Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9291
ISSN on-line: 1807-8648
Doi: 10.4025/actascihealthsci.v35i2.12769

Physical and chemical analysis of commercial nystatin

Edeilza Gomes Brescansin[1*], Márcia Portilho[1] and Francisco Benedito Teixeira Pessine[2]

*[1]Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brazil. [2]Universidade de Campinas, Campinas, São Paulo, Brazil. *Author for correspondence. E-mail: egbrescansin@uem.br*

ABSTRACT. Nystatin (NYS) is a fermentation-produced antibiotic of the polyene group. Commercial NYS is a mixture of compounds named NIS A1, A2 and A3. Current analysis undertook physical and chemical analyses in two samples of commercial nystatin (NYS I and NYS II). Moisture (Karl Fischer), spectroscopic (IR, UV and fluorescence) and thermal analyses (TGA and DSC) were conducted. The moisture was respectively 9.2% and 8.8% for samples I and II. Absorption spectrum in the UV/VIS region had a vibronic structure with three $\lambda_{max.}$. The mirror image rule was not complied with in the fluorimetric analysis. Spectroscopy analysis in the IV region indicated that samples showed spectra similar one to another, analogous to crystal type A. Thermal analyses by DSC provided a wide, single endothermal peak and, therefore, similarities among the samples. DTG shows that samples undergo decomposition at three phases within the temperature range under analysis. Results show that samples are impure and not polymorphic, constituted by a mixture of the compounds A1, A2 and A3.

Keywords: nystatin, physical and chemical analyses, analytical control.

Análises físico-químicas da nistatina comercial

RESUMO. A nistatina (NIS) é um antibiótico do grupo dos poliênicos produzido por fermentação. A NIS comercial consiste numa mistura de compostos denominados NIS A1, A2 e A3. O presente trabalho teve por objetivo realizar análises físico-químicas de duas amostras de nistatina comercial (NIS I e NIS II). Foram realizadas análises de umidade (Karl Fischer), espectroscópicas (IV, UV e fluorescência) e térmicas (TGA e DSC). A umidade encontrada foi de 9,2% e 8,8% para as amostras I e II, respectivamente. O espectro de absorção na região do UV/VIS apresentou estrutura vibrônica com três $\lambda_{máxs.}$. Na análise fluorimétrica não se observou obediência à regra da imagem espelho. A análise por espectroscopia na região do IV mostrou que as amostras apresentam espectros semelhantes entre si, análogos ao cristal do tipo A. As análises térmicas por DSC apresentaram um pico endotérmico, largo, único, sugerindo semelhanças entre as amostras. O DTG indica que as amostras sofrem decomposição em três fases no intervalo de temperatura estudado. Dos resultados obtidos, concluiu-se que que as amostras são impuras e não polimorfas, constituindo-se de uma mistura dos compostos A1, A2 e A3.

Palavras-chave: nistatina, análises físico-químicas, controle analítico.

Introduction

Nystatin (NYS) or fungicidin (molar mass 926.13 g mol^{-1}) is an antibiotic of the polyene group extracted from *Streptomyces nursei* microorganism cultures. The compound was isolated in 1950 by Hazen and Brown, researchers at the Division of Laboratories and Research of the New York Health Department (MICHEL, 1972). Its structure comprises macrocyclic lactone, a hydroxyl tetraene dieno bonded to one or two sugar groupings, which also classify it as macrolide.

Commercial NYS is a mixture of closely related compounds. According to Porowska et al. (1972), the drug is actually a complex with three biologically active elements, called NYS A1, A2 and A3 (Figure 1) (MICHEL, 1972; POROWSKA et al., 1972). A1 is the main component of the complex and its structure consists of sugar amine, a d-mycosamine, linked to the oxygen of carbon 19 (MANWARING; RICHARDS, 1969; BOROWSKI et al., 1971). According to Zielinsky et al. (1979; 1987), NYS A2 has a very similar structure to that of A1. Certain differences may be seen in A2, such as in the antibiotic's aglycone, in the stereochemistry variations of the hydroxyl groups which lack a hydroxyl in carbon 10 of the macro cycle, and in the carbonyl position in carbon 15. A3 may have the above-mentioned sugar amine and another sugar radical, l-digitoxose, linked to carbon 35 (ZIELINSKY et al., 1979; ZIELINSKY et al., 1987). Compounds A1 and A2 were identified by Shenin et al. (1993) who analyzed pharmaceutical NYS samples at international standards. The structure of compound A2 was defined by Pawlak et al. (2005). The production method of commercial NYS is the cause of such complexity since

it is actually a fermentation process by which a mixture of the three components is obtained.

NYS is a highly efficient antibiotic in deep mycosis therapy, with fingistatic and fungicide activity on susceptible organisms. It is efficacious against fungus species of the genera *Candida*, *Cryptococcus*, *Aspergillus*, *Histoplasma*, *Blastomyces* and *Coccidioides*, but inefficient in protozoan cells and bacteria, with the exception of *Acheloplasma* grown in sterol, such as blue-green algae (GALE et al., 1972; GUNDERSON et al., 2000).

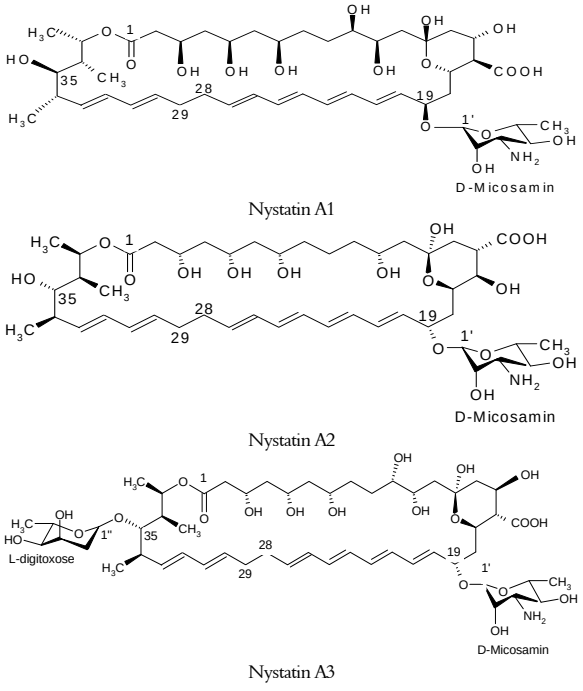


Figure 1. Molecular structure of NYS A1 (nystatin A1), A2 (nystatin A2) and A3 (nystatin A3) (BOROWSKI et al., 1971; ZIELINSKY et al., 1987; PAWLAK et al., 2005).

NYS and other polyene antibiotics activities are characterized by their bonding with ergosterol in the cytoplasm membrane of sensitive fungi. They cause changes in the membrane's permeability by forming intra-membrane pores and thus a loss of vital intra-cell compounds, such as ions and small molecules, and cell death (LEIBOVITZ, 2002). As a rule, polyene antibiotics have a greater affinity with fungus sterol, or ergosterol, than with mammal cells, or cholesterol. This is due to the fact that ergosterol has a harder structure than the cholesterol one. Bruheim et al. (2004) state that double links in the macrolactone ring of these molecules cause sterol-antibiotic interactivities. Lack of ergosterol in the bacteria's membranes may explain the incapacity of this antibiotic to trigger the growth of microorganisms.

Several authors have analyzed nystatin's physical and chemical characteristics (MICHEL, 1972). Studies have been conducted to define its molecular structure,stereochemistry and its overall characteristics. Current research undertakes the physical and chemical characterization of commercial NYS which will be used in future researches within the context of the development of pharmaceutical products. UV/VIS, infrared and fluorescence spectroscopic methods and thermal analysis, such as DSC and TGA, will be employed.

Material and methods

Materials

Commercial nystatins (NIS) NT/C 645743, title 6636 UI mg^{-1}, and 4102, title: 6372.0 UI mg^{-1}, were kindly donated by Medley and Cristália, respectively. They will be called NIS I and NIS II. Antibiotics were obtained from DSM Capua S.p.A., Italy.

Since the antibiotic was sensitive to light, its handling was undertaken at room illumination.

Methods

Moisture rate

Moisture rate in NYS samples was obtained according to Karl Fischer. Mean masses for NYS I of 36.2 mg and 30.9 mg for NIS II were employed. All analyses were conducted in triplicate.

Analysis of absorption spectroscopy in UV/V region

A stock NYS solution was prepared in methanol (1.3×10^{-4} mol L^{-1}) from which solutions 1.0×10^{-5} to 2.6×10^{-6} mol L^{-1} were prepared to obtain absorption spectra and the determination of molar absortivity (ε) in λ_{max} of NYS in methanol. All analyses were done in duplicate.

All absorption spectra in the UV/V region were obtained between 250 nm and 380 nm in quartz cuvettes (optic grade: 1.00 cm) with Teflon lid.

Analysis by fluorescence spectroscopy

Fluorescence spectra were obtained from NYS, used in the preparation of the calibration curse of the UV/V region (1.0×10^{-5} to 2.6×10^{-6} mol L^{-1}).

Two conditions were employed to obtain spectra:

Condition 1: Excitation and emission cleft size: 4 nm; λ_{exc}: 304 nm; scanning interval: 320 nm–550 nm.

Condition 2: Excitation and emission cleft size: 6 nm; λ_{exc}: 320 nm; scanning interval: 340 nm–620 nm.

Analyses were conducted at 25°C and glassware and cuvettes were previously cleansed with a sulfonitric mixture.

Analysis by infrared spectroscopy

NYS absorption spectra were obtained between 400 cm^{-1} and 4000 cm^{-1}, with suspension of drugs in mineral oil (Nujol).

Analysis by Differential Scanning Calorimetry (DSC) and Thermogravimetric Analyzer (TGA)

Analyses by DSC and TGA of NYS samples comprised the use of 3.5 mg for parcel of NYS I and 3.7 mg for NYS II. Samples were maintained in a desiccator and in a fridge till analysis. Experiments were undertaken in the following conditions: argon flow: 80 mL $min.^{-1}$; heating rate: 15°C $min.^{-1}$; maximum heating rate: 250°C.

Results and discussion

Antibiotics NYS I and II are micronized samples, manufactured by DSM Capua S.p.A., Italy.

Analysis of moisture rate

Analyses of moisture rate following Karl Fischer revealed a rate of 9.2% for NYS I and 8.8% for NYS II. Moisture was high when compared to rates in the Mexican (SECRETARÍA DE SALUD, 1988) and American (UNITED STATES PHARMACOPEIA, 2007) pharmacopeias. According to the literature, maximum moisture should be 5%. The correction of drug masses used in the different assays followed from these data.

Since preliminary spectroscopic analyses for NIS_s I and II in the UV/Vis and fluorimetric regions showed similar spectra for the two compounds, only the spectroscopic analysis of NYS I will be shown.

NYS spectroscopic characterization in the UV/VIS region in methanol

Absorption spectra in the UV/VIS region of NYS I solutions in methanol (2.6 x 10^{-6} to 1.0 x 10^{-5} mol x L^{-1}) were registered as overlying (Figure 2) so that changes in position, intensity and profile of absorption bands could be observed owing to NIS interactivity with the solvent and possible formation of aggregates.

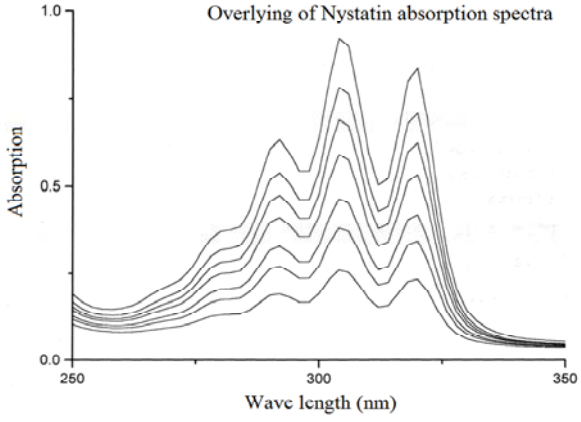


Figure 2. Absorption spectra of NYS I in the UV/V region in methanol, at 25°C (2.6; 3.9; 5.3; 6.6; 7.9; 9.2 x 10^{-6}; 1.0 x 10^{-5} mol L^{-1}) at 25°C.

A spectrum with vibronic structure with three λ_{max} was reported at 292 nm, 304 nm and 320 nm, with the number of joined doubles present in the macrolactone ring. So that the correlation between concentrations and reading in the absorbance of the three λ_{max} could be evaluated, calibration curves for λ_{max} were prepared and rates of molar absortivity calculated (ε) by Origin 5 (Figures 3, 4 and 5).

Figure 3 shows the calibration curve for NYS I solutions in λ_{max} 292 nm.

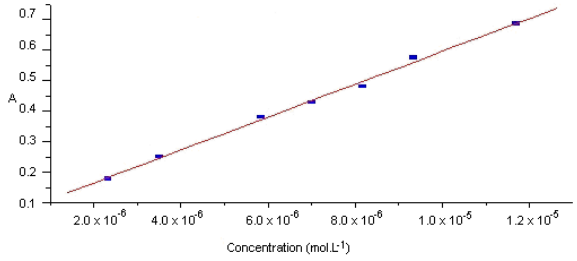


Figure 3. Calibration curve for NYS I solutions (λ_{max} 292nm).

Table 1 shows linear regression data for type Y = A + B * X, with correlation coefficient 0.9982 and the rate of molar absortivity (ε) of 53814 L. mol^{-1} cm^{-1}.

Table 1. Data for linear regression of calibration curve (λ_{Max} 292 nm).

Parameters	Rates	Corr. Coeff.
A	0.0578 ± 0.0109	0.9982
B	53814.2373 ± 1455.4081	

Figure 4 shows calibration curve of NYS I for λ_{max} 304 nm.

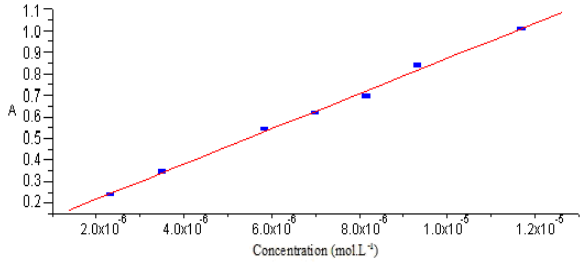


Figure 4. Calibration curve for NYS I solutions (λ_{max} 304nm).

Table 2 shows linear regression data for the calibration curve of NYS I solutions at 304 nm. Correlation coefficient and ε rate were 0.9982 and 81597 L mol^{-1} cm^{-1}.

Table 2. Linear regression data of calibration curve (λ_{Max} 304 nm).

Parameters	Rates	Corr. Coeff.
A	0.0548 ± 0.01629	0.99822
B	81596.8984 ± 2178.6079	------

Figure 5 shows calibration curve for λ_{max} 320 nm.

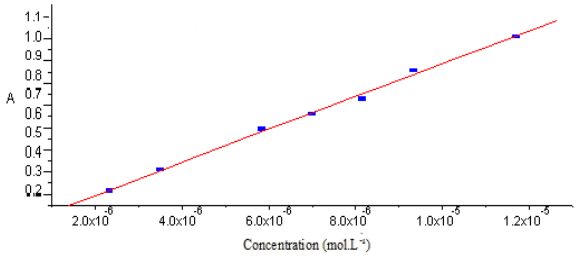


Figure 5. Calibration curve for NYS I solutions (λ_{max} 320 nm).

Table 3 shows linear regression data for this curve. Correlation coefficient was 0.9983 and ε was rated 73889 L mol^{-1} cm^{-1}.

Table 3. Linear regression data of calibration curve (λ_{Max} 320 nm).

Parameters	Rates	Corr. Coeff.
A	0.0483 ± 0.0146	0.99825
B	73888.8001 ± 1955.5780	------

All linear regression data were calculated by concentrations in mol L^{-1}.

NYS absorption spectra between 230 nm and 800 nm (data not shown) only showed bands in the region indicated in Figure 2, λ maximum of 292, 304 and 320 nm. According to Thomas et al. (1981), when antibiotic samples (tetraene compounds) are absorbed in 382 nm, the latter are contaminated by heptaene compounds. Bruheim et al. (2004) state that heptaene compounds are confirmed when absorptions in the UV/VIS region are verified in λ between 370 and 410 nm, which is not verified in current study. According to Coutinho and Prieto (1995), the drug in monomer state is present in the analysis of the NYS absorption spectra in the concentrations and solvent under analysis. Castanho et al. (1992) show that NYS fails to show any changes in the absorption spectra in concentrations when it is in the monomere state and in the aggregate in a water medium. It does not occur with Filipin, another polyene antibiotic.

Fluorimetric analysis of NYS

Figure 6 shows that emission spectrum is not structured as the absorption one (Figure 2). Compliance to mirror image rule is not observed.

Analysis of absorption spectra and emissions of polyene compounds reveals that fluorescence starts from symmetry state, albeit different from that associated with absorption spectra, since the transition of the smallest energy $S_0 \rightarrow S_1$ is not allowed. Absorption spectra represent excitation at a higher level, although emission still occurs as transition $S_1 \rightarrow S_0$ (LAKOWICZ, 1999).

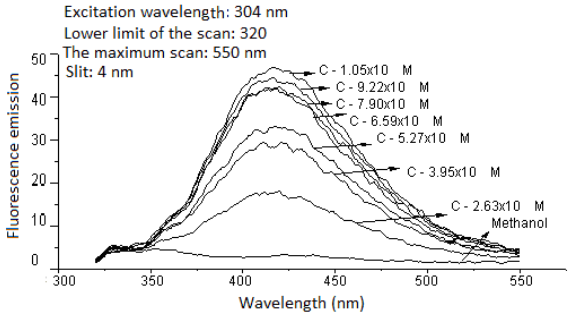


Figure 6. Fluorescence spectra of NYS I in MeOH ($1.05x10^{-5}$; 9.2; 7.9; 6.6; 5.3; 3.9; $2.6x10^{-6}$ mol L^{-1}) of pure methanol.

Spectroscopy analysis in the IR region

IR spectra were obtained (Figure 7) from samples NYS I and II. 'The Analytical Profiles of Drug Substances' (MICHEL, 1972) gives three infra-red spectra for the different types of NYS crystals, classified as A, B and C (Figure 8). The two samples provide similar spectra, analogous to type A, even though contamination with other types may have occurred.

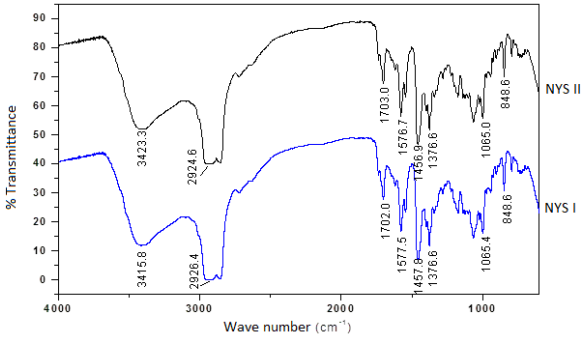


Figure 7. IR spectra from NYS I and NYS II samples

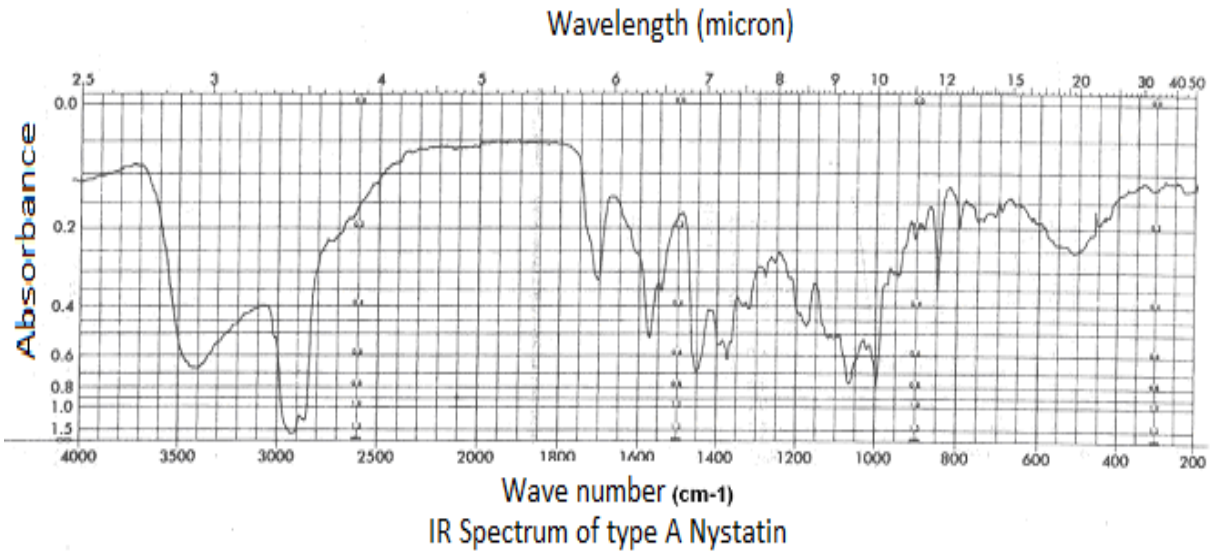
Table 4 shows the frequencies of the molecules' most characteristic vibrations.

Table 4. Absorption frequencies characteristic of NYS I and II bonding vibrations.

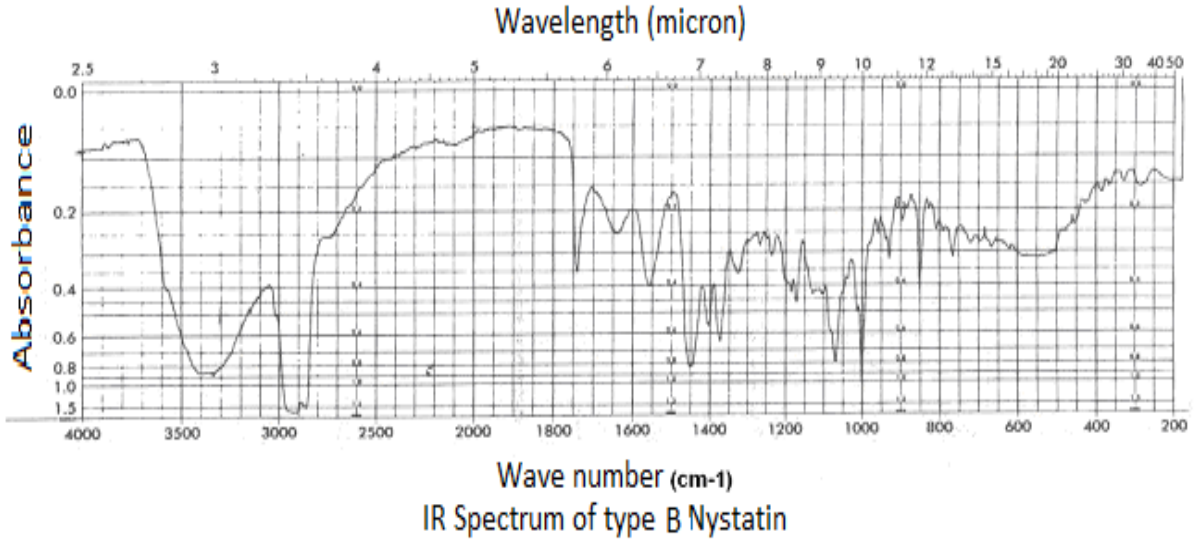
Frequency (cm^{-1})	Vibration mode
1065	Symmetrical stretching of CH_3
1376	Symmetrical deformation of CH_3
1577 and 1576	Carboxyl ion
1702	Lactone
3300 - 3500	NH, OH stretching

NYS analysis by DSC

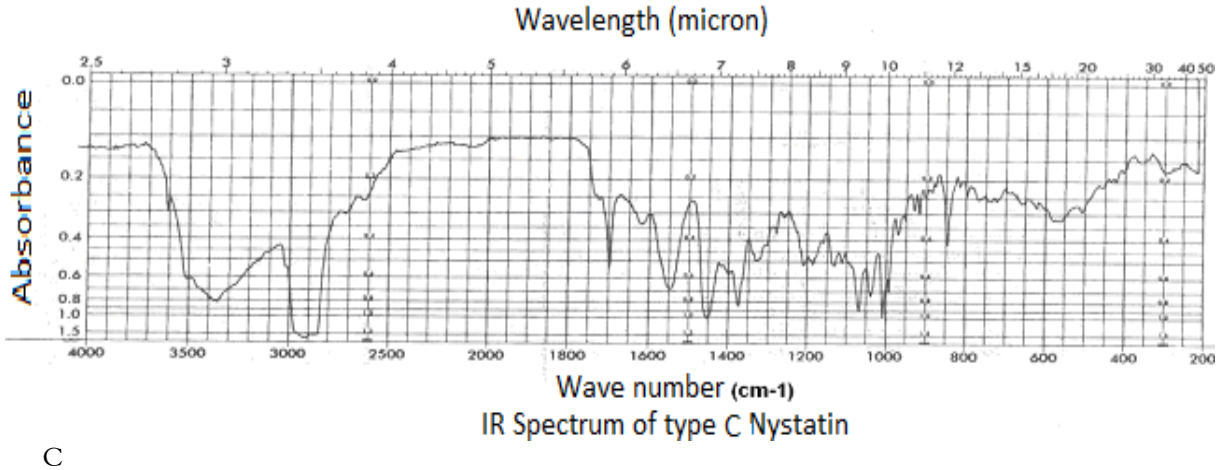
Analysis were undertaken at a temperature ranging between 50°C and 250°C. Figure 9 shows heating curve by DSC of NYS I (a) and II (b) samples, with a wide and single endothermal peak. The wide peak shows that samples are not pure, or rather, they are made up of a mixture of NYS A1, A2 and A3. Peak widening is also the result of a gradual decomposition occurring at over 160°C without merging (MICHEL, 1972).



A



B



C

Figure 8. NYS infra-red spectra of different types of crystallization of NYS A, B and C, (MICHEL, 1972).

However, a single peak probably shows the prevalence of compound A1 which is more abundant in the two samples.

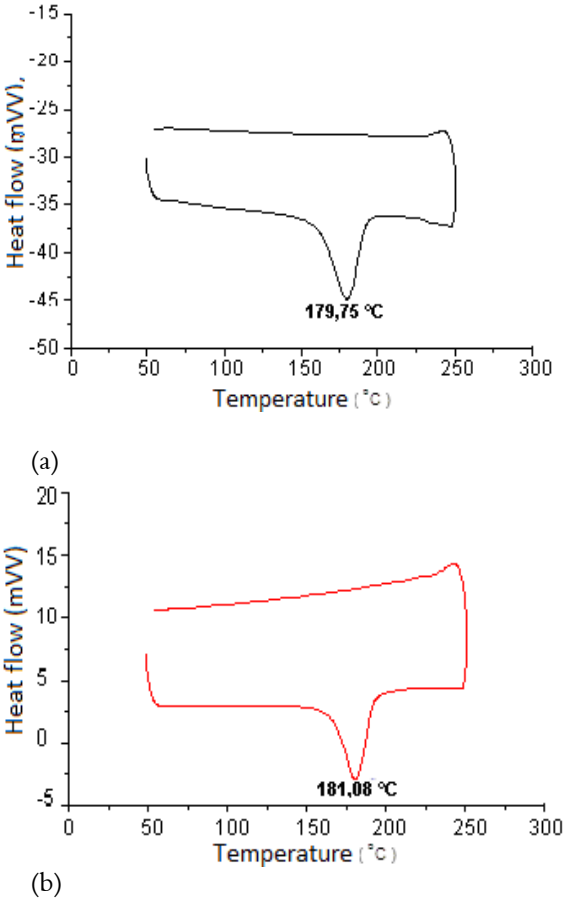


Figure 9. Heating curves provided by DSC for NIS I (a) and II (b) samples.

The determination of peak areas provides fusion enthalpy for the samples. Whereas enthalpy was 295.8 J g^{-1}. In the case of NYS I, it was 245.8 J g^{-1} for NYS II. The latter is thus impure. It should be underscored that the two heating curves by DSC provided a single endothermal peak without any polymorphism, or rather, there is only a crystalline structure for both samples.

Analysis of NIS by TGA

Thermogram of samples NIS I and II show that they are very similar, featuring the same mass loss profile (Figure 10).

Samples' DTGs show that NYSs decompose in three phases within the temperature interval under analysis (Figure 11). Corresponding temperatures for NIS I were 90.1°C, 171.0°C and 260.3°C; temperatures were 88.4°C, 173.6°C and 258.8°C for NYS II.

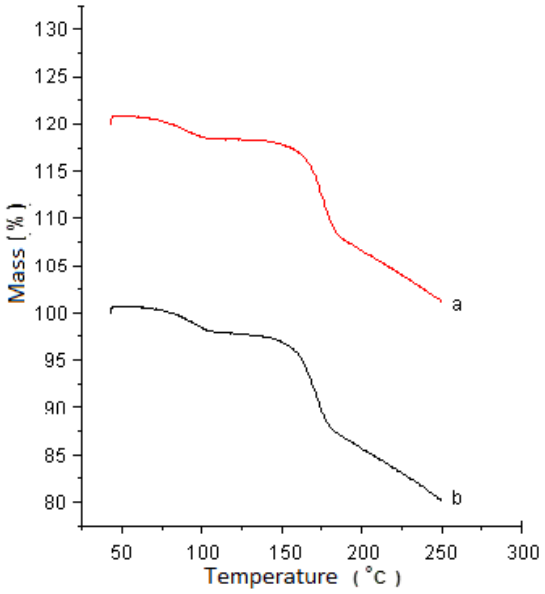


Figure 10. Heating curves by TGA of NYS parcels: NT/C 645743/ and 04102. Masses: 3.7 mg ; 3.5 mg, respectively for NYS I and NYS II. a: Sample Medley; b: Sample Cristália.

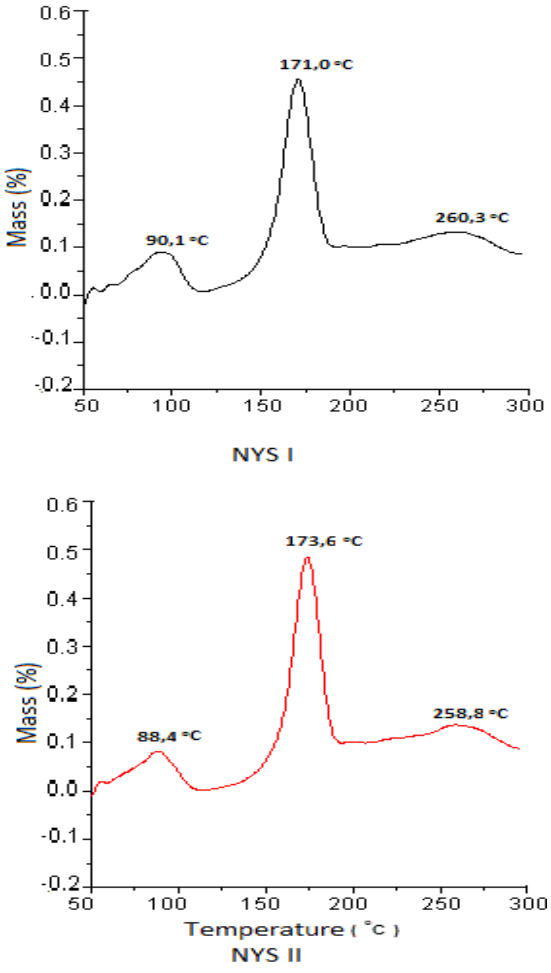


Figure 11. DTG curves of NIS. Masses: 3.7 mg (NIS I) and 3.5 mg (NIS II).

Conclusion

UV/VIS analyses demonstrated that samples were not contaminated by heptaene compounds. Samples' fluorescence derives from the symmetry state which is different from that associated with the absorption spectrum. Spectra from the IV region were similar and analogous to NYS A. Analyses of NYSs by DSC show wide and single endothermal peaks which indicate the lack of polymorphism and the impurity of samples. In fact, they are made up of NYS A1, A2 and A3, although the former predominates. This fact was expected due to the compound's characteristics, or rather, it has been produced by fermentation.

References

BOROWSKI, E.; ZIELINSKI, J.; FALKOWSKI, L.; ZIMINSKI, T.; GOLIK, J.; KOLODZIEJCZYK, P.; JERECZEK, E.; GDULEWICZ, M. The complete structure of the polyene macrolide antibiotic Nystatin A1. **Tetrahedron Letters**, v. 12, n. 8, p. 685-690, 1971.

BRUHEIM, P.; BORGOS, S. E. F.; TSAN, P.; SLETTA, H.; ELLINGSEN, T. E.; LANCELIN, J. M.; ZOTCHEV, S. B. Chemical density of polyene macrolides produced by *Streptomyces nursei* ATCC 11455 and recombinant strain ERD44 with genetically altered poluketide synthase NYSC. **Antimicrobial Agents and Chemotherapy**, v. 48, n. 11, p. 4120-4129, 2004.

CASTANHO, M. A. R. B.; COUTINHO, A.; PRIETO, M. Absorption and fluorescence spectra of polyene antibiotics in the presence of cholesterol. **Journal of Biological Chemistry**, v. 267, n. 1, p. 204-207, 1992.

COUTINHO, A.; PRIETO, M. Self-association of the polyene antibiotic nystatin in dipalmitoylphosphatidylcholine vesicles: a time-resolved fluorescence study. **Biophysical Journal**, v. 69, n. 6, p. 2541-2557, 1995.

GALE, E. F.; CUNDLIFFE, E.; REYNOLDS, P. E.; RICHMOND, M. H.; WARING, M. J. **Molecular basis of antibiotic action**. 2nd ed. Cambridge: John Wiley and Sons, 1972.

GUNDERSON, S. M.; HOFFMAN, H.; ERNST, E. J.; PFALLER, M. A.; KLEPSER, M. E. In vitro pharmacodynamic characteristics of nystatin including time-kill and postantifungal effect. **Antimicrobial Agents and Chemotherapy**, v. 44, n. 10, p. 2887-2890, 2000.

LAKOWICZ, J. R. **Principles of fluorescence spectroscopy**. 2nd ed. New York: Kluwer Academic, 1999.

LEIBOVITZ, E. Neonatal candidosis: clinical picture, management, controversies, consensus, and new therapeutic options. **Journal of Antimicrobial Chemotherapy**, v. 49, suppl. 1, p. 69-73, 2002.

MANWARING, D. G.; RICKARDS, R. W. The structure of the aglycone of the macrolide antibiotic Nystatin. **Tetrahedron Letters**, v. 10, n. 60, p. 5319-5322, 1969.

MICHEL, G. W. **Analytical profiles of drug substances**. New Jersey: Academic Press, 1972.

PAWLAK, J.; TABIN, P.; SOWIÑSKI, P.; BOROWSKI, E. The structure of nystatin A2. **Polish Journal of Chemistry**, v. 79, n. 10, p. 1673-1679, 2005.

POROWSKA, N.; HALSKI L.; PLOCIENNIK Z.; KOTIUSKO D.; MORAWASKA H.; KOWSZYK-GINDIFER Z.; BOJARSKA-DAHLIG H. Composition of polifungin, a new antifungal agent. **Recueil des Travaux Chimiques des Pays-Bas**, v. 91, n. 7, p. 780-784, 1972.

SECRETARÍA DE SALUD. **Farmacopea Nacional de los Estados Unidos Mexicanos**. Comisión Permanente de Farmacopea. México: Talleres Gráficos de la Nación, 1988.

SHENIN, Y. D.; BELAKHOV, V. V.; ARAVIISKII, R. A. Nystatin:methods of preparation, search for derivatives, and prospects for medicinal use, **Pharmaceutical Chemistry Journal**, v. 27 n. 2, p. 84-92, 1993.

THOMAS, A. H.; PHARM, B.; NEWLAND, P.; QUINLAN, G. J. Identification and determination of the qualitative composition of nystatin using thin-layer chromatography and high-performance liquid chromatography. **Journal Chromatography A**, v. 216, p. 367-373, 1981.

UNITED STATES PHARMACOPEIA. **The national formulary** – USP 30, NF 25. Rockville: United States Pharmacopeial Convention Inc., 2007.

ZIELINSKY, J.; GOLIK, J.; PAWLAK, J.; BOROWSKI, E.; FALKOWSKI, L. The structure of nystatin A3, a component of nystatin complex. **The Journal of Antibiotics**, v. 41, n. 9 p. 1289-1291, 1987.

ZIELINSKY, J.; JERECZEK, E.; SOWINSKI, P.; FALKOWSKI, L.; RUDOWSKI, A.; BOROWSKI, E. The structure of a novel sugar component of polyene macrolide antibiotics: 2,6-dideoxy-L-ribohexopyrane, **The Journal of Antibiotics**, v. 32 n. 6, p. 565-568, 1979.

Received on March 3, 2011.
Accepted on March 1, 2012.