve and calculations of niacin concentrations of samples. Further statistical evaluation is carried out by the use of statistical software. Flowsheets of the conventional microbiological tube assay and of the microplate assay are shown in Fig. 1.

MATERIALS AND METHODS

Lactobacillus plantarum strain ATCC 8014 was obtained from American Type Culture collection, Rockville, MD, USA, and cryopreserved for long-time storage. The niacin (nicotinic acid) used for standards was Niacin USP Reference Standard obtained from United States Pharmacopeial Convention Inc., Rockville, MD, USA. A standard stock solution of a concentration of 100·0 mg niacin standard per 1000 ml ethanol (96%) was prepared. The stock solution was controlled by being assayed as a calibrator by the conventional microbiological method. The stock solution is stable for six months at 5°C.

The assay medium was prepared according to Official Methods of Analysis of the AOAC, Method No. 944·13. (AOAC, 1990). Samples were three different infant-food formulas, three breakfast cereals, and three feeds. Extractions of niacin from samples were performed according to Official Methods of the AOAC (AOAC, 1990). These extractions were used for further testing in the conventional microbiological assay as well as in the microplate assay. All reagents used were of analytical grade.

Conventional microbiological assay

The conventional microbiological assay was performed as described in Official Methods of Analysis of the AOAC, Method No. 944·13 (AOAC, 1990). Growth response was read by a spectrophotometer (Model 24 Beckman Inc., Palo Alto, CA, USA) at 650 nm. The standard curve and concentrations of samples were computed by a locally developed computer program called VITA (National Food Agency of Denmark, Søborg, Denmark).

Culture microplate assays

A working standard solution of niacin was prepared by diluting the standard stock solution in water to a concentration of 60 ng ml^{-1} niacin. Sample extracts, the working standard solution, the assay medium, and water were filtered through a 0·2 μm-pore-size sterile filter into sterile containers. The 48-well culture microplates were sterile 48-well Cell Culture Clusters (cat. no. 3548), and the 96-well culture microplates were Tissue Culture Clusters (cat. no. 3595), purchased from Costar Corporation, Cambridge, MA, USA.

In this study, pipetting was performed automatically by a Biomek 1000 Automated Laboratory Station (Beckman Inc., Palo Alto, CA, USA). For comparative studies, manual pipetting was done by using a Multipette 4780, a Multidispenser with plus/8 adaptor, and Combitips (Eppendorf, Hamburg, Germany) and a Digital Multichannel Pipette (Flow Laboratories Ltd, Ayrshire, UK).

Preparation of niacin depleted Lactobacillus plantarum *culture*

The stock culture was *L. plantarum* strain ATCC 8014. The agar was Bacto Micro Assay Culture Agar (MACA) from Difco Laboratories, Detroit, MN, USA, and prepared as suggested by the manufacturer. Liquid culture medium with (culture medium 1) and without niacin (culture medium 2) was prepared as prescribed in Official Methods of Analysis of the AOAC, Method No. 944.13 (AOAC, 1990). Cells from the stock culture were transferred with an eye needle into fresh culture medium 1 and incubated for 24 h at 37°C to recover the bacteria. Cells from this medium were subsequently spread on MACA-agar plates and incubated for 24 h at 37°C. After incubation, cells of *L. plantarum* from one colony were transferred to sterile culture flasks containing 20 ml of culture medium 2 in order to deplete the culture of niacin. These were incubated for 24 h at 37°C. Subsequently, the pH was adjusted to 6(5·5–6·5) with 1 M NaOH, and the content of the culture flasks was mixed with an equal volume of sterile glycerol (87% v/v). This mixture was dispensed in 1-ml aliquots into sterile cryotubes (Nunc A/S, Roskilde, Denmark) and stored at 80°C or in liquid nitrogen for a long time (this was the working culture). The remaining inoculum from each 20-ml container was spread on agar plates (MACA) to ensure the purity of the culture and finally tested by the API test (API 50 CH, BioMerieux SA, Montalieu-Vercieu, France).

The 48-well culture-microplate-assay procedure.

The working standard solution with 60 ng ml^{-1} niacin was added to the wells in sixfolds in a volume ranging from 0 to 500 μl. Samples were diluted to contain between 0 and 60 ng ml^{-1} niacin. Wells for samples contained in fourfolds 250 and 500 μl of the diluted sample. The volume of standards and samples was adjusted to 500 μl by adding sterile water. Assay medium (25 ml) was inoculated with thawed working culture (500 μl) of the niacin-depleted *L. plantarum* culture. Finally, inoculated medium (500 μl) was added to each well, which resulted in a final volume of 1 ml in each well. Wells for the standard curve then contained from 0 to 30 ng niacin per well.

The completed culture plates were incubated at 37°C, 5% CO_2, 98% r.h. for approximately 22 h in a CO_2

incubator, model IG 150 (Jouan, Saint Herblain, France).

The 96-well culture-microplate-assay procedure.
Working standard solution with 60 ng ml^{-1} niacin was added to the wells in eightfolds in a volume ranging from 0 to 100 μl. Samples were diluted to contain between 0 and 60 ng ml^{-1} niacin. Wells for samples contained in fourfolds 50 and 100 μl of diluted sample. The volume in each well was adjusted to 100 μl by adding sterile distilled water. Sterile assay medium (12 ml) was inoculated with thawed working culture (100 μl) of the niacin-depleted *L. plantarum*. Finally, inoculated assay medium (100 μl) was added to each well. Plates were incubated at 37°C, 5% CO_2, 98% r.h. for approximately 22 h in the CO_2 incubator.

Reading of growth response

The growth response in the 48-well plates was read by VDIP. The growth response in the 96-well plates was read by VDIP and by ELISA reader (Titertek Multiscan Plate Reader, Flow Laboratories Ltd, Ayrshire, UK) at 630 nm.

Digital images of the microplates were recorded by using a video camera, Panasonic CCTV model WV-CD 20/G (Matsushita Communication Industrial Co. Ltd, Japan). The lens in this is an Ernitec 12·5–75 mm zoom (Ernitec A/S, Herlev, Denmark). The camera is placed 80 cm above a light box, Color Control (Just, Weilheim, Germany) on a copy stand. Two blue filters, Cokin A020 and A021 (Cromofilter SA, Paris, France), are attached to the lens. The arrangement is shown in Fig. 2. After a calibration phase, the image of each well is converted into a grey tone level (number) proportional to the light intensity of the well. In order to calibrate, an empty microplate is placed on the light box.

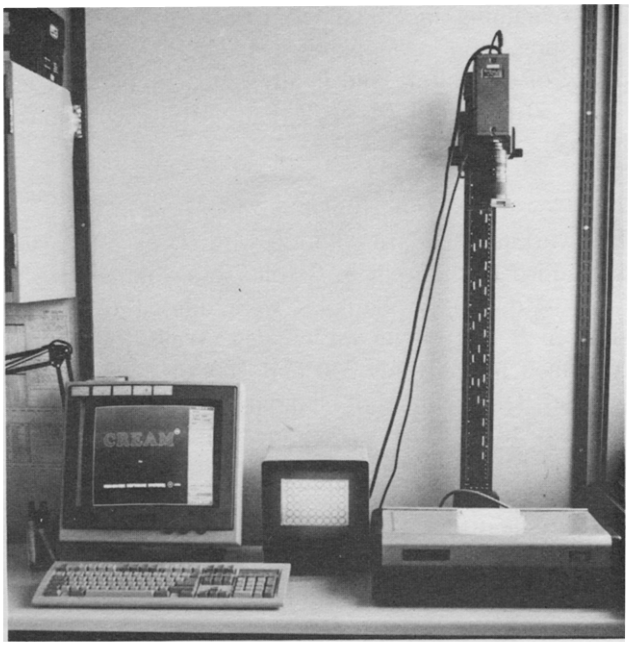

Fig. 2. Video-image processing. From left: display and keyboard, monitor, light box, and video camera on copy stand.

An appropriate shutter reading and focusing are set, and, before the image of the empty plate is stored, the user-defined parameters (plate format and plate position) are given to the CREAM software, EIA module (Kem-En-Tec A/S, Copenhagen, Denmark), capable of scanning 24-, 48-, and 96-well plates. The final step in the calibration phase is to save an image of a plate with a coloured film, Rosco Supergel 06 (Rosco, Hollywood, CA, USA) in the lid, representing the maximum colour intensity of the wells being measured. After the calibration, the plate with samples is placed on the light box. The light shining through each well is proportional to the bacterial density in the well. An image of the plate with samples is recorded and stored.

The digitalisation of the image is done by a framegrabber (a video digitiser board), DT 2853 (Data Translation, Inc., Marlboro, MA, USA) installed in an IBM-compatible personal computer. The framegrabber subdivides the field of vision of the video camera, which in this case is a grey-level image of the plate, into 512 × 512 picture elements (pixels). The CREAM software then further subdivides the image of the 48-well plate, at first into small areas of 7 × 7 pixels (for 96-well plates, this area is 3 × 3 pixels) in the centre of each well and subsequently into areas of 13 × 13 (for 96-well plates, this area is 9 × 9) pixels in each well and assigns a value (pixel value) between 0 and 250 for each pixel in the well. The value of a black pixel would be 0 and the value of a very bright pixel would be 250. Intermediate grey tones would be assigned values ranging from 0 to 250. The values of optical density for each well are then calculated as an average of the pixel values across the well, interpolated according to the calibration. Pixel values varying more than 3 grey-tone values from the average of the values of the first 7 × 7 pixels are outlied. The time for digitalisation of one image is 1/25 second.

After the calibration phase and scanning of the sample plate, the digitised image of the sample plate is then further processed by the CREAM software. The values for each well are displayed on the screen, a standard curve is plotted by cubic-spline curve-fitting, and concentrations of samples are computed. The time for scanning one microplate including calibration is about one minute. The data are then transferred to statistical software, Immunofit (Beckman Inc., Palo Alto, CA, USA) for further statistical analysis.

Statistics
The standard curve was fitted by a quadratic curvefit ($y = A + Bx + Cx^2$). The correlation coefficient for this curvefit was 0·99. The Immunofit program gives the opportunity to choose the curvefit giving the best correlation coefficient. The precision of the microplate assay was estimated by calculating the CV% for different samples. Comparison of the results of the microplate assays and the conventional microbiological assay was made by regression analysis. A test for significance of the correlation was made by using the Fisher Z transformation (Miller & Freund, 1977).

RESULTS AND DISCUSSION

Inoculum

Figures 3 and 4 show standard curves (as plotted by Immunofit) for the growth response of both the depleted and the non-depleted culture of *L. plantarum* in the 48-well plate assay. The depletion results in a lower value of the zero blank and an increased growth response to low concentrations of niacin, which results in improved sensitivity of the assay. The depleted culture is therefore preferable to the non-depleted.

Inoculation of the assay medium is performed directly after thawing of the culture, which thereby saves 24 h when compared with the conventional microbiological assay, in which the thawed working culture is pre-incubated in liquid culture medium 1 for 24 h. The addition of 100–600 μl of inoculum was tested for the 48-well assay, and the addition of 30–500 μl of inoculum was tested for the 96-well assay (data not shown). The experiments showed that the steepest standard curve, that is, the highest degree of sensitivity, was obtained by adding 500 μl of inoculum to 25 ml of assay medium for use in the 48-well plate assay and 300 μl of inoculum to 12 ml of assay medium for use in the 96-well plate assay. These combinations of volumes of inoculum and assay medium were used in the succeeding assays.

Shaking of plates prior to reading

The effect of shaking the plates on a microplate shaker prior to reading of the growth response was examined. Intra- and inter-assay variation with and without shaking of plates prior to reading is shown in Table 1. Intra- and inter-assay variation for the 48-well plate assay decreased if no shaking of plates was carried out prior to reading of growth response. No significant difference between shaking and no shaking of plates before taking a reading of the 96-well plates was found.

After incubation of the plates, the growth response of *L. plantarum* in each well could be seen as cells sedimented in a smooth layer suitable for measuring and relating to the niacin concentration in the well. Shaking of the 48-well plates resulted in a cluster of cells in the centre at the bottom of each well, with the rest of the cells dispersed in the liquid in the well. This uneven distribution of cells is unsuitable for taking measurements. Shaking of the 96-well plates resulted in

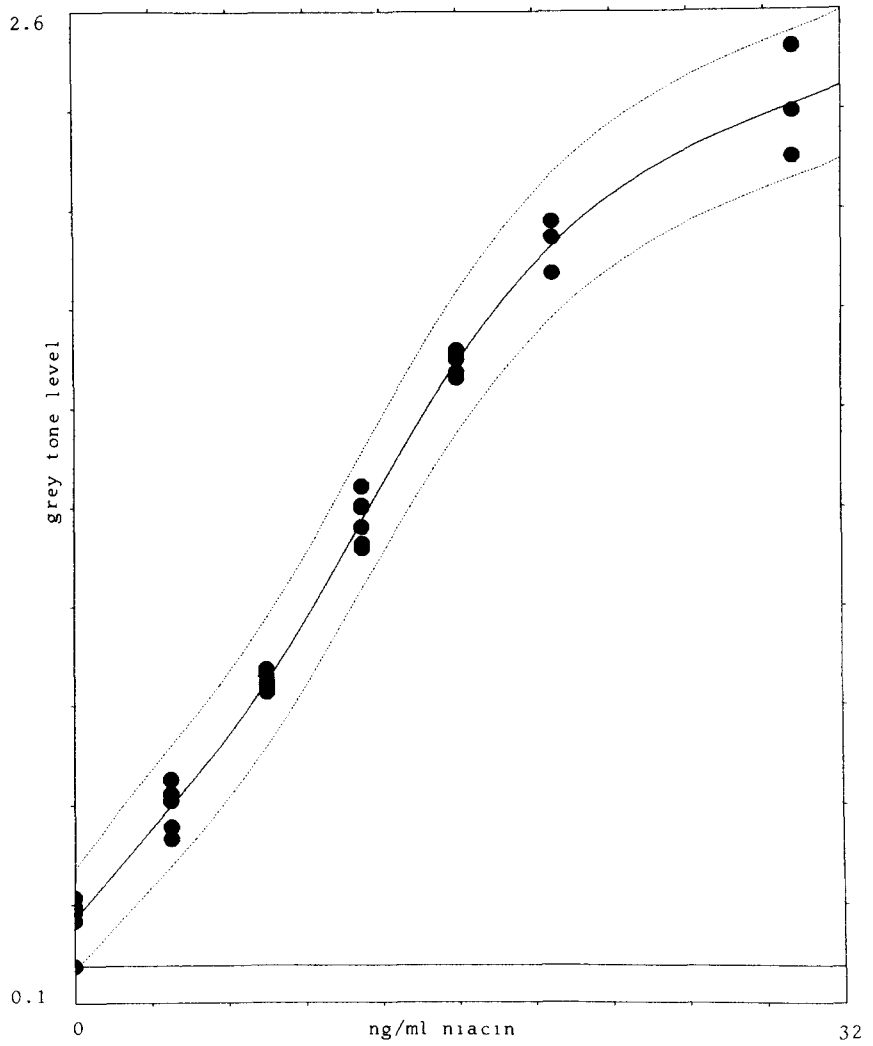

Fig. 3. Standard curve for the response of the depleted culture of L. plantarum in the 48-well plate assay.

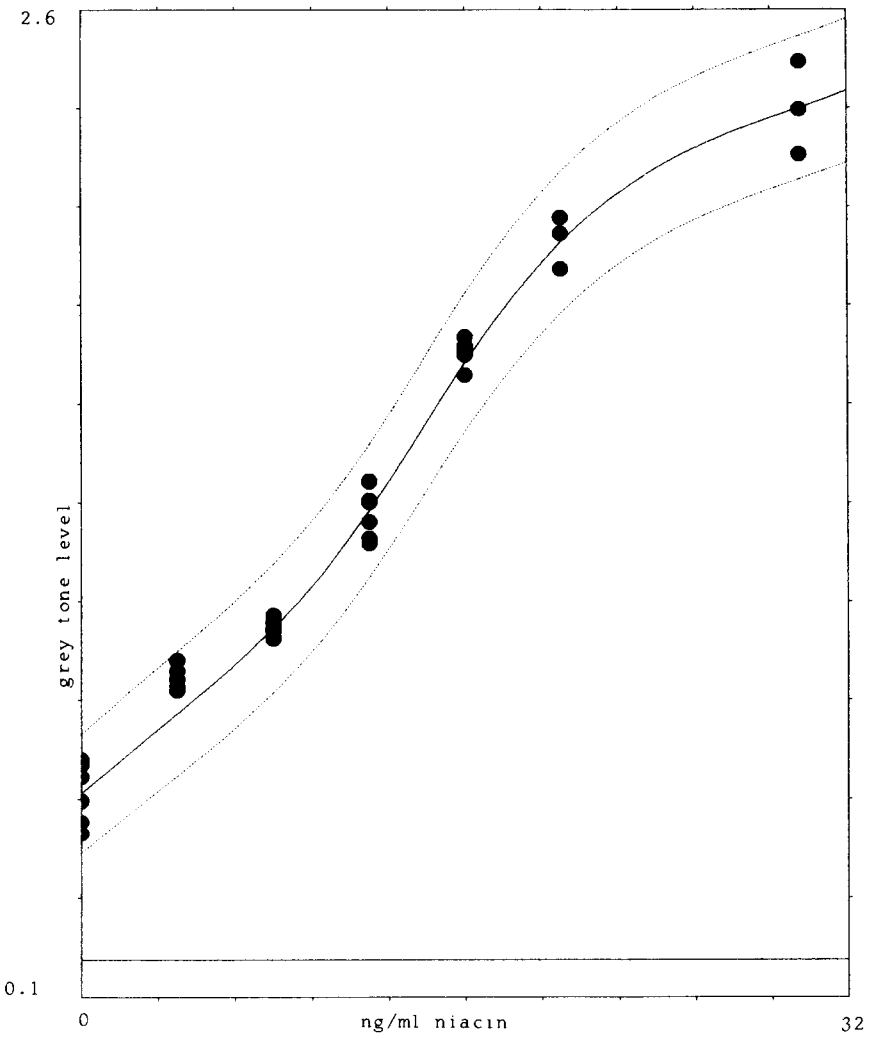

Fig. 4. Standard curve for the response of the non-depleted culture of *L. Plantarum* in the 48-well plate assay.

an even distribution of cells in the liquid in each well. This even distribution of cells in the wells made it possible to read the plates after shaking.

It was concluded that no shaking of the 48-well plates before reading was preferable.

Mode of pipetting

Manual and automatic (Biomek 1000) methods of pipetting were compared. The average relative intra-assay variation of ten different concentrations of standard in the 48-well assay performed manually ranged from 6 to 16%, and from 3 to 9% ($n = 8$ for each concentration) for the assay performed automatically. The precision was improved by using the laboratory robot, and time spent on tedious pipetting was avoided.

Reading of growth response

The CREAM software gives the user the opportunity to measure plate formats of 24, 48, and 96 wells. Other software capable of scanning microplates is available.

The higher degree of precision in the 48-well assay compared with the 96-well assay may be due to micro-environmental differences in general as well as to the fact that the larger volume of the wells in the 48-well plates may improve pipetting precision slightly. The light path and the number of pixels measured are other possible sources of variation. The light path in the liquid in each well is 10 mm in the 48-well plates and

Table 1. Precision as estimated by the 48-well assay- with and without shaking of plates

Shaking			No shaking		
	Intra-assay			Intra-assay	
Mean[a,b]	SD	CV%	Mean[a,b]	SD	CV%
10	1·7	17·0	10·1	0·8	7·92
11	1·9	17·3	10·7	0·6	5·61
12	1·8	15·0	12·6	0·8	6·35
	Inter-assay			Inter-assay	
Mean[b,c]	SD	CV%	Mean[a,b]	SD	CV%
9	1·9	21·1	9·6	0·4	4·17
10	3·6	36·0	10·7	0·3	2·80
13	4·3	33·0	12·5	0·4	3·20

[a] $n = 6$.
[b] Niacin concentration in ng/ml.
[c] $n = 4$.

3·5 mm in the 96-well plates. The measurement of growth response in the 96-well assay may be optimised by using the maximum amount of liquid in each well, corresponding to a light path of 10 mm. The picture of one well in a 48-well plate consists of 169 (13 × 13) pixels, whereas the picture of one well in a 96-well plate consists of 81 (9 × 9) pixels. This difference in measured area is probably of great importance for the precision of the measurement. The significance of the light path and of the number of pixels in the measurements is currently being investigated. Another possibility of optimising during the calibration phase is to use a film in the plate lid that better represents the appearence of maximum growth response. The film used in these experiments was Rosco 06, which is slightly straw-coloured and bright. Furthermore, the use of a camera of a higher quality may improve the results.

The growth response in the 96-well plates can be read by an ELISA reader. Using an ELISA reader for measuring growth response in the 96-well plates improved the intra-assay variation from 21% (when read by VDIP) to 8% (average of ten different concentrations of sample, $n = 8$ for each concentration). The 48-well assay showed a higher degree of precision.

The VDIP system has several other applications in addition to the scanning of microplates. The price of the VDIP system, including software for microplate reading, corresponds to the price of an ELISA reader.

Precision, accuracy, recovery and detection limit

Table 2 shows the precision as estimated at different niacin concentrations by the 48-well assay. The CV values range from 3·66 to 8·46% for the intra-assay variation and from 1·24 to 4·17% for the inter-assay variation. Table 3 shows the precision as estimated by the 96-well plate assay. The CV values range from 16·7 to 27·5% for the intra-assay variation. The intra-assay variation in the conventional microbiological assay ranges from 1·59 to 14·0% ($n = 7$) and from 2·20 to 21·4% ($n = 2$) for the inter-assay variation.

Table 2. Precision as estimated by the 48-well assay

	Intra-assay	
Mean[a,b]	SD	CV%
10·1	0·8	7·92
10·7	0·6	5·61
10·9	0·6	5·50
12·6	0·8	6·35
13	1·1	8·46
19·0	0·8	4·21
19·1	0·7	3·66
21·2	0·9	4·25
32	1·6	5·00
	Inter-assay	
Mean[a,b]	SD	CV%
9·6	0·4	4·17
10·0	0·4	4·00
10·7	0·3	2·80
12·5	0·4	3·20
20·8	0·8	3·85
32·3	0·4	1·24

[a] $n = 6$
[b] Niacin concentration in ng/ml.

Table 3. Precision as estimated by the 96-well assay

	Intra-assay	
Mean[a,b]	SD	CV%
8	2·2	27·5
9	1·5	16·7
11	2·5	22·7
12	3·1	25·8
15	2·6	17·3
19	4·2	22·1
22	5·4	24·5
33	6·2	18·7

[a] $n = 4$.
[b] Niacin concentration in ng/ml.

The regression analysis of 40 samples assayed by the conventional microbiological assay and by the 48-well assay is shown in Fig. 5. The correlation was highly significant ($r = 0{\cdot}985$, $p < 0{\cdot}001$). The regression analysis of 37 samples assayed by the conventional microbiological assay and by the 96-well assay is shown

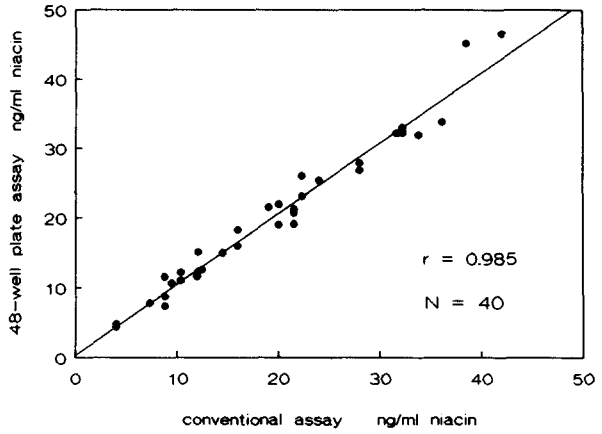

Fig. 5. Regression analysis of 40 samples assayed by the conventional microbiological assay and by the 48-well plate assay.

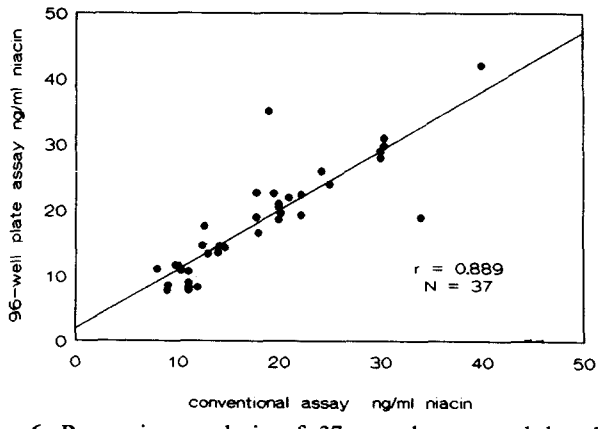

Fig. 6. Regression analysis of 37 samples assayed by the conventional microbiological assay and by the 96-well plate assay.

Table 4. Analytical recovery of added standard for the 48-well assay and the 96-well assay

48-well assay		
expected (added) ng/ml	recovered[a] ng/ml	% recovery[b]
4·00	3·91	97·8
6·00	5·97	99·5
12·0	12·1	101
18·0	18·0	100
24·0	25·6	107
30·0	30·1	100
Average		101 ± 3
96-well assay		
expected (added)	recovered[c] ng/ml	% recovery[b]
4·00	3·59	89·8
6·00	6·82	114
8·00	8·03	100
12·0	11·9	99·2
16·0	16·7	104
20·0	24·8	124
Average		105 ± 12

[a] $n = 6$.
[b] % recovery = (recovered/expected) × 100.
[c] $n = 8$.

in Fig. 6 and the correlation was significant ($r = 0.889$, $p < 0.001$) for this assay.

The recovery of added standard at six different concentrations was on average 101 ± 3% ($n = 6$) for the 48-well assay and 105 ± 12% ($n = 6$) for the 96 well assay. This difference may be due to micro-environmental differences (Table 4).

The detection limit (Rodbard *et al.*, 1978) for the 48-well assay was found to be 0·5 ng niacin per well.

CONCLUSION

The 48-well microplate assay presented here has the obvious advantage of miniaturisation, such as ability to adapt to automation, speed of operation and reading, and minimum reagent costs. Furthermore, the incubation time is shortened from 48 to 24 h, and the sensitivity is improved by the use of a niacin-depleted culture. The microplate assays formerly described have been applied on clinical samples. This assay has proved suitable for the quantitation of niacin concentrations in samples of foods and feeds. The assay shows acceptable reproducibility and good recovery of added standard, and the results correlate well with those of the conventional assay. The assay is well suited for analysing a large number of samples, it is within the competence of any routine laboratory, and it is therefore a highly recommendable alternative to the conventional microbiological assay.

To our knowledge, this is the first presentation of the VDIP used for quantitation of microbiological growth response.

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