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Direct determination of sorbitol and sodium glutamate by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) in the thermostabilizer employed in the production of yellow-fever vaccine

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ABSTRACT

Reference methods for quality control of vaccines usually require treatment of the samples before analysis. These procedures are expensive, time-consuming, unhealthy and require careful manipulation of the sample, making them a potential source of analytical errors. This work proposes a novel method for the quality control of thermostabilizer samples of the yellow fever vaccine employing attenuated total reflectance Fourier transform infrared spectrometry (ATR–FTIR). The main advantage of the proposed method is the possibility of direct determination of the analytes (sodium glutamate and sorbitol) without any pretreatment of the samples. Operational parameters of the FTIR technique, such as the number of accumulated scans and nominal resolution, were evaluated. The best conditions for sodium glutamate were achieved when 64 scans were accumulated using a nominal resolution of 4 cm^{-1}. The measurements for sodium glutamate were performed at 1347 cm^{-1} (baseline correction between 1322 and 1369 cm^{-1}). In the case of sorbitol, the measurements were done at 890 cm^{-1} (baseline correction between 825 and 910 cm^{-1}) using a nominal resolution of 2 cm^{-1} with 32 accumulated scans. In both cases, the quantitative variable was the band height. Recovery tests were performed in order to evaluate the accuracy of the method and recovery percentages in the range 93–106% were obtained. Also, the methods were compared with reference methods and no statistical differences were observed. The limits of detection and quantification for sodium glutamate were 0.20 and 0.62% (m/v), respectively, whereas for sorbitol they were 1 and 3.3% (m/v), respectively.

1. Introduction

The yellow fever vaccine distributed by the World Health Organization (WHO) comes from the 17D strain of the yellow fever virus. It is a lyophilized vaccine of the attenuated virus, formulated with a thermostabilizer solution composed by sodium glutamate, sorbitol and hydrolyzed gelatin [1,2].

The thermostabilizer has an important impact on the thermolability of the vaccine after its reconstitution, which significantly influences the potency, one of the most critical features of any vaccine. Furthermore, the thermostabilizer influences the cryoprotectant features. Despite the fact that the mechanism of action of these compounds is not well known, sorbitol (Fig. 1A) is primarily employed to protect the infectivity of the virus during the freeze-drying process [1], constituting a critical component of the thermostabilizer and, as a consequence, of the yellow fever vaccine. In turn, sodium glutamate (Fig. 1B) provides the protective effect of the thermostabilizer to viral suspension, i.e., it is employed to maintain the title of the vaccine [2]. Another important component of the thermostabilizer is the hydrolyzed gelatin, which improves the physical cohesion of the vaccine. This important component interferes with reconstitution of the vaccine, since the gelatin can absorb five to six times its weight in water [3]. In this scenario, it is important to highlight that the quality control of the yellow fever vaccine depends on the quality control of the thermostabilizer.

In quality control of vaccines, there is special interest in knowing the matrix of the sample (excipients) and not just the analyte. Frequently, it is necessary to know the ratio of some compounds presents as well as the way that they interact. In this sense, it is extremely important to assess the composition of pre-vaccine materials during the formulation step.

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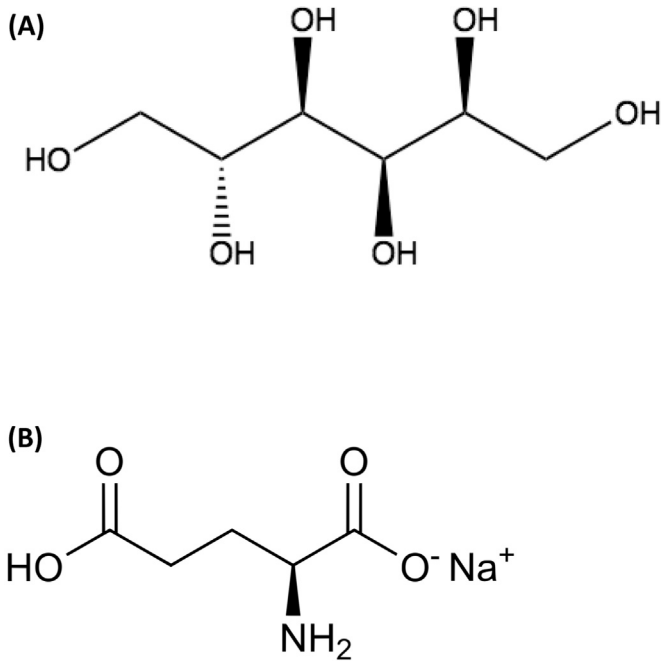

Fig. 1. Molecular structures of the substances analyzed in this work: (A) sorbitol and (B) sodium glutamate.

Reference methods for quality control of vaccines traditionally involve some pretreatment of the samples, being necessary the application of extraction, dialysis or digestion steps. Also, it is important to mention the need for using solvents, which are, in general, toxic. Therefore, these methodologies are expensive, tedious, unhealthy and generate large amounts of chemical and biological wastes. Furthermore, they are a potential source of analytical error [4].

Classically, mid-infrared spectrometry has been employed in the identification of functional groups in organic molecules. However, its use in quantitative analysis has strongly increased in the last two decades, especially after the popularization of Fourier transform-based instruments, which provide fast measurement of the whole spectrum and an advantageous signal-to-noise ratio when compared to dispersive instruments. Fourier transform infrared spectrometry (FTIR) has already been employed in the direct quantification of some components in several types of samples such as pesticide formulations [5–9], cosmetics [10,11], foodstuffs [12–14] and others [15–17]. Special attention has been given to the analysis of pharmaceutical products, especially in the quantification of their active ingredients [18–22]. To the best of our knowledge, there is no work in the literature reporting the use of FTIR for the quality control of vaccines and/or their raw materials.

The main objective of this study was to develop a simple, fast and selective method for the direct determination of sodium glutamate and sorbitol in samples of the thermostabilizer employed in the manufacture of yellow-fever vaccine using attenuated total reflectance Fourier transform infrared spectrometry (ATR–FTIR).

2. Experimental

2.1. Apparatus

A Thermo-Nicolet Fourier transform infrared spectrometer (Madison, WI, USA), model 4700, was employed for the acquisition of all spectra. It was equipped with an ECT (electronically temperature controlled) EVERGLO™ source, a KBr beam-splitter and a DTGS (deutered triglicine sulfate) detector. The attenuated total reflectance (ATR) accessory used in the measurements was equipped with a diamond crystal as the reflection element. The spectrometer was provided with a purge system (dry air) to minimize the effect of the room moisture on the measurements.

The spectra were measured by introducing the liquid sample directly onto the diamond crystal, which required only one or two drops of sample. The samples did not undergo any treatment before measurement and the background was established using deionized water.

The HPLC-based method described in the European Pharmacopoeia was employed as the reference method for the quantification of sorbitol [23]. HPLC analyses were conducted with a liquid chromatographic system from Waters, model Alliance 2695 (Milford, MA, USA). The chromatographic system was equipped with a refractory index detector, model 2414, which was maintained at 40 °C. The separation was carried out with a Sugar Pak SC1011 Shodex (300 × 8 mm, 6 mm i.d.) analytical column, at 80 °C. We still used a guard column, Sugar SC1011P Shodex Pak (50 × 6 mm, 6 mm i.d.) to avoid any damage of the analytical column. The elution was performed in isocratic mode using deionized water as eluent, which was pumped at 0.8 mL min^{-1}. The injection volume was 20 μL.

The enzymatic method was employed as the reference method in the determination of sodium glutamate. It is based on the reaction of glutamate with glutaminase, which promotes the dehydrogenation of glutamate, thus reducing NAD^+. The reaction occurs in a single step and results in the formation of α-ketoglutarate, NH_4^+ and NADH. The NADH formed was monitored spectrophotometrically at 340 nm [24].

The determination of sodium glutamate in the samples by the reference method was carried out with a commercial kit manufactured by Sigma-Aldrich (test code GLN-1). The sample was prepared as follows: 1.0 mL of sample was transferred to a 250 mL volumetric flask and the volume was completed with deionized water. We transferred an aliquot of 250 μL of the diluted solution to a test tube and added 640 μL of deionized water. The tubes were capped and stirred. Afterwards, we added 20 μL of glutaminase and stirred the tubes again. After 40 min, the absorbance at 340 nm was measured with a Hitachi model U5100 spectrophotometer (Tokyo, Japan) using a 10-mm quartz cuvette.

2.2. Reagents and materials

All solutions were prepared with deionized water obtained in a Direct-Q 3 system supplied by Millipore (Molsheim, France). The samples were diluted with deionized water before their measurement by FTIR.

For the formulation and preparation of the synthetic thermostabilizer samples, we used sodium glutamate monohydrate from Merck KGaA (Darmstadt, Germany) and analytical-grade sorbitol from Roquette Freres (Vecquemont, France).

Five synthetic samples (S_6, S_7, S_8, S_9 and S_{10}) of the thermostabilizer were prepared using a 25% (m/v) solution of hydrolyzed gelatin supplied by the Cultures and Solutions Preparation Laboratory at FIOCRUZ (Brazil). This laboratory is responsible for routine preparation of the thermostabilizer used in the preparation of the yellow fever vaccine provided to the population in Brazil. The 25% *m*/*v* solutions were diluted to 9% *m*/*v* (with deionized water), which is the final concentration of gelatin present in the real samples of thermostabilizer. Besides the mentioned synthetic samples, five real thermostabilizer samples (S_1, S_2, S_3, S_4 and S_5) were randomly selected for testing the methodology.

2.3. General procedure

The spectra of the thermostabilizer samples were measured by pipetting one or two drops of the liquid sample directly onto the ATR crystal of the FTIR spectrometer. The spectra were acquired using the operational conditions optimized in this work. Calibration curves were constructed using aqueous solutions of sodium glutamate (1–10% m/v) and sorbitol (5–25% m/v) and their spectra were measured using the same operational conditions (number of accumulated scans, nominal resolution and baseline correction range) employed in the measurement of the samples.

3. Results and discussion

The present work was developed taking into consideration three steps. In the first step, the spectra of individual components were compared with the spectrum of the thermostabilizer in order to identify possible bands to be used for quantification purposes. Once the spectral bands were chosen, the FTIR method was optimized by investigating the effect of operational parameters (nominal resolution and number of scans) on the sensitivity and selectivity of the method. Finally, the developed method was employed in the analysis of real and synthetic samples and its analytical features were determined.

3.1. Spectra of individual components and thermostabilizer

As mentioned previously, the first step of this work was to obtain the spectra of the analytes (glutamate and sorbitol) in aqueous medium and compare them with the spectrum of the thermostabilizer. The results are shown in Fig. 2. As expected, the spectra of sorbitol and sodium glutamate presented characteristic absorption bands that are also present in the spectrum of the thermostabilizer. In the case of sorbitol, the most important bands presented maximum absorption at 890, 1046, 1084 and 1411 cm^{-1}. The bands located at 1046 and 1084 cm^{-1} were attributed to C–OH stretching vibrations, whereas the bands with maxima at 890 and 1411 cm^{-1} appeared due to the in-plane and out-of-plane bending vibrations of O–H bonds, respectively [25]. The spectrum of glutamate presented characteristic bands at 1347, 1402 and 1557 cm^{-1}. The bands at 1402 and 1557 cm^{-1} were attributed to the symmetrical and asymmetrical stretching of C–O bonds in carboxilate ion, respectively, whereas the band at 1347 cm^{-1} probably occurred due to the C–N stretching [26,27]. It was also possible to note the presence of the bands for gelatin, which were more intense at 1663 and 1552 cm^{-1}. These bands were attributed to amide I and amide II, respectively, since gelatin is a mixture of peptides and proteins obtained from partial hydrolysis of collagen.

Initially, in order to evaluate the possible use of these bands for quantification purposes, calibration curves were prepared with aqueous solutions of both sodium glutamate (1–10% m/v) and sorbitol (5–25% m/v). The signals were measured in terms of area and height and the baseline was always corrected using two points under the bands, which were established in accordance with the characteristic of each band. The results are shown in Table 1.

Although excellent correlations were observed between analyte concentration and the absorbance signal for all situations tested, this parameter alone could not be used to confirm the suitability of the bands for their use for quantification, since it did not take into account the possible interference of gelatin absorption on the analyte signals or any overlapping among bands of different components. Therefore, in order to verify the suitability of each band for the quantification of sodium glutamate and sorbitol in the thermostabilizer, a synthetic sample of the thermostabilizer with known concentrations of both sodium glutamate (6.9% m/v) and sorbitol (16% m/v) was prepared and analyzed using the different selected bands. The final concentration of gelatin in the synthetic sample was 9.0% m/v, which is the exact concentration of gelatin in the real thermostabilizer samples. The analytical curve method was employed as the calibration strategy. The results obtained in the analysis of the synthetic thermostabilizer, and their respective errors (in relation to the expected value), are displayed in Table 2.

Satisfactory results were only observed when the determinations were carried out at 1347 cm^{-1} for glutamate and 890 cm^{-1} for sorbitol, and when measurements were performed in terms of band height. We believe that the use of area for quantification purposes was not suitable due to some overlapping among bands of different substances. The errors observed in the analysis of the synthetic sample were always positive, reinforcing the idea that gelatin also absorbs radiation in most of the spectral region chosen for measuring, and interferes with the quantification of sodium glutamate and sorbitol. With these results, we selected the bands

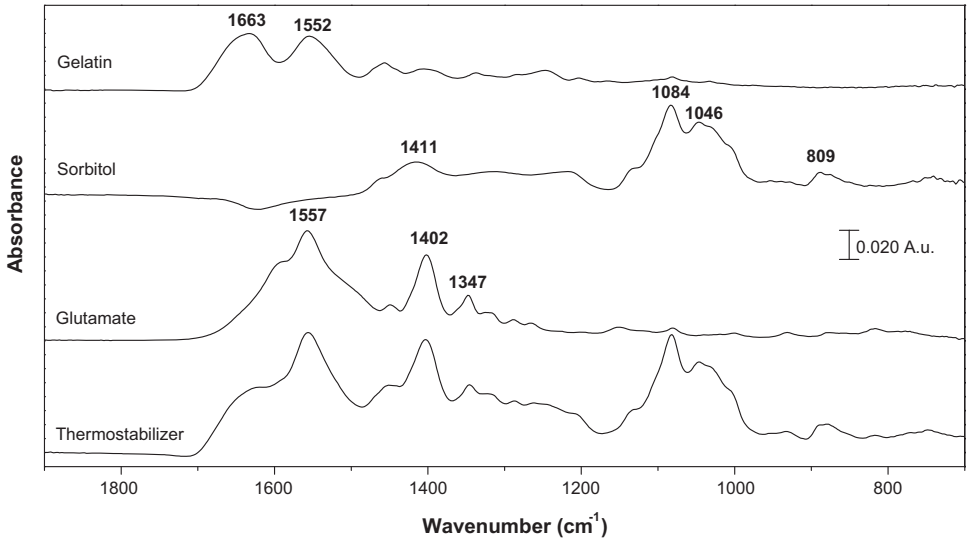

Fig. 2. Individual spectra in water, in the range of 1900–700 cm^{-1}, of all components of the thermostabilizer and the spectrum of a real sample of thermostabilizer. The spectra are drifted for better viewing. The concentration of the analytes in the solutions were 6% m/v for sodium glutamate and 15% (m/v) for sorbitol. The concentration of hydrolyzed gelatin in the solution was 9% (m/v).

Table 1
Evaluation of the analytical characteristics of different bands for the determination of sodium glutamate and sorbitol in thermostabilizer of the yellow fever vaccine.

Analyte	Measurement mode	Band at (cm^{-1})	Baseline correction (cm^{-1})	Analytical curve	r^2
Glutamate	Height	1347	1332–1371	$A=0.0014C-0.0408$	0.9999
		1402	1371–1436	$A=0.0043C-0.0199$	0.9999
		1557	1463–1723	$A=0.0067C-0.0083$	0.9999
	Area (under)	1347	1332–1371	$A=0.0235C-0.0732$	0.9999
		1402	1371–1436	$A=0.124C-0.0417$	0.9999
		1557	1463–1723	$A=0.641C-0.0231$	0.9999
Sorbitol	Height	890	838–910	$A=0.00063C-0.374$	0.9998
		1046	990–1063	$A=0.00100C-0.376$	0.9995
		1084	1062–1118	$A=0.00189C-0.024$	0.9999
		1415	1354–1492	$A=0.00102C-0.005$	0.9999
	Area (under)	890	838–910	$A=0.0212C-0.319$	0.9990
		1046	990–1063	$A=0.0550C-0.341$	0.9992
		1084	1062–1118	$A=0.0506C-0.044$	0.9999
		1415	1354–1492	$A=0.0680C-0.018$	0.9999

Table 2
Evaluation of the different bands for the determination of sodium glutamate and sorbitol in the thermostabilizer of the yellow fever vaccine. Calibration curves of sodium glutamate (1–10% *m*/*v*) and sorbitol (5–25% *m*/*v*) were employed for their quantification if the sample.

Analyte	Measurement mode	Band at (cm^{-1})	Baseline correction (cm^{-1})	Concentration of the analyte found (% *m*/*v*)	Error (%)
Sodium glutamate	Height	1347	1332–1371	7.1 ± 0.2	2.8
		1402	1371–1436	9.9 ± 0.4	44.1
		1557	1463–1723	9.7 ± 0.3	41.3
	Area (under)	1347	1332–1371	7.8 ± 0.3	13.9
		1402	1371–1436	10.7 ± 0.4	55.1
		1557	1463–1723	9.3 ± 0.4	34.3
Sorbitol	Height	890	838–910	16.0 ± 0.5	0.40
		1046	990–1063	17.6 ± 0.4	10.0
		1084	1062–1118	20.5 ± 1.9	28.0
		1415	1354–1492	31.5 ± 0.8	97.0
	Area (under)	890	838–910	19.2 ± 0.5	20.0
		1046	990–1063	18.6 ± 0.4	16.0
		1084	1062–1118	19.7 ± 2.5	23.0
		1415	1354–1492	26.9 ± 1.6	68.0

at 1347 cm^{-1} and 890 cm^{-1} for the quantification of sodium glutamate and sorbitol, respectively, using peak height as the quantitative variable.

3.2. Optimization of operational conditions

Having established suitable bands for the determination of glutamate and sorbitol in the thermostabilizer samples by FTIR, we optimized two parameters of particular importance in measurements by FTIR: (i) the nominal resolution and (ii) the number of accumulated scans. These parameters were optimized in order to perform the measurements with maximum sensitivity and in the minimum time.

The use of high values of nominal resolution (lower resolution) should be avoided because, in this situation, high data spacing is observed, which can lead to the deformation of the absorption bands. However, it must be considered that the time required to record the spectra decreases with the increase of the nominal resolution value. On the other hand, the number of accumulated scans influences the baseline noise, thus affecting the limits of detection and quantification.

The influence of these parameters was evaluated by comparing the slopes of the analytical curves obtained with standard solutions of sodium glutamate (1–10% *m*/*v*) and sorbitol (5–25% *m*/*v*). The nominal resolution was tested between 2 and 16 cm^{-1}, whereas the number of accumulated scans was tested in the range 16–100. The data obtained in the experiments are shown in Fig. 3.

As can be seen in Fig. 3A , the nominal resolution presented remarkable influence on the sensitivity of sodium glutamate measurements. Maximum sensitivity was observed when a nominal resolution between 2 and 4 cm^{-1} was employed, independent of the number of accumulated scans. In order to ensure maximum sensitivity and a reasonable time of acquisition of sodium glutamate spectra, a nominal resolution of 4 cm^{-1} and 64 scans were set for the method.

A different behavior was observed for sorbitol (Fig. 3B). The nominal resolution and the number of accumulated scans did not present significant influence on the sensitivity. Therefore, a nominal resolution of 2 cm^{-1} and 32 scans were chosen for the method to make the process faster.

3.3. Analytical characteristics of the method

3.3.1. Limits of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is defined as the lowest concentration of a given analyte that generates an analytical signal significantly different from the blank signal [28]. According to IUPAC, this condition is satisfied when the signal-to-noise ratio is 3. Therefore, the LOD was estimated using the following expression: $LOD=3\sigma/S$, where σ is the noise estimate and S is the sensitivity, represented by the slope of the analytical curve. For the noise estimate, we used the standard deviation obtained from 10 measurements of the first point of the analytical curve constructed for each analyte. The same procedure was applied in the

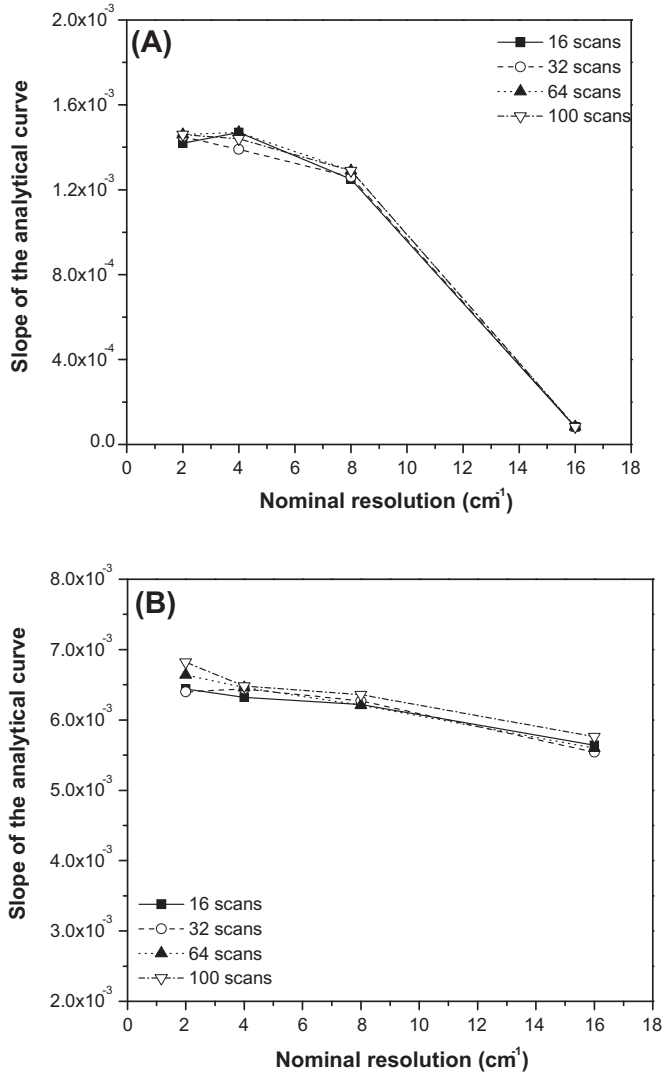

Fig. 3. Effect of the nominal resolution and the number of accumulated scans on the sensitivity of the proposed method for (A) sodium glutamate and (B) sorbitol. The absorbance values were measured, in terms of band height, at 890 and 1347 cm^{-1} for sorbitol and sodium glutamate, respectively. The analytical curves were constructed with sorbitol (5–25% m/v) and sodium glutamate (1–10% m/v) aqueous solutions.

determination of the limit of quantification (LOQ), but using a signal-to-noise ratio of 10. Then, the LOQ was calculated using the expression $LOQ = 10\sigma/S$.

An analytical curve for glutamate was constructed in the range 1–10% (m/v) to obtain sensitivity. For sorbitol, the analytical curve was constructed in the range 5–25% (m/v). As mentioned previously, the noise estimate was performed by calculating the standard deviation of 10 independent measurements of the solutions with the lowest concentration of each analyte. In this condition, the LOD for glutamate and sorbitol were 0.2 and 1% (m/v), respectively, and the LOQ were 0.7 and 3.3% (m/v), respectively.

3.3.2. Precision

The precision of the method was evaluated taking into consideration two aspects: (i) the repeatability and (ii) the intermediary precision. The repeatability of the method was evaluated by the analysis of six independent aliquots of one sample in the same conditions. The intermediary precision was assessed by analyzing one sample on two different days by two different analysts. The results were evaluated using the coefficient of variation. Satisfactory repeatability was observed in the determination of glutamate and sorbitol. In the case of glutamate the coefficient of variation was 1.6%, whereas in the case of sorbitol it was 3.1%.

In the evaluation of intermediary precision, the results obtained by the two analysts were firstly compared using Student-t test (95% confidence level). No statistical differences were observed for either glutamate and sorbitol determinations. Once we had verified that the performance of the analysts was similar, the coefficients of variation were calculated and they were 2.7% for glutamate and 3.5% for sorbitol.

3.3.3. Accuracy

The accuracy of the proposed method was evaluated following two approaches. In the first approach, a recovery test was performed with four samples of thermostabilizer. Then, the results obtained with the proposed method were compared with the results obtained with the reference methods, for 10 samples (five synthetic and five real samples).

The recovery test was carried out by spiking the samples with 4% and 10% (m/v) of each analyte. They were then analyzed by the proposed method and the values compared with those determined for the unspiked samples. The results obtained in the recovery test are shown in Table 3. As can be seen in Table 3, satisfactory results were observed in the recovery test. For glutamate, the recovery percentages varied in the range 95–106%, whereas for sorbitol the recovery percentages were in the range 93–106%. These results proved that no matrix interferences were present in the determination of the analytes by the proposed method.

In order to confirm the accuracy of the method, another experiment was performed, taking the reference methods for comparison. In the case of glutamate, the UV enzymatic method [24] was taken for reference. For sorbitol, the determination was performed by the recommended HPLC-UV method [23]. The results are shown in Table 4.

The difference between the results obtained by the proposed and reference methods were tested using the paired Student-t test (95% confidence level). For glutamate, the calculated value of t was 2.11 and for sorbitol the t value was 1.56. As the critical value of t, in both cases, is 2.26 (9 degrees of freedom) we concluded that there was no statistical difference between the results obtained by the two methods. These results demonstrated that the ATR–FTIR method proposed in this work is accurate enough to perform the

Table 3
Recovery percentages observed in the tests performed with the thermostabilizer samples. The samples were diluted 1:1 with purified water before analysis. Recovery percentages were derived from the concentrations determined by the analytical curve method.

Sample	Analyte added	Concentration added (% m/v)	Recovery (%)
S_2	Sodium glutamate	4	98
		10	98
S_3	Sodium glutamate	4	98
		10	95
S_4	Sodium glutamate	4	106
		10	101
S_5	Sodium glutamate	4	102
		10	98
S_2	Sorbitol	4	100
		10	97
S_3	Sorbitol	4	102
		10	106
S_4	Sorbitol	4	93
		10	104
S_5	Sorbitol	4	100
		10	99

Table 4
Results obtained in the determination of sodium glutamate and sorbitol in the thermostabilizer samples by the proposed method. Results are expressed as mean ± standard deviation ($n=3$).

Sample	Sodium glutamate determined by the proposed method (% *m*/*v*)	Sodium glutamate determined by the reference method (% *m*/*v*)	Sorbitol determined by the proposed method (% *m*/*v*)	Sorbitol determined by the reference method (% *m*/*v*)
S_1	5.52 ± 0.10	5.77 ± 0.17	15.9 ± 0.2	15.8 ± 0.1
S_2	4.16 ± 0.05	4.29 ± 0.06	15.3 ± 0.4	15.0 ± 0.1
S_3	5.16 ± 0.08	5.27 ± 0.15	15.8 ± 0.2	15.3 ± 0.1
S_4	5.28 ± 0.22	5.58 ± 0.06	17.5 ± 0.3	17.1 ± 0.3
S_5	5.81 ± 0.11	5.93 ± 0.19	16.7 ± 0.3	16.1 ± 0.1
S_6	5.35 ± 0.02	5.47 ± 0.19	15.4 ± 0.1	15.4 ± 0.1
S_7	5.88 ± 0.08	5.70 ± 0.27	15.2 ± 0.1	15.2 ± 0.1
S_8	5.46 ± 0.09	5.46 ± 0.46	15.4 ± 0.2	15.5 ± 0.1
S_9	5.48 ± 0.08	5.39 ± 0.30	15.6 ± 0.1	15.6 ± 0.1
S_{10}	5.56 ± 0.04	5.56 ± 0.40	15.3 ± 0.2	15.4 ± 0.1

determination of both glutamate and sorbitol in the thermostabilizer samples.

4. Conclusions

The results obtained in this research showed that a fast, simple and efficient determination of sodium glutamate and sorbitol in pre-vaccine (thermostabilizer) samples of yellow-fever vaccine can be achieved by attenuated total reflectance Fourier transform infrared spectrometry (ATR–FTIR). The developed method required no manipulation of samples, making the procedure easy to implement for routine quality control of pre-vaccine samples.

The absorption bands of sodium glutamate and sorbitol observed at 1347 and 890 cm^{-1}, respectively, provided enough sensitivity and selectivity for determination of the analytes present in the thermostabilizer, even in the presence of other excipients such as hydrolyzed gelatin. The proposed method can be considered an excellent alternative to the reference methods, based on chromatographic and UV spectrophotometric measurements.

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