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Journal of Chromatography A, 911 (2001) 119–126

JOURNAL OF CHROMATOGRAPHY A

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Identification and quantification of base and nucleoside markers in extracts of *Ganoderma lucidum*, *Ganoderma japonicum* and *Ganoderma* capsules by micellar electrokinetic chromatography

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Received 31 May 2000; received in revised form 5 December 2000; accepted 5 December 2000

Abstract

The present paper describes the development of a micellar electrokinetic chromatographic method for the determination of nucleoside (adenosine, uridine) and base (uracil) markers in aqueous extracts of *Ganoderma* medicinal preparations. The markers were successfully separated within 10 min using an 80 m*M* borate buffer, with 25 m*M* sodium dodecyl sulfate adjusted to pH 9.0, an operating voltage of 22 kV, temperature of 20°C and a hydrodynamic injection time of 5 s. Separations were carried out in a fused-silica capillary with peak detection by direct UV at 254 nm. Following semi-validation of the method, with each analyte showing a good linear relationship over a 0.2 to 20 ppm concentration range (correlation coefficients from 0.9986 to 0.9998), the amounts of the three markers in the various forms of *Ganoderma* were easily determined using a relatively simple extraction procedure.

Keywords: *Ganoderma* spp.; Pharmaceutical analysis; Nucleosides; Uracil; Adenosine; Uridine

1. Introduction

The fruiting bodies of *Ganoderma lucidum* and *Ganoderma japonicum*, both mushroom-like fungi, are regarded as a noble Chinese medicine in eastern countries with proven effects on cancer, hypertension, hepatitis and hypercholesterolemia [1,2]. Recent studies have shown many interesting biological properties including anti-tumour and anti-inflammatory effects as well as cytotoxicity to hepatoma cells and inhibition of platelet aggregation [3]. Like many traditional Chinese medicines, products of *Ganoderma* are complex mixtures of carbohydrates, proteins, nucleic acids, nucleosides, triterpenoids, steroids, sterols, fatty acids, betaine, vitamins, furans and other trace elements [5]. The water-soluble fraction of *Ganoderma* suppresses platelet aggregation with an inhibitory substance having been identified as adenosine [3]. The extract has also been shown to contain uridine and uracil, both of which are capable of lowering the elevated serum aldolase level of mice suffering from experimental myotonia [4]. All three of these bioactive compounds play several key roles in energy transformation and regulate many metabolic pathways. As Chinese literature has shown these compounds to be present

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)01241-3

in various forms of *Ganoderma*, and to be of pharmacological importance, they were selected as the markers for this investigation.

Capillary electrophoresis (CE) is a recently developed analytical technique that offers numerous advantages over more traditional modes of analysis such as high-performance liquid chromatography (HPLC). Short analysis times, micromolar requirements of analytes and reagents, improved separation efficiencies and the overall versatility of the various types of CE have led to increasing applications to the quality assurance of drugs, with good results already shown in the analysis of Chinese herbs [6–10]. To date, there have been no CE methods developed for the determination of any of the components of *Ganoderma*.

Micellar electrokinetic chromatography (MEKC) is a mode of CE that is capable of separating neutral species as well as charged analytes. The buffer in MEKC contains a surfactant, in this case sodium dodecyl sulfate (SDS), at a concentration in excess of its critical micelle concentration whereupon the SDS monomers aggregate into spherical micelles. As the micelles have a hydrophobic interior, they serve as a non-polar pseudostationary phase within the capillary column [11].

Qualitative and quantitative analysis of the components of any pharmacologically active material is of paramount importance to ensure safe usage. The objective of this investigation was to develop for the first time an accurate MEKC method for the determination of the selected base and nucleoside marker content in the aqueous extracts of *Ganoderma lucidum*, *Ganoderma japonicum* and two commercially available brands of *Ganoderma* capsules, with the hope of utilising these markers as some form of quality control. The conditions of buffer composition, pH, temperature, voltage, surfactant concentration and injection time were all optimised in order to develop the most suitable environment for the separations.

2. Experimental

2.1. Apparatus

A Beckman P/ACE 5000 CE system (Beckman Instruments, Fullerton, CA, USA) was used throughout the study. The system was composed of an autosampler, a high-voltage power supply and a UV–visible detection system operated at a wavelength of 254 nm. Separations took place in a fused-silica capillary of 57 cm (50 cm from inlet to detector)×75 μm I.D., also manufactured by Beckman. A personal computer controlled the P/ACE system, with data analysis performed on Beckman P/ACE Station software.

2.2. Chemicals

Analytical reference standards of uracil, adenosine and uridine were purchased from Sigma (St. Louis, MO, USA). SDS was obtained from Bio-Rad (Hong Kong, China). Sodium tetraborate-10-hydrate, pH buffer solutions and hydrochloric acid (analytical grade) were obtained from Riedel-de Haen (Seelze, Germany). Sodium hydroxide was obtained from Pharmacos (Southend on Sea, UK). *Ganoderma lucidum*, *Ganoderma japonicum* and *Ganoderma* capsules were bought from a local traditional medicine shop (Hong Kong, China). Milli-Q deionised water was used throughout the study (Millipore, Bedford, MA, USA).

2.3. Procedure

2.3.1. Optimisation of conditions

Prior to running any standard or sample solutions through the CE system, the capillary was rinsed with 0.1 *M* NaOH followed by Milli-Q water and finally the buffer solution in use. Each of these solutions was rinsed through for 2 min in order to regenerate and equilibrate the capillary. Unless otherwise stated, the operating conditions for the system were a voltage of 25 kV, temperature of 25°C and an injection time of 10 s.

A mixed standard solution was prepared of approximately 6 mg of each of the markers – uracil, adenosine and uridine – accurately weighed out into a 50-ml volumetric flask and dissolved in water. Initially, separation of the standard mixture was performed by CE with varying borate concentration; 50 m*M*, 60 m*M*, 70 m*M* and 80 m*M* dissolved in water. Once the optimum borate concentration had been determined, the effect of surfactant concen-

tration was investigated. Solutions were prepared of 12.5 m*M*, 25 m*M*, 50 m*M* and 70 m*M* SDS in 80 m*M* borate buffer. The next factor to be looked at was the effect of pH, with 80 m*M* borate–25 m*M* SDS buffers prepared at pH 8.6, 8.8, 9.0, 9.2, 9.4 and 9.6 by dropwise addition of NaOH or HCl. For further optimisation, instrumental parameters were varied and the effects noted, i.e., operating voltage (in the range 15 to 25 kV), temperature (18 to 26°C) and injection time (5 to 11 s). Spiking of the mixed standard solution was performed to resolve any difficulties in peak identification.

2.3.2. Method repeatability/linearity

Once the optimum operating conditions had been established, validation of the method was performed to a certain extent. The mixed standard solution was injected 10 times and repeatability determined for each of the analyte peaks in terms of the relative standard deviation (RSD) of migration times and corrected peak areas, i.e., peak area divided by migration time of peak. A 20-ppm solution of uracil standard was prepared by dissolving 1 mg of solid in 50 ml of water. Five other different concentrations were prepared from this solution by sequential dilution down to 0.2 ppm and the solutions run on the MEKC system under optimum conditions in order to produce a calibration curve. This process was repeated with 20 to 0.2 ppm standard solutions of adenosine and uridine.

2.3.3. Sample analysis

Sample preparation was as follows. With respect to *Ganoderma lucidum* or *Ganoderma japonicum*, the fruiting body of the fungus was cut into small pieces before being mixed in a blender. Around 10 g of the fine particles was accurately weighed out and placed in a boiling apparatus along with 400 ml of water. The apparatus was covered with aluminium foil and the mixture boiled for 5 h. The resultant extract was spun in a centrifuge at 600 rpm for 10 min. Any non-dissolved particles were removed by filtration and the filtrate placed in a refrigerator to freeze it, before being freeze–dried in a vacuum concentrator. For MEKC analysis, around 0.1 g of the freeze–dried powder was accurately weighed out into a 50-ml volumetric flask and dissolved to volume in water. The solution was ultrasonicated for 15 min and injected into the CE system following final filtration through a 0.25-μm disc filter.

Two types of commercially available *Ganoderma* capsules (brands A and B) were also analysed. Around 0.04 g of brand A capsule was accurately weighed out and dissolved in 50 ml of water. A 0.1-g amount of brand B capsule was also dissolved in 50 ml of water, and both solutions were ultrasonicated and filtered prior to MEKC analysis.

3. Results and discussion

3.1. Optimisation of conditions

The effects of increasing the borate concentration on resolution between the three marker peaks and overall run time (in terms of the migration time of uridine, the last peak to migrate) were initially considered. Uracil consistently migrated within the least amount of time, followed by adenosine and finally uridine. Whilst the resolution of the peaks is acceptable at all concentrations (always >2.0) and the migration time increases as the borate concentration increases, 80 m*M* was determined to be the optimum concentration as this provided a much smoother baseline and greater system stability. Addition of the SDS surfactant, essential as peak splitting is a major problem without it, also showed marked effects on both resolution and migration time. The optimum SDS concentration was determined to be 25 m*M*, providing short analysis times as well as more than adequate resolution of peaks. The buffer composition selected for subsequent analysis was 80 m*M* borate and 25 m*M* SDS.

Increasing the pH of the buffer from 8.6 to 9.6 led to a slight overall increase in the migration time of the analytes. The resolution of uracil from adenosine generally decreased with increasing pH, while resolution of uridine from adenosine increased. As pH increases the electroosmotic flow (EOF) in the system will increase due to greater dissociation of the silanol groups on the inner capillary wall, thus producing a greater zeta potential that is directly proportional to the EOF. Counter-acting this effect is the increased deprotonation of the analytes at higher pH values resulting in increased negative charges on the markers and decreased apparent electrophoretic

mobilities. At pH 9.0, the peaks were sharp and well resolved within a reasonable length of time (less than 10 min). At other pH values the peak shapes were quite poor so 9.0 was selected as the optimum value.

Investigation into the effect of increasing the operating voltage from 15 to 25 kV produced expected results. The larger the voltage the greater the EOF and hence marked decreases in migration time were observed. The peaks were resolved satisfactorily no matter what the applied voltage was, despite the effect on resolution being somewhat erratic. However at larger values the high potential difference can lead to excessive Joule heating in the system and affect repeatability. Therefore 22 kV was selected as the optimum value to provide short run times and excellent separation of peaks.

As with voltage, increasing the temperature in turn increased the EOF by reducing the viscosity of the buffer and thus reduced migration times. Despite optimum resolution of peaks being achieved at a temperature of 26°C, splitting of the peaks became a major problem under such conditions. Once again this was probably a consequence of band broadening due to Joule heating in the system, and 20°C was chosen as the optimum temperature value in order to maintain system stability.

In hydrodynamic injections, a longer injection time leads to greater sample amounts being placed in the capillary. As the injection time was increased, the increased sample loading led to very noticeable band broadening and subsequent losses in resolution between the analyte peaks. From investigation it became obvious that 5 s was the optimum injection time.

Overall, the optimum conditions determined for the separation of the markers in *Ganoderma* aqueous extracts were: buffer composition of 80 m*M* borate, 25 m*M* SDS, pH 9.0, operating voltage of 22 kV, temperature of 20°C and an injection time of 5 s. A typical electrokinetic chromatogram of a mixed standard injection under these conditions is shown in Fig. 1.

3.2. Method repeatability/linearity

Repeatability of the system running under optimum conditions for 10 injections of the mixed standard solution produced results as shown in Table 1. The migration time for each peak did not vary to any great extent, indicating that the method was suitable for the identification of any of the selected markers in real samples. The RSD values for corrected peak areas were quite high, up to around 7.5%, indicating a certain amount of instability such as capillary degradation between injections. However establishing linear responses over a concentration

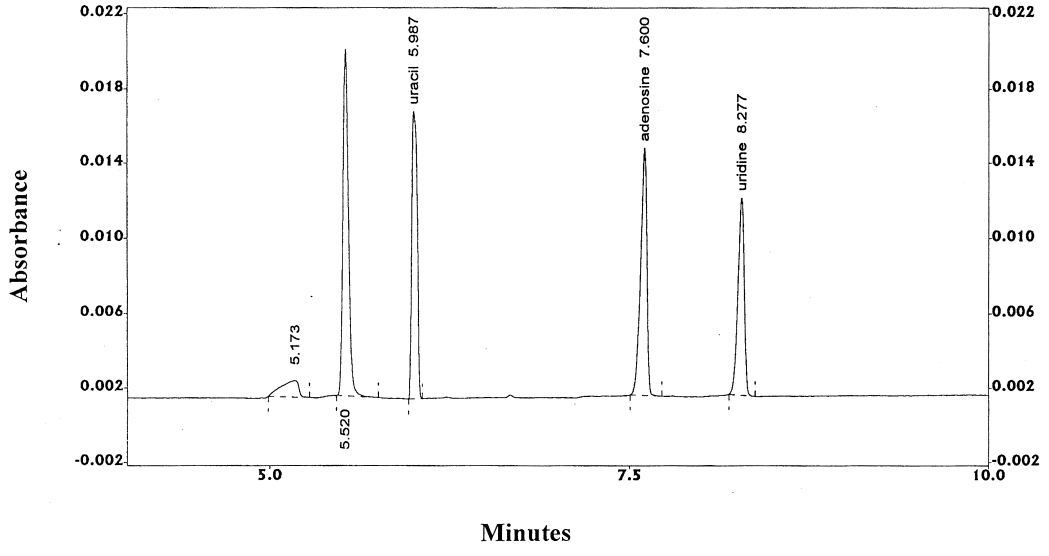

Fig. 1. Electrokinetic chromatogram of mixed marker standard for *Ganoderma* analysis.

Table 1
Relative standard deviation (RSD) of marker migration times and peak areas for 10 mixed standard injections[a]

Marker peak	Mean migration time (min)	RSD of migration time (%)	Mean corrected peak area	RSD of corrected area (%)
Uracil	5.87	2.52	6030	7.44
Adenosine	7.12	0.72	4670	4.21
Uridine	7.89	2.13	3330	3.36

[a] Experimental conditions: 80 m*M* borate, 25 m*M* SDS buffer at pH 9.0, 22 kV operating voltage, temperature 20°C, 5-s hydrodynamic injection.

range for uracil, adenosine and uridine further supported validity of the method. The following straight-line equations and correlation coefficients were obtained for these three analytes over the 0.2 to 20 ppm concentration range: uracil, $y=154.69x-1.5003$, $R^2=0.9996$; adenosine, $y=104.13x+9.4732$, $R^2=0.9986$; uridine, $y=137.38x-17.055$, $R^2=0.9993$; where y=corrected peak area and x=standard concentration (ppm). Number of data points=5 in all cases.

3.3. Sample analysis

With these equations, quantification of the amount of markers in the different forms of *Ganoderma* was possible. Electrokinetic chromatograms of *Ganoderma lucidum*, *Ganoderma japonicum*, *Ganoderma* capsule brands A and B are shown in Figs. 2–5, respectively. The optimum experimental conditions described previously were applied in all cases.

Sample spiking was performed in order to resolve any difficulties in peak identification. Taking into consideration the differences between sample and standard preparations, the concentrations of the nucleosides or nucleotides present in the raw materials (flowering part of the fungi or the capsules) were calculated. The results are shown in Table 2. In all cases the limit of quantification for each peak was set at the lowest concentration analysed in the linearity analysis, i.e., 0.2 ppm. Therefore limit of detection was taken as three-tenths of this value, 0.06 ppm. This equated to a μg/g detection limit of 1.66 for markers in *Ganoderma lucidum*, 0.337 for *Ganoderma japonicum*, 75.00 for brand A capsules and 30.00 for brand B.

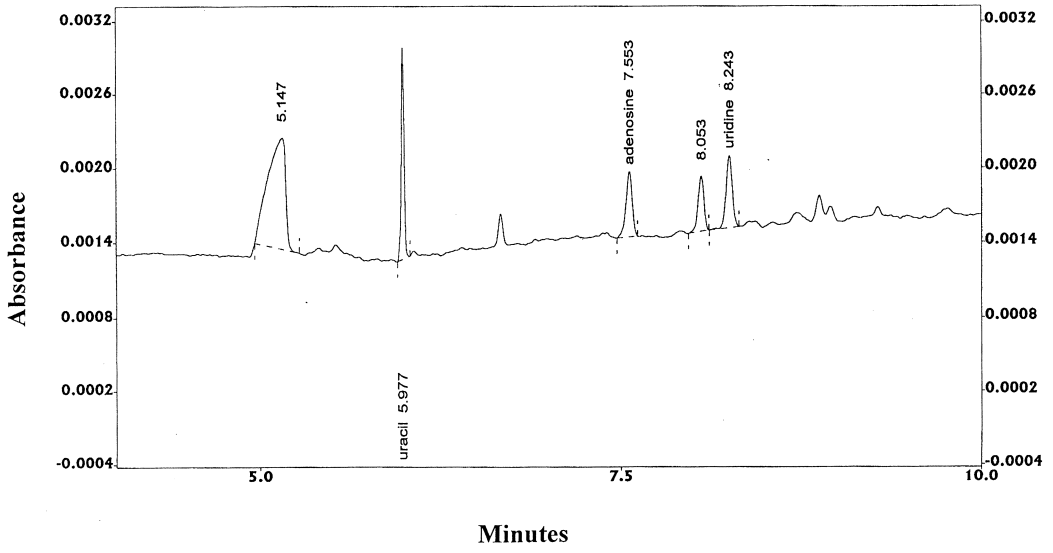

Fig. 2. Electrokinetic chromatogram of water extract of *Ganoderma lucidum*.

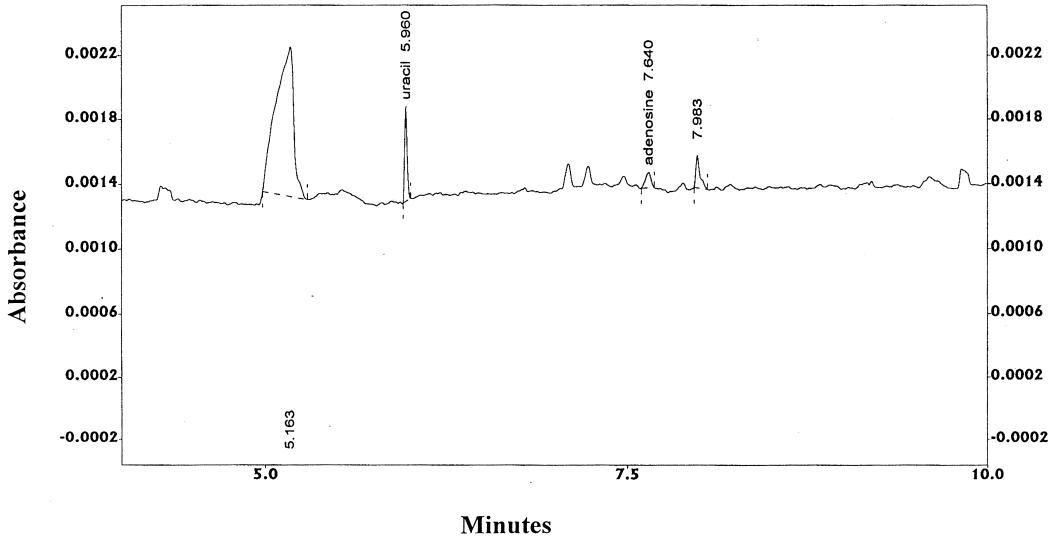

Fig. 3. Electrokinetic chromatogram of water extract of *Ganoderma japonicum*.

From the table, it is obvious that the brand A *Ganoderma* capsules are a much more potent source of the selected markers compared to the naturally found *lucidum* and *japonicum* fungi. Reasons for why none of the markers are present in the brand B *Ganoderma* capsules are unknown, but they are most likely removed during the manufacturing process of the capsules. The fact that none of the selected components were detected in brand B capsules only indicates that, with respect to these markers and their pharmacological activities, the capsules are of comparatively lower quality. However, it may be that the

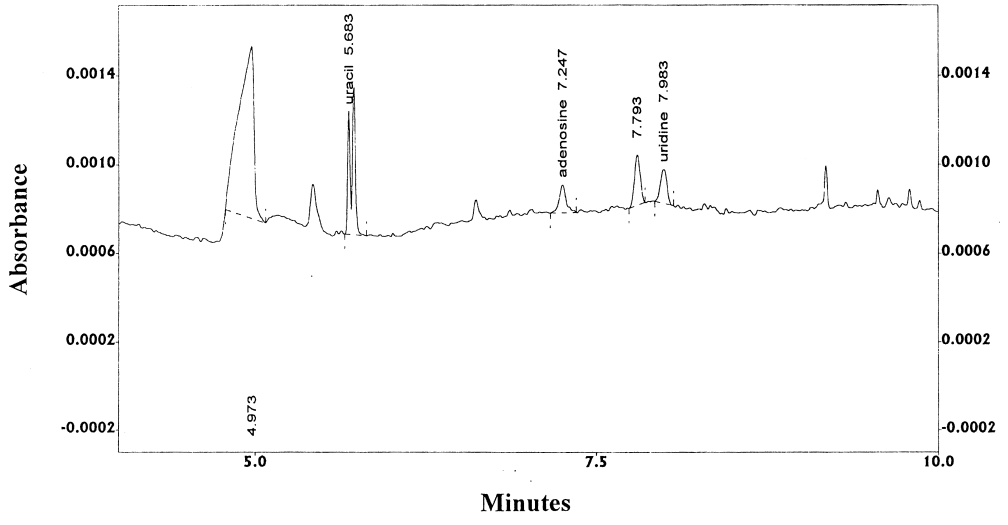

Fig. 4. Electrokinetic chromatogram of water extract of *Ganoderma* brand A capsules.

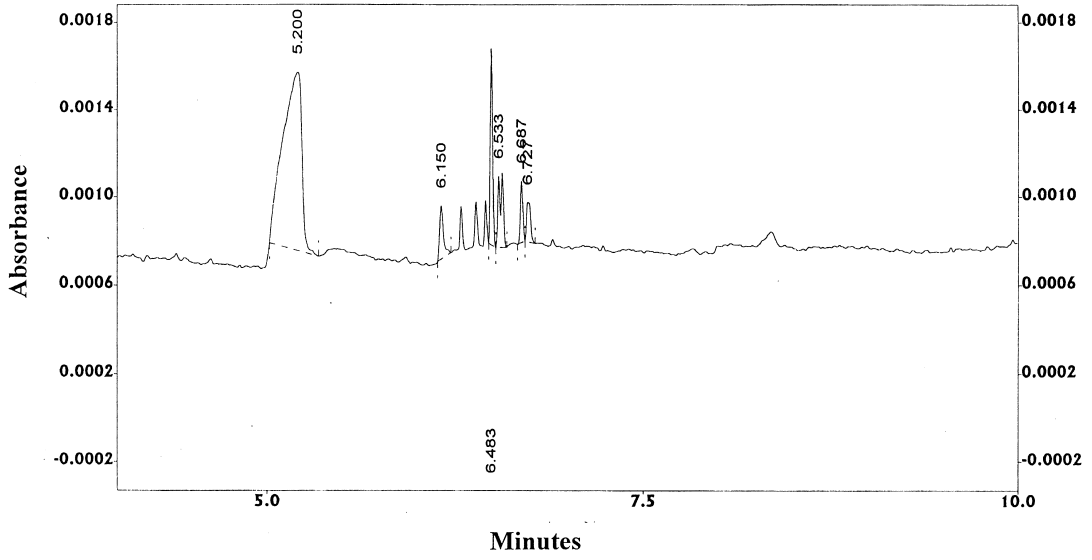

Fig. 5. Electrokinetic chromatogram of water extract of *Ganoderma* brand B capsules.

manufacturers of brand B capsules have processed and extracted the *Ganoderma* in such a way as to enhance other bioactive effects.

4. Conclusions

In conclusion, the presently developed MEKC method for determination of the selected base and nucleoside markers in commercially available forms of the traditional Chinese medicine *Ganoderma* appears to be sensitive, selective and accurate. Furthermore, the extraction method for actual samples was relatively simple and efficient. A great deal more method validation work is necessary, with sample purification and enrichment prior to analysis certainly being possibilities for further improvement of methodology. However, the overall aim of this investigation was to successfully demonstrate the application of an as yet untried analytical technique – MEKC – to *Ganoderma* preparations. The time of having a complete quantitative profile of *Ganoderma* or any other traditional Chinese medicine is certainly a long way off due to the incredibly complex nature of such products. Hopefully the production of more data will eventually lead to some form of quality

Table 2
Quantity of the markers determined in water extracts *Ganoderma lucidum*, *Ganoderma japonicum* and *Ganoderma* capsules[a]

Type of *Ganoderma*	Uracil concentration (μg/g)	Adenosine concentration (μg/g)	Uridine concentration (μg/g)
Lucidum	18.7	4.65	ND
Japonicum	11.5	9.12	7.91
Brand A capsules	1950	523	801
Brand B capsules	ND	ND	ND

[a] Experimental conditions: 80 m*M* borate, 25 m*M* SDS buffer at pH 9.0, 22 kV operating voltage, temperature 20°C, 5-s hydrodynamic injection.

ND, Not detected.

criteria being specified for some or all of the pharmacologically active components in *Ganoderma* formulations.

Acknowledgements

The authors would like to acknowledge the financial support of Vida Laboratories Ltd. (Hong Kong SAR, China) to D.J.H. The financial support from the Pharmaceutical and Chemical Technology Centre Ltd. as well as the support from the Innovation and Technology Commission are both acknowledged for the Biotechnology internship and training of C.W.N.

References

[1] C. Jinghua, Z. Jinping, L. Zhnag, Y. Nakamura, T. Norisuye, Polym. J. 30 (1998) 838.
[2] Y.C. Fan, B.L. Chun, Process Biochem. 33 (1998) 547.
[3] A. Shimizu, T. Yano, Y. Saito, Y. Inada, Chem. Pharmacol. Bull. 33 (1985) 3012.
[4] J.G. Yu, Y.F. Zhai, Yao Hseuh Hseuh Pao 14 (1979) 374.
[5] Y. Naio, Z. Kasai, Sentankai Kenkyu KoKoKu 2 (1984) 1.
[6] C.Y. Wang, H.Y. Huang, K.L. Kuo, Y.Z. Hsieh, J. Chromatogr. A 802 (1998) 225.
[7] J. Yang, H. Long, H. Liu, A. Huang, Y. Sun, J. Chromatogr. A 811 (1998) 274.
[8] Y.R. Ku, Y.S. Chang, K.C. Wen, L.K. Ho, J. Chromatogr. A 848 (1999) 537.
[9] T. You, X. Yang, E. Wang, Anal. Chim. Acta 401 (1999) 29.
[10] M.L. Ling, J. Chromatogr. A 673 (1993) 219.
[11] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.