ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 437–451

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

A method for measuring potency of narasin extracts using near-IR spectroscopy

Daniel L. Morris Jr [a], Robert A. Forbes [b,*]

[a] *Rose–Hulman Institute of Technology, 5500 Wabash Avenue, Terre Haute, IN 47803-3999, USA*
[b] *Lilly Research Laboratories, A Division Of Eli Lilly and Company, Indianapolis, IN 46285, USA*

Received 12 April 2000; received in revised form 27 July 2000; accepted 27 July 2000

Abstract

We present a method for extracting the active component from granulated narasin samples using chloroform with subsequent quantitation by near-infrared absorption spectroscopy (NIRS). A multiple linear regression (MLR) calibration equation was developed using a set of 41 calibration samples. The potencies obtained using NIR analysis exhibit no larger than an 8% (3.03 mg/g) error when compared to results based on the primary HPLC reference method. We estimate the detection limit using this method to be 400 ppm narasin (20 mg/g potency), and the standard deviation for five independent extractions of the same sample is ≈ 24 ppm (≈ 1.2 mg/g potency or approximately 1%). We also present the results from a robustness study based upon a full factorial experimental design in which we varied extraction and measurement parameters. This study indicates that sample mass causes the most variation in the results. Bottle-to-bottle variations in the chloroform used for the extraction also proved significant. Variations in sample batch, number of spectral scans, and the interactions between *sample batch*soneration time*, *no. Scans*time in NIR*, and *sample batch*sample mass* were borderline significant. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Transmission near infrared (NIR) spectroscopy; Analytical method validation; Narasin; Multiple linear regression (MLR); Pharmaceutical analysis; Ionophore; Chemometrics

1. Introduction

Near-infrared (NIR) spectroscopy is emerging as a useful and versatile analysis technique. Its applications to materials analysis, especially pharmaceuticals, is ubiquitous [1–4]. Although somewhat limited, the selectivity of NIR spectra often allows detection of specific compounds in complicated matrices without a prior separation step, thereby making it a possible alternative to chromatographic assays [5]. NIR absorptions are attributed mainly to overtones and combinations of mid-IR vibrational bands involving N–H, O–H, and C–H bonds in molecules [6]. The strong signals from O–H groups make it a useful technique for identifying water [7] and alcohol [8] in samples as well as differentiating hydrated from anhydrous crystalline forms of a compound [9]. NIR spectroscopy can also be used to distinguish

* Corresponding author. Tel.: +1-765-8324394; fax: +1-765-8324799.

0731-7085/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(00)00469-6

amongst primary, secondary, and tertiary amines [10]. In general, NIR spectroscopy lends itself well to acquiring qualitative information about analytes that may not absorb in the visible or ultraviolet regions of the electromagnetic spectrum.

NIR spectroscopy can be employed for quantitative sample analysis. However, NIR spectroscopy is generally employed as a secondary method, requiring calibration against a primary method. Development of this calibration is not always straightforward. Robust calibration requires incorporation of many standards representative of the samples to be analyzed. It can take time to acquire enough representative standards for incorporation in a calibration set. Also, building the calibration methods and equations is not trivial. It is often necessary to verify that a particular band is specific to a given analyte, and calibration equations must often take into account matrix effects. However, advancements in instrument design, computers, and operating/analysis software have made NIR analysis a favorable technique for obtaining quantitative information for complex samples.

NIR measurements are relatively fast and require minimal, if any, sample preparation or pretreatment. For instance, it is possible to monitor water content and active components in pharmaceuticals directly through packaging materials [11]. Powder samples can also be analyzed directly with little or no sample preparation using reflectance measurements. Obviously, these qualities make NIR spectroscopy very valuable in process monitoring and/or quality-control [12–16].

However, there are problems associated with performing NIR measurements directly on solid samples. One specific problem with the application of NIR reflectance measurements to solid powders is non-uniform particle sizes. Small particles attenuate incoming light more than large particles, thereby introducing a positive systematic error in reported results. Segregation of small and large particles within a solid sample also affects the reproducibility of such measurements. One way of eliminating the non-uniformity of particle sizes in solids is to mill samples prior to NIR analysis. However, sample to sample differences in initial particle size along with non-uniform feed rates into the mill can lead to variation in particle size of the milled sample. Also, the time required for mill clean-up between samples decreases throughput. Many different chemometric methods, such as standard normal variate (SNV) or multiplicative scatter correction (MSC), have been developed to reduce the impact of variation in sample particle size, but these techniques may not completely eliminate the problem [17].

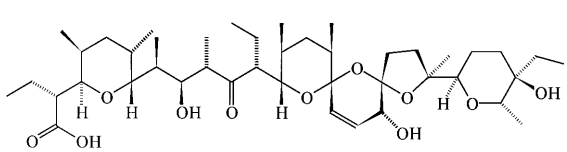

Fig. 1. Structure of narasin.

A viable alternative to reflectance analysis is to dissolve the sample in a solvent, and perform a NIR absorption measurement in a liquid sample cell. This eliminates the particle size problem entirely. This approach has not been commonly employed with NIR, perhaps due to its rather low sensitivity, although this should not be a problem for quantitation of the major component in a potency determination, provided the analyte exhibits reasonable solubility.

The active ingredient in granulated narasin is a polyether antibiotic produced by fermentation of a strain of *Streptomyces aureofaciens* [18]. In the case of the granulated narasin samples, the active ingredient must be extracted since the entire sample is not soluble. Also note that narasin (Fig. 1) does not possess a chromophore. Therefore, direct absorption measurements of narasin content cannot be performed in the UV or visible regions without prior labeling or derivitization. However, narasin lends itself well to NIR absorption. Provided that the extraction step does not require an inordinately long period of time, it is still possible to take advantage of the short measurement times that NIR spectroscopy offers. We present a method for extracting the active narasin component from granulated samples followed by potency measurements using NIR absorption spectroscopy. The NIR results are validated against lab values acquired using an established primary HPLC method.

2. Experimental

2.1. Instrumentation

NIR spectra were acquired using a Foss NIRSystems (Silver Spring, MD) model 6500 grating instrument equipped with a Multimode™ liquid/solid sample accessory with a transmission detector. All measurements were performed using a 2 mm path-length cuvette (Foss NIRSystems, Cat #: NR-7063-2) at a temperature of 37°C. Spectra were acquired over the 400–2500 nm range with the NSAS™ version 3.52 software provided with the instrument. A total of 32 scans were averaged for each spectrum. Prior to calibration development, the spectra were pre-processed with NSAS by conversion to the second derivative, utilizing a three point digital smoothing (moving average with a *gap* of zero and a *segment* size of 6 nm). The resulting second derivative spectra of the absorbance values exhibited both positive and negative "bands" related to the concentration of the NIR active components in the samples.

2.2. Calculations

During development, the performance of a given calibration model with respect to fitting the calibration spectra was assessed by the statistical measures provided by NSAS [19], the correlation coefficient (*R*, or *multiple R* when more than one wavelength term is utilized in the calibration) and the standard error of calibration (*SEC*) given by:

$$R = [\sum(Y_{\mathrm{NIRS}} - \overline{Y})^2 / \sum(Y_{\mathrm{REF}} - \overline{Y})^2]^{1/2} \quad (1)$$

and:

$$SEC = [\sum(Y_{\mathrm{NIRS}} - Y_{\mathrm{REF}})^2 / (n - m - 1)]^{1/2} \quad (2)$$

And similarly, the performance of the calibration models with respect to fitting the validation set spectra was assessed the standard error of prediction (*SEP*) given by:

$$SEP = [\sum(Y_{\mathrm{NIRS}} - Y_{\mathrm{REF}})^2 / (n - 1)]^{1/2} \quad (3)$$

where: Y_{NIRS} = NIRS predicted result for the sample, Y_{REF} = reference method result for the sample, $\overline{Y}$ = average of reference method results for samples, n = number of samples, m = number of independent variables.

In addition to the calculations provided with the NSAS software, the software package, JMP™ version 3.2.2 (Copyright ©1989–1997, SAS Institute Inc.) for Windows 95™ was utilized to perform other standard statistical analyses.

To establish the calibration curves to quantify narasin from the NIR spectra, a stepwise multiple linear regression (MLR) approach was utilized [20]. A variant of this approach, suggested by the instrument vendor, was to utilize a ratio of two wavelengths as each term for the MLR equation [21]. With this approach, the wavelength (λ_1) with the highest correlation to the analyte, while considering the spectral noise (sensitivity parameter), was selected as the numerator. During the next iteration, an optimal denominator (λ_2) for the first wavelength was sought. Selection of further wavelengths and/or ratios was continued until the improvements to the model were considered to be minimal. The general form of the calibration equation using this approach was:

$$c = a0 + a1\ \frac{\lambda 1}{\lambda 2} + a2\ \frac{\lambda 3}{\lambda 4} + a3\ \frac{\lambda 5}{\lambda 6}\ + \ldots \quad (4)$$

where a_0 was the intercept, and a_1, a_2, and a_3 were the constants resulting from the linear regression correlation to the lab reference method results, and λ_1 symbolizes the absorbance at the first wavelength. In addition to automatic selection of wavelengths, calibrations were developed by constraining terms to known absorbance peaks.

2.3. Materials and extraction method

Eli Lilly and Company provided samples of granulated narasin and a crystalline narasin reference standard. Granulated narasin samples (0.5 g) were weighed to the nearest 1 mg on an analytical balance (Mettler, model AE200) and transferred to 25 ml volumetric flasks. Approximately 20 ml of ACS grade chloroform (Mallinckrodt, [67-66-3], stabilized with 0.5–1% ethanol) was added to each flask. The flasks were stoppered and soner-

ated in an ultrasonicator for 20 min. Samples were allowed to cool for approximately 5 min and diluted to volume with chloroform. Immediately following dilution, the extracted samples were filtered through syringe filters (Gelman, 0.45 μm pore size, 20 mm diameter). The samples were placed into the cuvette immediately after filtering in order to minimize chloroform evaporation upon standing. Samples were allowed to sit it the sample cell holder for approximately 1 min in order to reach 37°C prior to acquisition of spectra.

2.4. Laboratory reference method

Granulated narasin samples were tested for narasin potency versus a crystalline reference standard with a high performance liquid chromatography method which utilized post-column derivatization with vanillin reagent (HPLC/PCD) [22]. This method was the laboratory reference method for analysis of samples employed as standards for NIRS calibration development.

3. Results and discussion

3.1. Choice of solvent

This particular assay requires a solvent that would allow: (1) efficient extraction of narasin from granulated samples; and (2) analysis of NIR bands arising from narasin. Most organic solvents are not quiet in the NIR region of the spectrum because they contain C–H bonds. The only common NIR solvents that do not exhibit any strong absorptions in the wavelength range from 1000 to 3000 nm are carbon tetrachloride and carbon disulfide. The hazards involved in working with these solvents make them undesirable for routine daily NIR analyses. Water also gives strong bands in the NIR region (2760, 2700, 1900, and 1390 nm). These factors, combined with the requirement of narasin solubility, limit the choice of available solvents. Narasin was found to exhibit acceptable solubility in chloroform, making this solvent appropriate for the extraction process. While chloroform also exhibits health hazards, they can be minimized by working in a hood and exercising appropriate safety measures.

3.2. Validation of selectivity

In order to determine if chloroform would obscure the NIR bands from narasin, we obtained the NIR spectrum of narasin reference standard dissolved in chloroform (2 ppt concentration) and subtracted the chloroform background. Comparison of the NIR spectra of the narasin standard (with chloroform subtracted) with neat chloroform in Fig. 2 shows that chloroform does not interfere substantially with *all* of the bands indicative of narasin in the 2200–2350 nm region. (The chloroform spectrum has been scaled down to 1/100 of its actual intensity for the sake of comparison.) While chloroform exhibits a very intense band at approximately 2350 nm, there are still two reasonably intense narasin bands visible at 2275 and 2300 nm. (Subtraction of the strong chloroform band at 2350 nm gives rise to the noise observed at 2350 nm in the narasin spectrum.) In Fig. 3A, we compare the NIR absorption spectrum of chloroform with a granulated narasin sample that has been extracted into chloroform. The chloroform background has not been subtracted from the spectrum of the extract. Acceptable calibration models could be developed from primary, first derivative and second derivative preprocessing treatments. We chose to work with second derivative spectra since this treatment optimally enhanced the spectral differences between the solvent and the extract, as shown in Fig. 3B. While chloroform exhibits absorption bands at 2275 and 2300 nm, the bands arising from narasin are still visible as they 'ride' on top of the solvent bands.

3.3. Development of a calibration model

As mentioned earlier, one of the disadvantages of quantitative analysis using NIR spectroscopy is the significant time involved in developing a calibration model. In this study, a calibration equation was developed using crystalline narasin reference standard dissolved in chloroform. Five standards were prepared over the 2–36 ppt con-

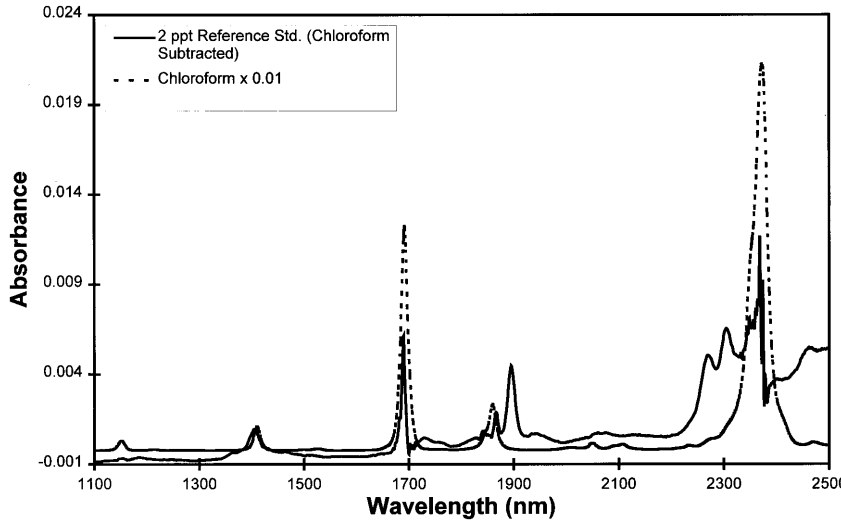

Fig. 2. NIR Spectra of 2 ppt narasin reference standard (with chloroform subtracted) and chloroform (dotted line, scaled by a factor of 0.01).

centration range. The second derivative spectra of these standards over the 2200–2325 nm region are presented in Fig. 4. A calibration curve was developed based on one wavelength (the C–H combination band at 2270 nm). The resulting calibration curve is presented in Fig. 5. The multiple R value for the fit is one with a standard error of $\pm 4.95 \times 10^{-5}$ ppt. The non-zero intercept is explained by the absorbance contribution from the chloroform/ethanol.

Granulated narasin samples differ from the narasin reference standard used for developing the calibration model. Typical narasin content of the granulated samples is around 10% by mass with the sample matrix containing significant amounts of oil, inorganics and mycelial matter. We plot the second derivative NIR absorption spectra of chloroform, narasin reference standard in chloroform, and varying amounts of an oil reference sample added to chloroform in Fig. 6. One can observe the increased contribution that the oil makes to the 2350 nm band intensity. It also appears that the intense 2350 nm band from the oil makes a small contribution to the 2270 nm band used in the previously described calibration curve (Fig. 5).

In order to examine possible matrix effects, we spiked a laboratory prepared matrix blank with narasin reference standard, followed by extraction with chloroform. These results were compared to a granulated narasin sample that was also spiked with narasin reference standard and extracted using chloroform. We plotted the second derivative of the 2270 nm band intensity as a function of the concentration of added narasin reference standard for both the matrix blank and the granulated narasin sample in Fig. 7. We have also included the calibration curve based upon the pure reference standard for comparison. Note that the slopes of all three plots were identical, and that the curve for the spiked matrix blank overlapped the calibration curve. The curve for the spiked narasin sample was offset, presumably due to the original narasin content of the sample (150 mg/g). These plots seemed to indicate that the sample matrix did not interfere in the analysis of narasin.

However, when a set of extracted narasin samples was compared to the calibration curve constructed using the narasin reference standard, the results for the extracted samples were on the order of 100% too high. This discrepancy could be explained by the fact that the matrix blank used in this study was prepared by removing the narasin from a typical sample with a methanol/water mixture. The oil component of the matrix was also removed by the methanol/water extraction. Therefore, while it appeared that the

methanol insoluble portion of the sample did not contribute a significant matrix effect, the results from Fig. 6 indicated that the oil did indeed made a contribution, and that the offset of the spiked narasin samples in Fig. 7 must also be due to the oil in the matrix. For this reason, it was concluded that the calibration model must be based on granulated narasin samples in order to compensate for the oil in the sample matrix.

We also noticed that as a given bottle of chloroform aged, the results obtained for extracted samples became consistently lower. Upon examination, it was determined that the NIR spectrum of chloroform changed with time. Fig. 8 shows the change in the intensity of the 2270 nm band from chloroform with time. Note that we observed no difference between chloroform samples that came from different bottles opened within 24 h of one another (Fig. 8, Bottle 1 and Bottle 2). However, the spectra exhibited a clear change as a given chloroform sample (the newer bottle in this case) is allowed to sit open in a hood from 15 min to 1 h. The effect was directional, repeatable, and much larger than could be explained by instrument drift. Evaporation of chloroform itself would not account for this change with time. However, chloroform has been stabilized with ethanol (0.5–1% by volume). Fig. 9 contains NIR spectra of chloroform, a narasin sample extract in chloroform, and absolute ethanol (multiplied by a

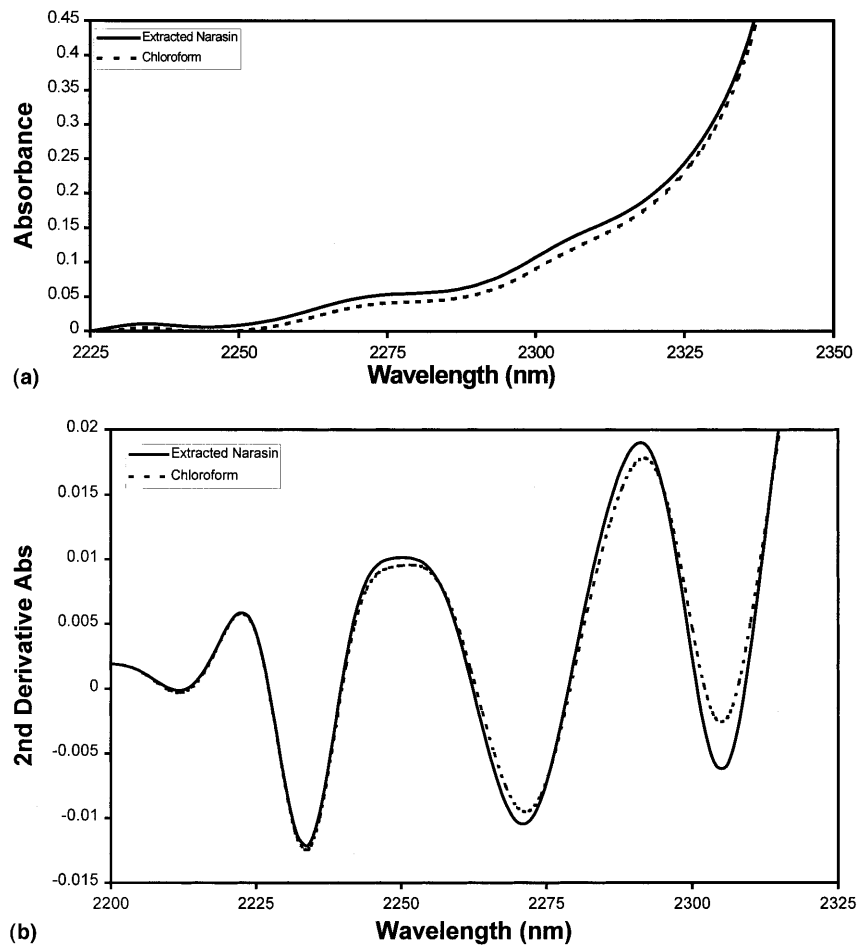

Fig. 3. (A) NIR absorption spectra of narasin extract and chloroform. (B) Second derivative absorption spectra of narasin extract and chloroform.

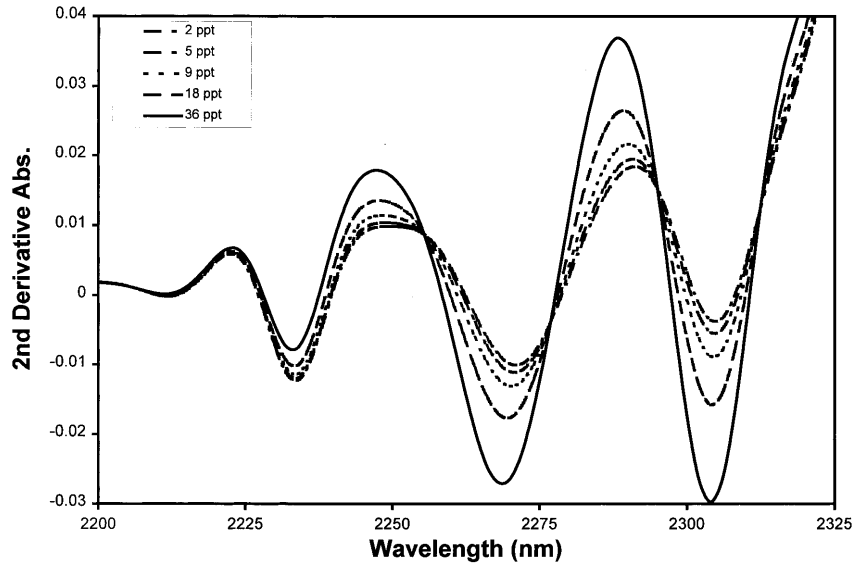

Fig. 4. Second derivative NIR spectra of narasin reference standards in chloroform.

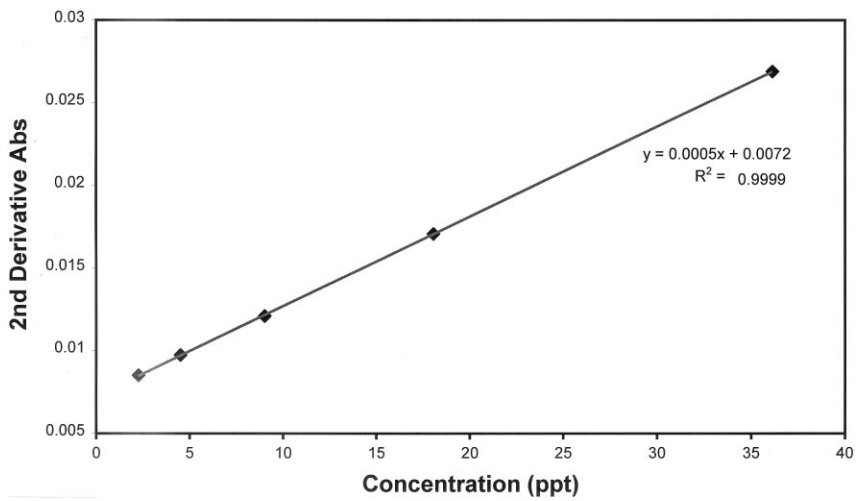

Fig. 5. Calibration curve from second derivative absorption spectra in Fig. 4 (MLR calibration model employing only the 2270 nm narasin band).

factor of 0.1 for scaling purposes). It was not surprising that ethanol has a very strong band around 2270 nm due its methylene group. Given the intensity of this band in absolute ethanol, one would expect this band to be visible even at a concentration of 0.5–1% in chloroform. We suggest that evaporation of ethanol stabilizer from chloroform caused the intensity changes in the 2270 nm band. Chloroform and ethanol form an azeotrope that exhibited a positive deviation from Raoult's Law when the composition was 93.10/6.90% chloroform/ethanol by weight [23]. The mass percentage of ethanol in commercially available chloroform was about 0.8%. At this composition, one component will evaporate preferentially over the other. Our data suggest that the more

volatile component was ethanol. Given the intensity of the NIR band from ethanol at 2270 nm, even a small change in the ethanol content of the chloroform will change the intensity of this band noticeably. Therefore, we suggest that as a chloroform sample ages, small amounts of ethanol evaporate from the solution, causing the 2270 nm band to decrease in intensity. Since the narasin band "rides" on top of this background, the results for the extracted narasin samples read consistently low. Krikorian described a procedure for removal of the ethanol stabilizer using molecular sieves [24]. However, as an alternative, we attempted to take this effect into account as we assembled the calibration set and developed the calibration model.

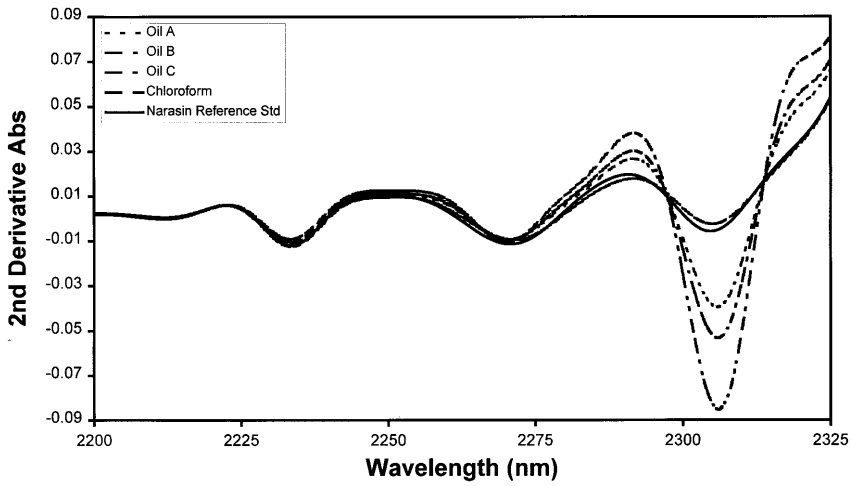

Fig. 6. Second derivative absorption spectra of narasin reference standard in chloroform, chloroform, and chloroform to containing varying amounts of oil (concentration of sample c > sample b > sample a).

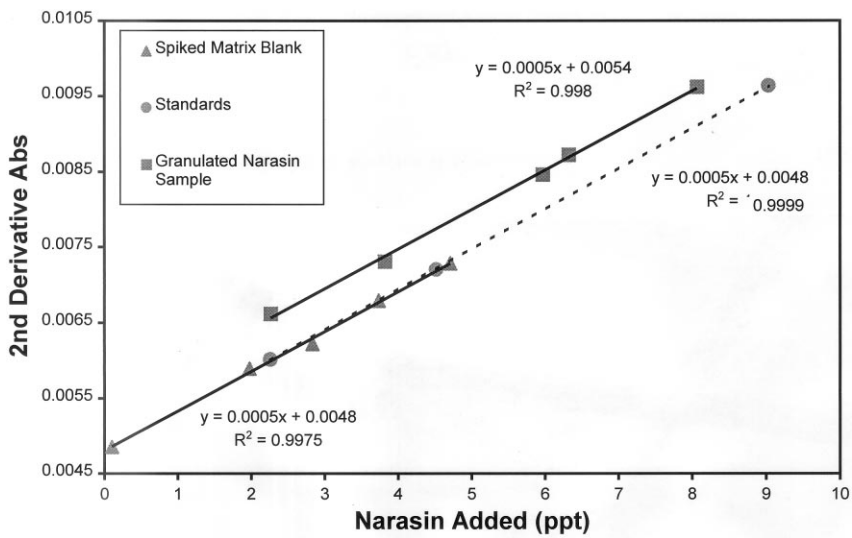

Fig. 7. Matrix effect study performed by spiking matrix blank (triangles) and a narasin sample (squares) with narasin reference. Shown for comparison is the calibration using narasin reference in chloroform. The second derivative absorbance of the 2270 nm band is plotted.

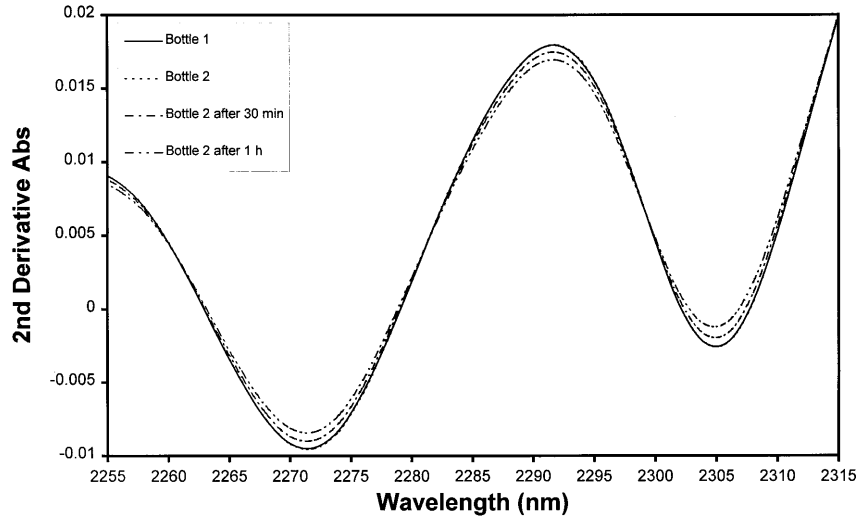

Fig. 8. Spectral changes in chloroform as a function of time

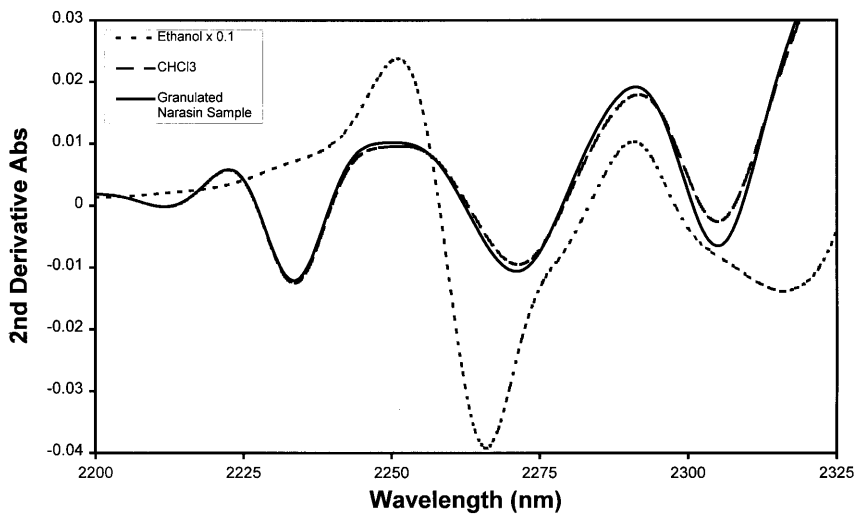

Fig. 9. Second derivative absorption spectra of extracted marasin, chloroform, and absolute ethanol ($\times$ 0.1).

In an effort to assemble a set of calibration samples representative of a wide range of variation in matrix (especially oil content) and narasin percentage, granulated narasin samples with compositions ranging from 12 to 15% by mass, and ranging in age from days to 3 months, were selected. The 41 different samples chosen for the calibration set were extracted as described in the experimental section. In order to extend the range of the calibration curve, we also performed the extraction procedure on varying sample masses (0.1, 0.2, 0.3, 0.4 and 0.5 g) taken from two of the calibration samples. The total number of data points included in the calibration curve was 50. The extractions were performed on several different days using different bottles of chloroform. Ten of these calibration points were obtained by extracting the samples with chloroform that had been allowed to sit open to the atmosphere for about 24 h. This was done in an attempt to build

robustness into the calibration model to compensate for the change in the 2270 nm band (believed to arise from ethanol) with time.

We used simple MLR analysis to develop a calibration model based on the second derivative spectra of the calibration set. In Table 1, we have compared several of the calibration models developed. The calibration equation that provided the best fit to the calibration and the validation spectra was the linear combination of the ratio of the 2456 and 1760 nm bands, the ratio of the 2260 and 2038 nm bands and the intensity of the 2230 nm band. The equation, complete with coefficients, was:

$$1.5(2456\ \text{nm}/1760\ \text{nm}) - 28.0(2260\ \text{nm}/2038\ \text{nm}) + 1352.6(2230\ \text{nm})$$

The multiple R value for the fit to the calibration samples was 0.9961 with a standard error of ± 0.0572 ppt (or ± 3 mg/g potency). The band at 2456 nm appeared to arise from a relatively weak narasin band while the 1760 nm band appeared to exhibit contributions from both narasin and oil in the sample matrix. The 2260 nm band appeared just to the blue of the 2270 nm band, which yielded the excellent calibration curve using the crystalline reference standard (Fig. 5). Referring to the spectrum of absolute ethanol in Fig. 9, one can see that the NIR band due to ethanol was shifted slightly to the blue of the 2270 nm narasin band. Therefore, we suggest that the 2260 nm wavelength accounted for the variation in the chloroform spectrum due to evaporation of the ethanol and its subsequent contribution to the narasin sample spectrum. The 2038 nm band also appeared to arise from narasin while the 2230 nm band appeared in chloroform, narasin, and oil (Fig. 6). We note that the calibration equation weighted the 2230 nm band heaviest with less emphasis on the 2260 nm/2038 nm ratio. The least heavily weighted term was the 2456 nm/1760 nm ratio.

Table 1
Comparison of different calibration models for quantitation of narasin in granulated samples using NIRS

Calibration model	Calibration set	
	Multiple R	SEC
2456 nm + 2260 nm	0.9924	0.0783
2456 nm/1760 nm	0.9889	0.0938
2456 nm + 2260 nm + 2228 nm	0.9956	0.0603
2456 nm + 2260 nm + 2228 nm + 1762 nm	0.9966	0.0535
2456 nm/1760 nm + 2260 nm/2038 nm + 2230 nm	0.9961	0.0572
2268 nm + 2454 nm/2284 nm	0.9937	0.0715
2268 nm + 2456 nm/1658 nm + 2260 nm	0.9923	0.0800
2268 nm/2304 nm + 2456 nm/2304 nm	0.9575	0.184
2456 nm + 2268 nm/2282 nm	0.9884	0.0971

3.4. Validation of accuracy

A set of 18 granulated narasin samples, which were not part of the calibration set, was chosen in order to test the accuracy of the calibration model. The accuracy samples encompassed a concentration range of 12–15% narasin by mass and were believed to represent differences in the matrix (especially oil content). The accuracy samples were extracted according to the procedure outlined in the experimental section. Multiple bottles of chloroform were utilized during extraction of the accuracy samples. A comparison of the values reported by the NIR method with the laboratory reference method values has been presented in Fig. 10 where we plotted the NIR values of potency vs the laboratory reference values. The correlation coefficient (adjusted R^2) for the fit was 0.807 with a $+0.935$ mg/g bias in the NIR results when compared to the HPLC laboratory values. The smallest percentage error between the NIR values and the laboratory reference values in this accuracy set was 0.5%. The largest percentage error between the NIR values and reference values of narasin content was 8%. The vast majority (15 out of 18) of the NIR potencies was low when compared to the reference potencies. It is important to note that the reference potencies were experimentally determined, contributing measurement variability on the order of 2% COV. The mean difference between the NIR values and the laboratory reference values was 3.03 mg/g. This difference was statistically significant at the 95%

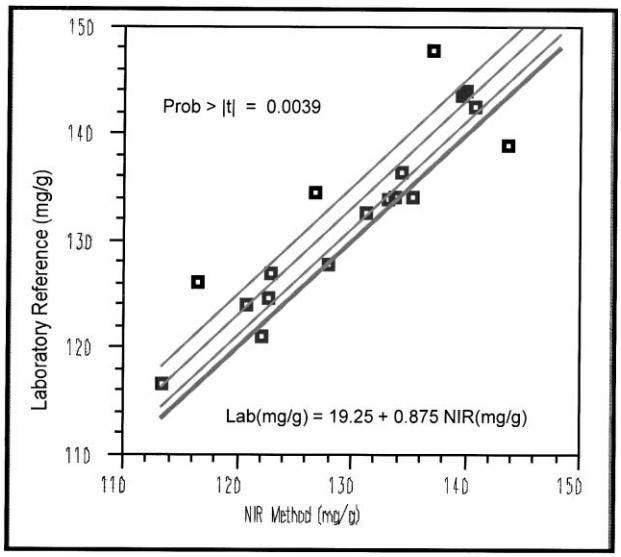

Fig. 10. Paired Student's t-test performed on narasin potency assay results for laboratory reference method vs the NIR method for 18 accuracy samples. Note that the expected fit (dark solid line) falls outside the 95% confidence interval drawn around the best-fit line (three lighter solid lines).

confidence ($\alpha = 0.05$) level. This result underscores the importance of validation of method accuracy with an independent set of samples prior to routine use of the method.

3.5. Precision and limit of detection

Five 0.5 g portions of a specific accuracy sample, with a reference potency of about 125 mg/g, were extracted and analyzed in order to test the precision of this method. The average NIR value returned for the potency of this sample was 129.2 mg/g (3% error relative to the HPLC value) with a standard deviation of ± 1.2 mg/g. The detection limit of this method was estimated by choosing another accuracy sample, with about 135 mg/g potency, extracting 0.5 g into 25 ml of chloroform, and performing sequential 10–25 ml dilutions on the extract. The spectra of these diluted samples (over the 2260 to 2280 nm region) have been presented in Fig. 11. Note that there was little discernible difference in intensity between the spectrum of the 200 ppm extract and that of chloroform. Therefore, we have reported a limit of detection of 400 ppm (20 mg/g) narasin for this method. At the estimated detection limit, the calibration equation reported a potency value of 135.2 mg/g (when corrected for dilution).

3.6. Validation of ruggedness

In order to determine the parameter(s) that has the greatest effect(s) on this particular assay, we performed a robustness study using a two level,

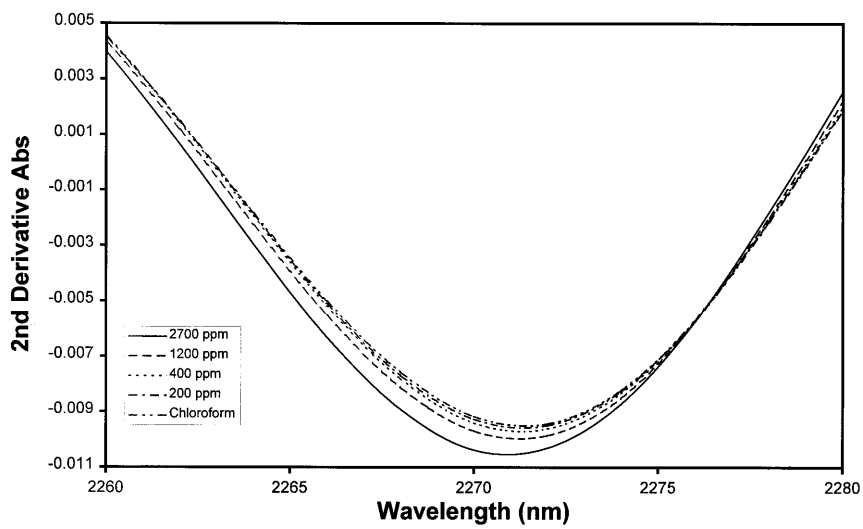

Fig. 11. Detection limit study.

Table 2
Results of components of variance analysis of results obtained in the factorial experimental design for narasin potency by NIRS

Parameter	Estimate	Sum of squares	Prob>|t|
Sample mass	+8.2	2125.5	<0.0001
$CHCl_3$ bottle	−1.1	41.0	0.0439
Sample batch*soneration time	−1.1	36.6	0.0545
No. scans*time in NIR	+1.1	36.1	0.0557
Sample batch*sample mass	−1.0	32.0	0.0689
Sample batch	+0.9	28.5	0.0834
No. scans	−0.9	28.5	0.0834
Time in NIR	+0.4	5.4	0.4203
Soneration time	+0.1	0.5	0.8137

full factorial experiment. We examined the significance of variations in sample batches, sample masses, chloroform bottles, soneration times, number of spectral scans, and time spent in the thermostatted sample cell holder prior to acquiring spectra for the NIR potency values. The robustness study was performed on two sample batches with reference potencies of about 132 mg/g. The parameter of *sample mass* was set at either 0.25 or 1 g (values that are to either side of the 0.5 g used for all other extractions) and extracted into 25 ml of chloroform. The other parameters studied were the particular *chloroform bottle* (old vs new), *soneration time* (10 vs 30 min), *number of spectral scans* (16 vs 64), and the *time in the NIR* before spectra were acquired (1 vs 10 min). The variables of *sample mass* and *soneration time* were thought to be important since they should be directly related to efficiency of extraction of the narasin from the samples. The bottle from which chloroform was taken was an important parameter to study based on the spectral changes exhibited by chloroform with time. The number of spectral scans was varied to determine if spectral noise had a significant effect on the results since the NIR signal due to the extracted narasin was relatively weak ($\sim$0.1 absorbance unit for the 2270 nm band in a typical extract). The time spent in the thermostatted sample cell holder was studied in order to determine if significant evaporation of the chloroform took place at the 37°C temperature.

The ANOVA (Analysis of Variance) results from the robustness study have been presented in Table 2 for selected parameters (and combinations thereof) that appeared to produce statistically significant variations in the data at the 95% confidence level. The R^2 adjusted value for the ANOVA was 0.904. From Table 2, it was clear that differences in sample mass produced significant variation in the results (see Fig. 12A). We attributed this difference in precision to the fact that lower sample masses produced more dilute solutions, decreasing the signal-to-noise ratio. The mean for the 0.25 g samples was biased low, since the absorbance due to narasin was less discernible above the background (chemical noise due to chloroform/ethanol) than the higher concentrations. This reduced signal-to-noise ratio also resulted in less precise results when compared to results with the larger sample masses.

Variation in chloroform bottles (old vs new) appeared to be the next most significant parameter influencing this assay, indicating that the calibration model probably did not adequately account for the spectral changes in chloroform over time. We call the reader's attention to Fig. 12B which compares the results obtained using a bottle of chloroform that had been used for several days (-1) compared to a fresh bottle ($+1$). Note that only results from 1 g sample weights are shown since the variation in the results when using 0.25 g weights obscured this difference. Not only are the mean values of potency significantly different for the two chloroform bottles, but the variance of the results differed. The difference in precision between the chloroform bottles was initially puzzling and was not consistent with evaporation of ethanol from the chloroform since the mean potency value obtained with the old bottle was higher than that for the new bottle. One would expect this to be reversed if the old bottle contained less ethanol, due to evaporation, than the new bottle.

One explanation for the difference in variability with the different chloroform could be the way

the experiment was conducted. The robustness study was performed over a period of 2 days, blocking the experiments each day according to the chloroform bottle. On the first day, extractions of all samples were performed using only the new chloroform bottle. On day 1, however, the samples were not filtered immediately after soneration (for 10 or 30 min). The samples remained in the volumetric flasks after soneration and were filtered some hours later, just prior to NIR analysis. After day 1 was complete, we realized that allowing the samples to remain in the chloroform might extract additional narasin, masking the effect of short sonoration times. On day 2, when using chloroform from the old bottle, we adjusted the procedure. When the prescribed soneration time was completed, the samples were filtered immediately into rubber stoppered shell vials where they remained awaiting NIR analysis. Thus, the samples were not in contact with the chloroform for the same amount of time. Better precision was obtained for samples extracted on day 1, with the new chloroform bottle, since they were in contact with the chloroform for a longer, more consistent period of time. This would result in more complete and uniform extraction.

However, the more precise day 1 results were also lower, apparently contradicting more complete extraction. During day 1, the samples remained in tightly sealed volumetric flasks, minimizing evaporation of the solvent. During day 2, we observed evaporation of the solvent, indicated by swelling of the rubber stoppers, while the extracts remained in the shell vials awaiting analysis. Evaporation of the solvent while in shell vials would result in higher potencies, consistent with the results shown in Fig. 12B.

ANOVA results for the other parameters shown in Table 2 indicated borderline significance (p-values greater than 0.05 but less than 0.1) of *sample batch*, and its interactions with *sample mass* and *sonoration time*. This suggested that the true potencies of the two batches were different, and/or they behaved differently during extraction. *Number of scans* was borderline in terms of introducing significant variation. The time spent in the thermostatted sample compartment before a scan was acquired and the soneration times for the samples did not appear to introduce significant variation in the results. This indicated 10 min of sonoration was sufficient for extraction, and one minute was sufficient time for the samples to equilibrate to temperature. While the *time in NIR*

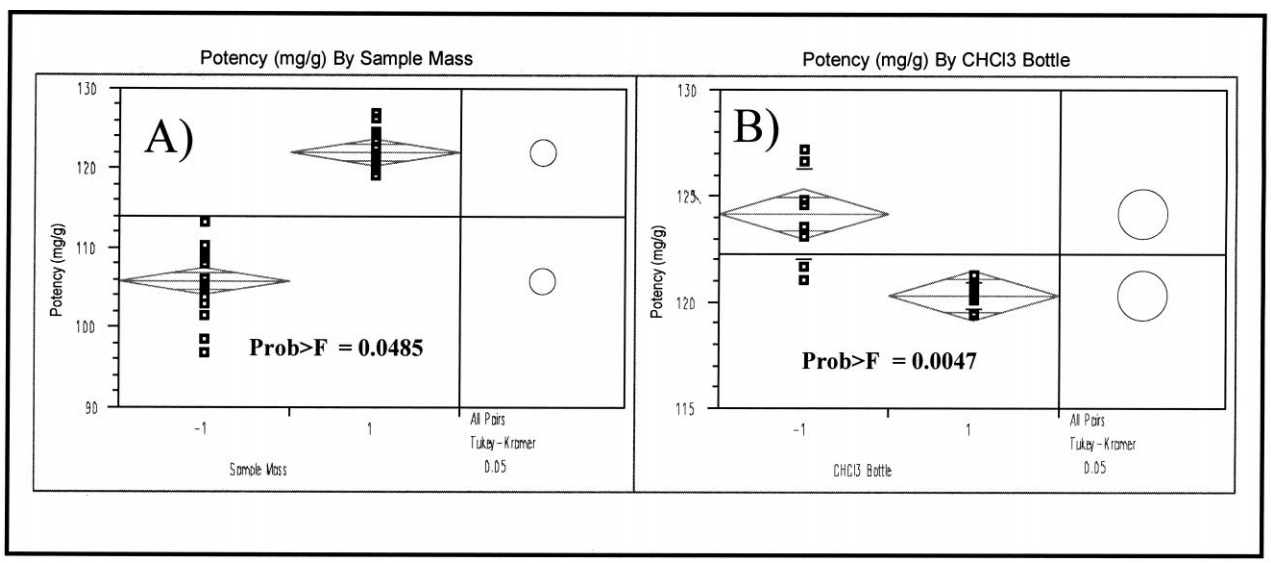

Fig. 12. Plot of potency results vs experimental conditions for robustness study. (A) All study results for 0.25 g (− 1) and 1 g (+ 1) plotted. (B) Only results obtained with 1 g sample weight plotted vs old chloroform bottle (− 1) and new chloroform bottle (+ 1).

parameter was not statistically significant by itself, the borderline significance of the *no. scans*time in NIR* interaction could be explained if one considered the case in which the sample sat in the 37°C sample holder for 10 min followed by acquisition of 64 scans. The amount of time available for evaporation of chloroform (concentrating the extract) and evaporation of ethanol from the chloroform (affecting the intensity of the 2270 nm band) would be much greater than for samples held for 1 min followed by 16 scans.

4. Conclusions

We have shown that measuring narasin potency in granulated samples using a chloroform extraction followed by quantitation with NIRS is feasible. Matrix effects (specifically those due to oil) necessitate that the calibration model be developed using granulated narasin samples instead of pure reference standard. The MLR calibration model we developed based on 41 different narasin samples had a multiple R value of 0.9961 with a standard error in the fit of 0.0572 ppt $\approx$ 3 mg/g potency). The estimated detection limit for this assay was 400 ppm (20 mg/g potency), and the precision of the method was estimated to be $\approx$ 24 ppm ($\approx$ 1.2 mg/g potency). We attributed spectral changes in the spectrum of chloroform as a function of time to evaporation of ethanol stabilizer. Attempts to account for this change using the calibration model were less than successful. Upon comparison of the NIR values of potency to the HPLC reference method values, the set of 18 accuracy samples exhibits no larger than 8% error in potencies. This translated to a mean difference of 3.03 mg/g in potency values between the NIR and laboratory values. We noted, however, that this difference was statistically significant at the 95% confidence level. The cause for the 3 mg/g bias was not well understood. It was possible the matrix of the accuracy samples differed from the calibration samples, for example, different average oil concentration. To compensate for this, a larger set of calibration samples, with a wider range in oil and/or ethanol content, could be employed to develop a different, more robust, calibration model.

ANOVA results from the robustness study indicated that sample mass was the most critical parameter to control in this method, perhaps due to working at concentrations only five times the detection limit. The next most significant parameter was bottle-to-bottle variations in chloroform, apparently due to differences in ethanol stabilizer concentration. Parameters that had borderline significance were *sample batch*, *number of spectral scans* and the interactions of *sample batch*soneration time*, *number of scans*time in NIR*, and *sample batch*sample mass*.

In order to better understand the limitations of the method, work is currently under way to perform the extraction using deuterated methanol as the solvent. We also plan to perform the extraction using chloroform from which the ethanol stabilizer has been removed according to the procedure published by Krikorian [24]. Additional solvents that may be investigated include cyclohexane and freon. Alternative calibration approaches, such as the partial least-squares (PLS) method may be investigated with the objective of developing a calibration that is more selective for narasin in the presence of the matrix/oils.

Acknowledgements

The authors wish to acknowledge R. F. Eizember supporting this project and M. Z. Luo for providing scientific review of the manuscript prior to its publication.

References

[1] W.H. Kohn, A.N. Jerger, J Forensic Sci 37 (1992) 35–41.
[2] C.I. Gerhasuuer, K.A. Kovar, Appl Spectrosc 51 (1997) 1504–1510.
[3] F. Gonzalez, R. Pous, J Pharm Biomed Anal 13 (1995) 419–423.
[4] K.B. Bradfield, R.A. Forbes, J Near Infrared Spectrosc 5 (1997) 41–65.
[5] W.F. McClure, A.M.C. Davies, Mikrochim Acta 1 (1988) 93–96.
[6] D.A. Skoog, J.F. Holler, T.A. Nieman, Principles of instrumental analysis, 5th edn, Saunders College Publ, Philadelphia, 1998, pp. 422–424 Chapter 17.
[7] E. Spinner, Anal Chem 47 (1975) 849–852.

[8] R.A. Forbes, B.M. McGarvey, D.R. Smith, Anal Chem 71 (1999) 1232–1239.
[9] W. Plugge, C. Van der Vlies, J Pharm Biomed Anal 11 (6) (1993) 435–442.
[10] R.B. Stage, J.B. Stanley, P.B. Moseley, J Am Oil Chem Soc 49 (2) (1972) 87–89.
[11] X. Zhou, P. Hines, M.W. Borer, J Pharm Biomed Anal 17 (1998) 219–225.
[12] P.L. Walling, J.M. Dabney, J Soc Cosmet Chem 39 (1988) 191–199.
[13] P. Corti, E. Dreassi, G. Ceramelli, S. Mattii, Analyst 124 (1999) 755–758.
[14] J.D. Kirsch, J.K. Drennen, Appl Spectrosc Rev 30 (1995) 139–174.
[15] E. Dreassi, G. Ceramelli, P. Corti, M. Massacesi, P.L. Perruccio, Analyst 120 (1995) 2361–2365.
[16] Z. Schmilovitch, A. Hoffman, H. Egozi, R. Ben-Zvi, Z. Bernstein, V. Alchanatis, J Sci Food Agric 79 (1999) 86–90.
[17] M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, C. de la Pezuela, Applied Spectroscopy 51 (2) (1997) 240–246.
[18] D.H. Berg, R.L. Hamill, J Antibiotics 31 (1) (1978) 1–6.
[19] Reference Manual for Near Infrared Spectral Analysis Software (NSAS), Appendix 1, 16–17, NIRSystems, Inc., Silver Spring, MD (1993).
[20] Blanco, M.; Coello, J.; Iturriaga, H.; Maspoch, S.; Rovira, E., J Pharm Biomed Anal, 16, 255-262, (1997).
[21] Reference Manual for Near Infrared Spectral Analysis Software (NSAS), Chapter 3, 18–26, NIRSystems, Inc., Silver Spring, MD (1993).
[22] J.M. Rodewald, J.W. Moran, A.L. Donoho, M.R. Coleman, J AOAC Int 77 (1994) 821–828.
[23] Z.M. Kurtyka, J Chem Eng Data 16 (3) (1971) 310–312.
[24] S.E. Krikorian, Anal Chem 48 (1976) 190–191.