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PAPER

Characterization and identification of suspected counterfeit miltefosine capsules

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Recently, it was revealed that generic miltefosine capsules for the treatment of visceral leishmaniasis, a fatal parasitic disease, were possibly counterfeit products. Here we report on the methods to characterize and identify miltefosine in pharmaceutical products and the procedures that were used to assess the quality of these suspected counterfeit products. Characterization and identification of miltefosine were done with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), Fourier transform infrared (FT-IR) spectroscopy and near-infrared (NIR) spectroscopy. Moreover, a simple, rapid and inexpensive colorimetric test was developed and evaluated for the detection of miltefosine in pharmaceutical products that can be used in the field. The complementary analytical techniques presented here were able to determine qualitatively or (semi-)quantitatively the presence or absence of miltefosine in pharmaceutical preparations and could identify suspected counterfeit miltefosine capsules. This finding of a suspected counterfeit drug intended to treat a neglected disease in a resource-poor country emphasizes the urgent need to develop more simple inexpensive assays to evaluate drug quality for use in the field.

1 Introduction

Counterfeit and substandard drugs are an increasing public health problem worldwide, but appear most prevalent in resource-poor countries.[1,2] Antiparasitics and antibiotics are amongst the most affected classes of drugs, although accurate estimations of the prevalence of poor-quality medicines remain lacking.[3–5] Poor-quality anti-infectives containing a subtherapeutic amount of active compound not only pose a risk to the individual health of patients but also severely threaten the control efforts for tropical infectious diseases and may even contribute to emerging drug resistance.[5,6] Therefore, the development of (simple) methods to assess the quality of anti-infective medicines that are being used in resource-poor countries is urgently needed.[5,7,8]

Recently, abnormally high failure rates in hundreds of Bangladeshi visceral leishmaniasis patients were reported after the use of a locally procured pharmaceutical product labelled 'Miltefos', supposedly containing miltefosine.[6,9] Miltefosine (hexadecylphosphocholine, Fig. 1) is the first effective oral treatment option for the neglected tropical disease visceral leishmaniasis, which is caused by the protozoan *Leishmania* parasite and is inevitably fatal without proper treatment.[10,11] The quality of the 'Miltefos' product needed to be assessed to establish pharmaceutical equivalence to the genuine 'Impavido' product. Previously reported analytical techniques to identify and characterize poor-quality drugs include, amongst others, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS),[12–15] Fourier-transform infrared (FT-IR) spectrophotometry[16–19] and near-infrared (NIR) spectrophotometry.[20–26] NIR received particular attention because of its high throughput and non-invasive potential, leaving the integrity of the tested capsules or tablets intact. This paper presents a platform of these qualitative and quantitative analytical techniques for the characterization and identification of the active pharmaceutical ingredient miltefosine in pharmaceutical preparations, illustrated by comparison of the 'Miltefos' capsules to the genuine 'Impavido' product. Besides these complementary instrumental techniques, a new simple, rapid and inexpensive colorimetric test is presented for the detection of miltefosine *in situ* in the field.

2 Materials and methods

2.1 Sample collection

'Miltefos' drug samples were collected in Bangladesh by local Bangladeshi medical doctors, who were administering the drug

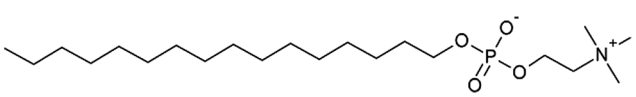

Fig. 1 Chemical structure of miltefosine (hexadecylphosphocholine).

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to patients admitted within the context of the Bangladeshi visceral leishmaniasis elimination programme, at two different locations in August and September 2008, respectively. Because there was no direct suspicion of a poor-quality product, no randomization or sample size assessment was applied for the sample collection. The two sets of samples were shipped from Bangkok, Thailand, and Dhaka, Bangladesh, respectively, at room temperature to our laboratory located in Amsterdam, The Netherlands, which received the samples on August 21 and September 5, 2008, respectively. Both sets of samples were packed in sealed and signed envelopes, to ensure that they had not been opened in between sending and receipt of the shipment.

2.2 Chemicals and reference standards

Miltefosine (hexadecylphosphocholine, 99.9% purity) was kindly provided by Zentaris GmbH, Frankfurt, Germany. Methanol and acetonitrile, both Supra-Gradient grade, and chloroform, HPLC grade, were from Biosolve Ltd, Amsterdam, The Netherlands. Triethylamine, acetic acid (100%), hydrochloric acid (25%), ammonia (25%), and 1,2-dichloroethane (100%), all of analytical grade, were from Merck, Darmstadt, Germany. Distilled water was from B. Braun Medical, Emmenbrücke, Switzerland. Ammonium ferrithiocyanate was prepared in our lab by dissolving ferric chloride hexahydrate (Sigma-Aldrich, Zwijndrecht, The Netherlands) and ammonium thiocyanate (Fluka, Buchs, Switzerland) in distilled water.[27] Lactose (100%) was from DMV Pharma, Veghel, The Netherlands. 'Impavido' ('50 mg Kapseln, Wirkstoff: Miltefosine', batch no. 2649, expiry date: November 2010) was obtained from Zentaris GmbH, through Paesel + Lorei Pharma Vertriebs- und Marketing GmbH, Duisburg, Germany.

2.3 Preparation of reference capsule contents and capsules

A reference standard within the 'Miltefos' matrix was prepared, in order to identify any matrix effects of the 'Miltefos' matrix (*e.g.* excipients) on the analyses and spectrophotometric identification of miltefosine. From a 'Miltefos 50' capsule with a label claim of 50 mg miltefosine, the total contents were collected (about 200 mg), an aliquot of 50 mg was discarded, replaced by a sample of 50 mg miltefosine reference standard and manually mixed by using a pestle and mortar. These spiked 'Miltefos' capsule contents were either extracted as explained in the following paragraph (for LC-MS/MS and FT-IR), used in whole (for FT-IR, NIR and the colorimetric test), or put back in the 'Miltefos' capsule case (for NIR). For NIR spectroscopy, additional 'Miltefos' capsule contents were spiked with 10 or 25 mg miltefosine reference standard.

2.4 Sample preparation and extraction of miltefosine

'Miltefos' capsules from two different batches with two different label claims were available for analysis: 'Miltefos 50' and 'Miltefos 10' with corresponding label claims of 50 and 10 mg miltefosine, respectively. All extractions and analytical tests described here were performed on both 'Miltefos' batches. For clarity, when results from both batches were similar, only results for 'Miltefos 50' capsules will be provided and will be referred to as 'Miltefos'. Only for the NIR analysis, additional results for the 'Miltefos 10' capsules will be provided.

'Impavido' and 'Miltefos' capsules were emptied and the total content of the capsules was weighed. From the total content of a capsule (about 200 mg), a portion of 50 mg was separated for extraction. The 50 mg portion was suspended in 1.25 mL methanol : water (1 : 1, v/v), then the sample was vortexed for 15 seconds and ultrasonicated for 30 min to make sure all miltefosine was extracted. Thereafter the sample was centrifuged (10 min at 15 000 rpm). The same extraction procedure was also performed with 1.25 mL of chloroform. For FT-IR spectra, the extracts in methanol : water (1 : 1, v/v) and chloroform were subsequently dried under a gentle stream of nitrogen at 40 °C.

2.5 Analysis of samples and equipment

2.5.1 Analysis with FT-IR. Absorbance spectra with a range of 650 cm^{-1} to 3700 cm^{-1} were taken on a FT-IR spectrophotometer (FTIR-8400S; Shimadzu, Kyoto, Japan) of the total capsule contents and their dried extracts in methanol : water (1 : 1, v/v) and chloroform (Table 1), and were compared to reference standards of miltefosine, different excipients and an in-house reference library. The number of scans was set at 16 with a resolution of 4.0 and a mirror speed of 2.8.

2.5.2 Analysis with LC-MS/MS. Capsule extracts in methanol : water (1 : 1, v/v) (Table 1) were diluted to reach a final nominal concentration of 10 μg mL^{-1} in order to avoid contamination of the apparatus and then 10 μL was directly injected into a Gemini C18 analytical column (150 mm × 2.0 mm I.D., 5 μm) within an HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) interfaced to an API2000 triple-quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) by an electrospray ionization source operated in positive ion mode. The apparatus was optimized for the detection and quantification of miltefosine as previously described.[28] The final nominal concentration in the diluted extracts was either 10 μg mL^{-1} or 2 μg mL^{-1} for the capsules with a miltefosine label claim of 50 mg or 10 mg, respectively. All samples were analyzed in duplicate. Using the multiple reaction monitoring (MRM) mode the specific mass transition for miltefosine (Q1: *m*/*z* 408.6 → Q3: *m*/*z* 124.8) was monitored for all diluted extracts. Flow injection analysis (FIA) was applied to identify any other components in the extractions: a 10 μL aliquot was directly injected into the mobile phase and subsequently into the source of the mass spectrometer without any separation on an analytical column. Cumulative scans (85 scans in total, MCA mode) in Q1 of the mass spectrometer were recorded over a mass range of *m*/*z* 30–850 during 1 minute; typically all ions had passed before 0.3 min.

2.5.3 Colorimetric analysis of miltefosine. A colorimetric assay based on the complexation of the zwitterionic miltefosine with the anionic ammonium ferrithiocyanate ($NH_4Fe[NCS]_4$) dye was developed.[29] The test was derived from an assay developed for the phosphatidylcholine lecithin, in which it forms a coloured complex with ammonium ferrithiocyanate, which can then be extracted in organic solvents.[27] This procedure was modified for miltefosine and 1,2-dichloroethane was used for extraction of the brown-red coloured complex. Absorption was

Table 1 List of analyzed samples and sample types per analytical technique as reported here

Technique	Sample type	Samples[a]
FT-IR	Total capsule contents	M50, Imp, M50 + 50
	Dried extract (methanol : water (1 : 1, v/v))	M50, Imp, M50 + 50
	Dried extract (chloroform)	M50, Imp, M50 + 50
LC-MS	Extract (methanol : water (1 : 1, v/v))	M10, M50, Imp, M50 + 10, M50 + 50
Colorimetry	Total capsule contents	M50, Imp
NIR	Total capsule contents	M10, M50, Imp, M50 + 10, M50 + 25, M50 + 50
	Total capsules contents inside capsule	M50, Imp, M50 + 50 in empty M50 capsule, Imp in empty M50 capsule

[a] Sample codes: M10 = 'Miltefos 10'; M50 = 'Miltefos 50'; Imp = 'Impavido'; M50 + 10/25/50 = 'Miltefos 50' spiked with 10, 25 or 50 mg miltefosine, respectively.

measured at the optimal maximal wavelength for the miltefosine–ferrithiocyanate complex of 460 nm. An amount of 8 mg of 'Impavido' or 'Miltefos 50' capsule content was dissolved in 200 μL water to which 200 μL 0.1 M $NH_4Fe[NCS]_4$ in water was added together with 1 mL of 1,2-dichloroethane. The solution was vortexed for 30 s and centrifuged for 1 min at 15 000 rpm to extract the coloured complex in the organic phase. The results were visually and spectrophotometrically assessed and compared to a reference standard of 2 mg mL^{-1} miltefosine in 1,2-dichloroethane.

2.5.4 Analysis with NIR. NIR measurements were performed on a NIRFlex N500 FT reflectance spectrometer (Büchi Labortechnik AG, Flawil, Switzerland) with a resolution of 4 cm^{-1} over a spectral range of 4000 to 10 000 nm using an indium–gallium–arsenide (InGaAs) detector. For each spectrum 32 scans were accumulated. Interpretation of the obtained spectra and further data exploration were performed with the chemometric software package NIRCal 5 (Büchi Labortechnik AG). Spectra were pretreated by application of multiplicative scatter correction (MSC) followed by taking the Savitzky–Golay first derivative. Data exploration was done using the unsupervised Principal Component Analysis (PCA) option in NIRCal in which variability between spectra is transformed and reduced into 'objective' uncorrelated orthogonal variables. These variables, or Principal Components (PCs), explain in decreasing order the highest (remaining) variability in the spectral patterns.[30] The 'loadings' of a PC correspond to the wavelengths responsible for the variability in absorbance explained by the PC. PCA yields PC 'scores' for each of the spectra which can be clustered and used for differentiation of the corresponding samples.[20]

The different capsule contents (listed in Table 1) were transferred and measured in glass vials. A pure reference standard of miltefosine and the excipients microcrystalline cellulose and lactose monohydrate were also analyzed. Per capsule type, spectra were obtained from 3 capsules. PCA was performed on these spectra, in which calibration was based on all sample types. The use of two PCs (PC1 and PC2) was considered sufficient for this analysis, since more than 95% of variation in the dataset was explained by these and all samples could be differentiated using these two PCs only.

The non-invasive abilities of NIR were explored by taking spectra of intact capsules without opening them (Table 1), with spectra of empty capsules as controls. An empty 'Miltefos 50' capsule refilled with either the contents of a 'Miltefos 50' capsule spiked with 50 mg miltefosine or 'Impavido' capsule contents was included as external reference. Per capsule type spectra were obtained from at least 2 capsules and 32 scans were accumulated for each spectrum. PCA was performed based on only two calibration classes: empty and full 'Impavido' capsules. A single PC was considered sufficient for this analysis, since all capsule types could be differentiated using this PC only.

3 Results and discussion

3.1 Sample description and physical appearance

Two different lots of 'Miltefos' capsules from Bangladesh were available for evaluation: 'Miltefos 50' (lot no.: 'SFD13 E0610') and 'Miltefos 10' (lot no.: 'SFD12 E0610'). The 'Miltefos' capsules were professionally packed in alu–alu blister packs containing 10 capsules, with a print on the back in both English and Bengali stating 'Miltefos 50, miltefosine 50 mg' or 'Miltefos 10, miltefosine 10 mg' in red/black and green/black colour (Fig. 2). The blisters were not packed in a box, but did come with a leaflet, stating general information on miltefosine.

Interestingly, after dispersing the capsule contents in water, methanol or a mixture, a clear difference was seen between the contents of the 'Miltefos' capsules and of the reference capsules ('Impavido'/'Miltefos' spiked with miltefosine). At sufficiently high concentrations (approximately >20 μg mL^{-1}) in polar

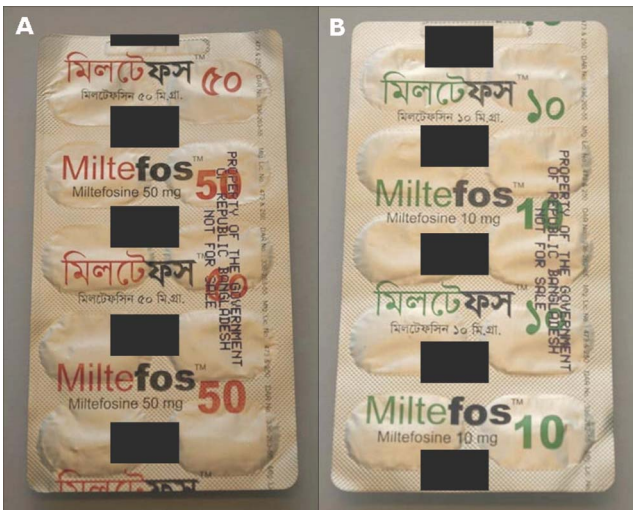

Fig. 2 Backs of blister package 'Miltefos 50, Miltefosine 50 mg' (A) and 'Miltefos 10, Miltefosine 10 mg' (B), respectively. The manufacturer's name and logo have been obscured.

solvents, dissolved miltefosine will cause a stable layer of foam on top of the solution when shaken or stirred. The solutions of the reference capsules displayed a large layer of foam after vortexing, which remained for several hours, indicating the presence of a surface active compound like miltefosine. However, the solutions of the dissolved 'Miltefos' capsules did not display any foaming, indicating the absence of a surface active compound in the solution.

3.2 Identification of miltefosine in the capsule contents

FT-IR, LC-MS/MS, a newly developed colorimetric method and NIR were used to identify miltefosine in the 'Miltefos' capsules.

3.2.1 Identification by FT-IR. FT-IR spectra were taken from the total contents of the different capsules, reference standard and excipients, plus dried extracts of the capsule contents (Table 1). A chloroform extraction of the capsule contents was attempted as well, because miltefosine is known to be soluble in chloroform. However, there was no residue after evaporation of the chloroform phase with the 'Miltefos' capsules, indicating that no miltefosine was present in the chloroform extract of the 'Miltefos' capsule contents.

Clearly distinguishable 'fingerprint' areas could be identified for miltefosine from the FT-IR spectra of the pure miltefosine standard, which are summarized in Table 2. These wavelength areas are specific for miltefosine and other long-chain phosphocholines.[31] Representative spectra of 'Impavido', 'Miltefos' and 'Miltefos' spiked with miltefosine are depicted in Fig. 3. Visual comparison of all spectra revealed that the miltefosine fingerprint areas are substantially different in all 'Miltefos' samples compared to the reference spectra of the pure reference, 'Impavido' and 'Miltefos' spiked with miltefosine. Only peak area IV (1100–1050 cm^{-1}) contains a peak both in the spectra of the 'Miltefos' samples and in the reference spectra. However, closer inspection of this peak reveals a completely different peak 'profile'.

FT-IR spectra of the dried extracts in methanol : water (1 : 1, v/v) of the total capsule contents showed that the miltefosine fingerprints (Table 2) were again clearly recognizable in the extracts of 'Impavido', 'Miltefos' spiked with miltefosine and miltefosine pure reference, but not in the dried extracts of 'Miltefos' (spectra not shown).

3.2.2 Identification by LC-MS/MS. The specific mass transition for miltefosine (Q1: *m/z* 408.6 → Q3: *m/z* 124.8) was monitored for all diluted extracts. In Fig. 4 are shown representative chromatograms of extracts of (A) 'Impavido', (B) 'Miltefos' and (C) 'Miltefos' spiked with miltefosine. The miltefosine peak present in the chromatograms is depicted in Fig. 4A and C, with a typical retention time of 2.8 min and a comparable peak area (average peak area: 1.1×10^6 cps, with a coefficient of variation of 11.4%). On the other hand, the chromatograms of the extracts of 'Miltefos' (Fig. 4B) do not show any peak at the miltefosine mass transition, indicating that miltefosine was not present in the 'Miltefos' capsules.

There was no (additional) ion suppression of matrix components in the 'Miltefos' capsules spiked with miltefosine compared to the 'Impavido' capsules. Both samples could accurately be quantified based on a calibration curve of miltefosine in methanol : water (1 : 1, v/v) and their deviation in the calculated concentration (back-calculated from the analyte peak area) was within ±15%.[28]

3.2.3 Colorimetric miltefosine test. A colorimetric detection test was developed to specifically detect and identify miltefosine using only small amounts of the total capsule contents of an 'Impavido' capsule and of a 'Miltefos' capsule. The 'Impavido' capsule contents showed a deep orange-red colouration of the lower organic liquid phase, indicating the formation of a complex between ferrithiocyanate and miltefosine (Fig. 5, vial 3). The dissolved 'Miltefos' capsule contents do not yield any red colouration of the lower organic liquid phase, indicating that there were no phospholipids or phospholipid derivatives such as miltefosine present in the 'Miltefos' capsule (Fig. 5, vial 4). However, a very slight purple hue was visible in the 'Miltefos' sample corresponding to a complex with maximal absorption at a wavelength of 490 nm or 510 nm, pointing at the formation of higher coordination complexes of Fe^{3+} with thiocyanate. These types of complexes which typically yield a purple hue were commonly observed in this test using other detergent-like compounds, *e.g.* Tween-20, Triton X-100 or sodium dodecylsulfate (results not shown). These other detergent-like compounds were therefore easily visually differentiated from miltefosine. The colorimetric assay showed a linear response over a range of 12.5–800 μg mL^{-1} miltefosine ($R^2 = 0.9981$) allowing its semi-quantitative use in combination with a set of miltefosine standards or possibly even a simple colour chart (results not shown).

Other additional colorimetric tests were developed in our lab, making use of either the formation of miltefosine micelles, the anionic properties of miltefosine, or the specific enzymatic degradation of miltefosine by phospholipase D (from *Streptomyces chromofuscus*) yielding choline which subsequently can be detected using choline oxidase (from *Alcaligenes* sp.) and horseradish peroxidase (results not shown).[29] The latter enzymatic test was very sensitive and selective for miltefosine, but is more expensive and less stable than the tests based on the zwitterionic properties of miltefosine and is therefore probably less suitable for use in resource-poor settings. The ammonium

Table 2 Miltefosine specific IR-spectral peak areas

Wavenumber (cm^{-1})	Code	Description
2950–2850	I	High double peak, corresponding with CH_2 stretching in the long hexadecyl-chain
1520–1490	II	Medium high peak, corresponding with CH_2 bending
1260–1230	III	Medium high peak, possibly corresponding with antisymmetric stretching of the phosphate group (P=O)
1100–1050	IV	High integral broad peak, possibly corresponding to the phosphate group (PO_2 or P–O–C bonds)
980–960	V	Medium high peak, possibly corresponding to the choline group (N–C)

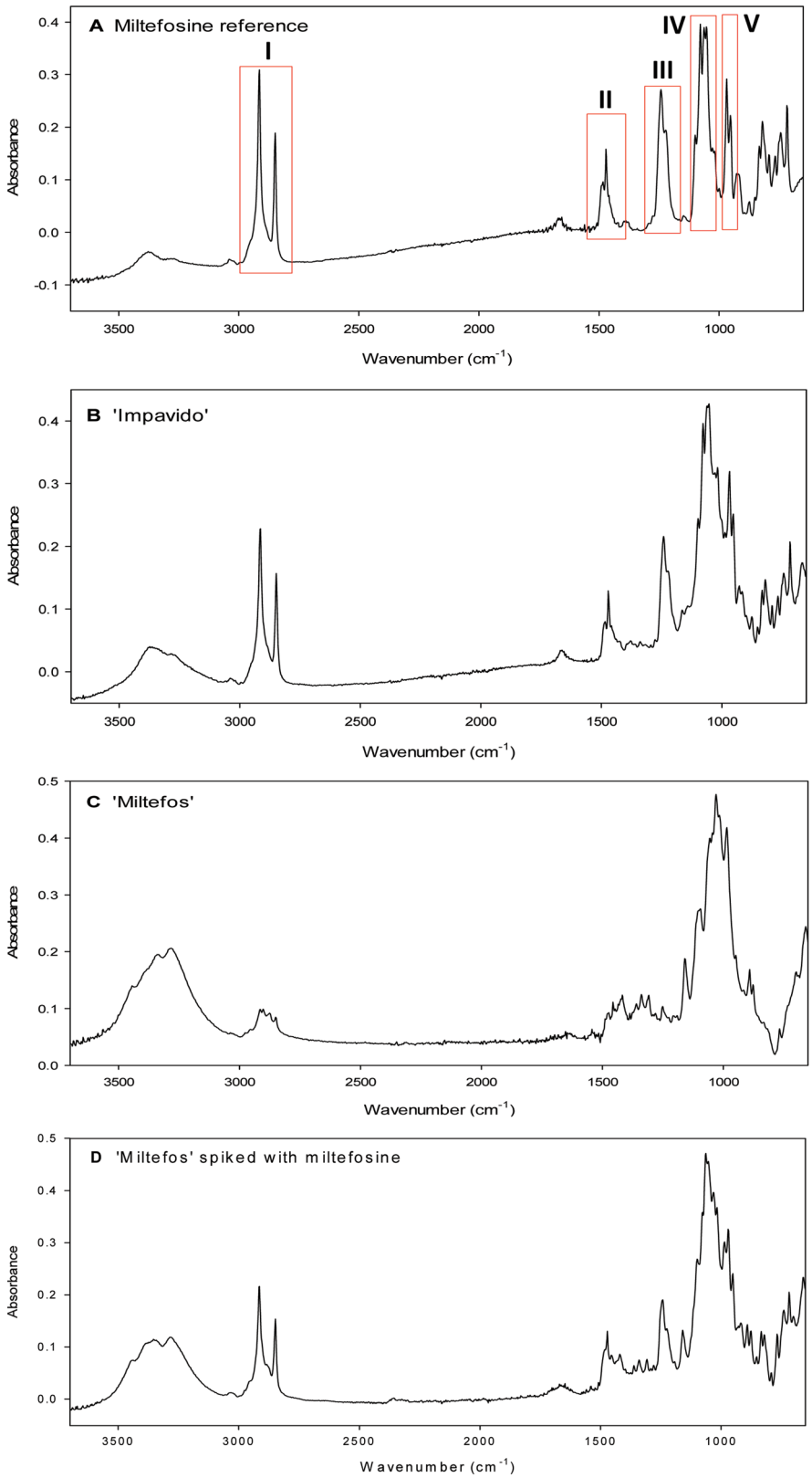

Fig. 3 Representative IR spectra of (A) the pure miltefosine reference standard (with miltefosine-specific peak areas framed) and the total capsule contents of (B) an 'Impavido' capsule, (C) a 'Miltefos' capsule and (D) a 'Miltefos' capsule spiked with miltefosine. The assignments of the framed peak areas in (A) are listed in Table 2.

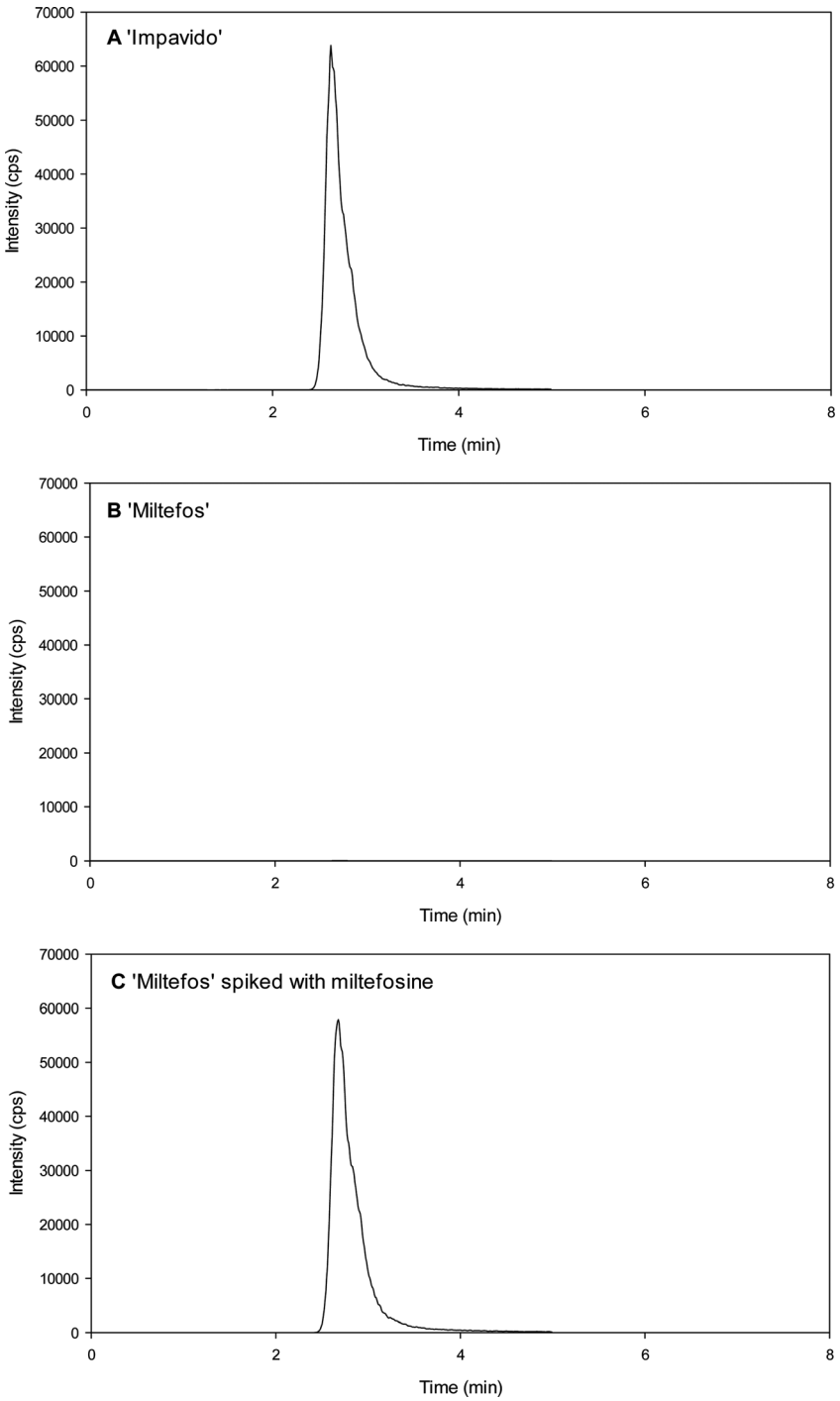

Fig. 4 Representative chromatograms with LC-MS/MS in MRM mode (mass transition Q1: *m/z* 408.6 → Q3: *m/z* 124.8) of the diluted methanol : water (1 : 1, v/v) extracts of various capsules: (A) 'Impavido' capsule, (B) 'Miltefos' capsule, and (C) 'Miltefos' capsule spiked with miltefosine.

ferrithiocyanate test presented here was the most selective one of the non-enzymatic tests.

3.2.4 Identification by NIR. The identification and detection of miltefosine-containing capsules in comparison to capsules not-containing miltefosine were evaluated by NIR spectroscopy. PCA analysis was performed on the NIR spectra of the capsule contents. The plot of PC1 scores (accounting for 90.1% of variability) *versus* PC2 scores (accounting for 7.3% of variability) is shown in Fig. 6. Clusters of the spectra for 'Impavido', 'Miltefos 50' and 'Miltefos 10' could distinctively be separated and PCA showed 100% predictability using a random validation set of 2 samples per sample category. Moreover, the 'Miltefos 50' capsule contents spiked with 10, 25 or 50 mg of miltefosine were separated along PC1 (Fig. 6). The capsule contents of 'Miltefos 50' spiked with 50 mg miltefosine had approximately the same

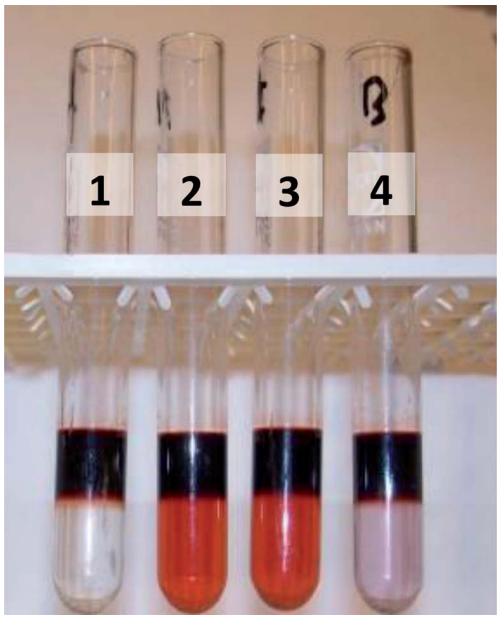

Fig. 5 Colorimetric test results for miltefosine utilizing the complex formation with ammonium ferrithiocyanate. Vial 1 contains a blank solution (water), vial 2 contains 2 mg mL^{-1} miltefosine in water, vial 3 contains 8 mg 'Impavido' capsule contents in water and vial 4 contains 8 mg 'Miltefos' capsule contents.

PC1 value as the 'Impavido' capsule contents which also contained 50 mg miltefosine. These results collectively indicate that NIR analysis provided sufficient relevant information regarding the composition of the samples in order to classify them in separate clusters and that the PC1 score is primarily associated with the amount of miltefosine present in the capsules. The 'loadings' of PC1 per scanned wavelength confirm this observation as these matched closely the profile of the miltefosine reference NIR spectrum (data and plot not shown).

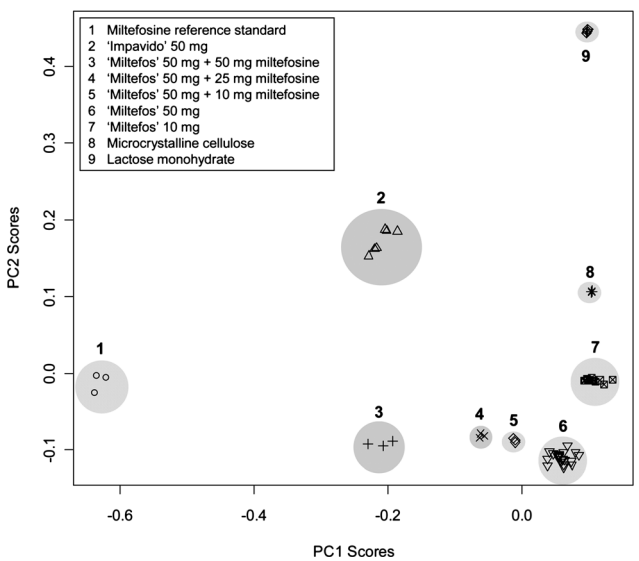

Fig. 6 Principal components analysis (PCA) score plot of the processed spectra taken from the capsule contents obtained by near-infrared (NIR) spectroscopy, which reveals distinct classes for 'Impavido' capsule contents, 'Miltefos' capsule contents and 'Miltefos' capsule contents spiked with different amounts of miltefosine (10, 25 or 50 mg). Clusters of spectra are indicated by grey circles. The PC1 score appears to be correlated with (the amount of) miltefosine in the right to left direction.

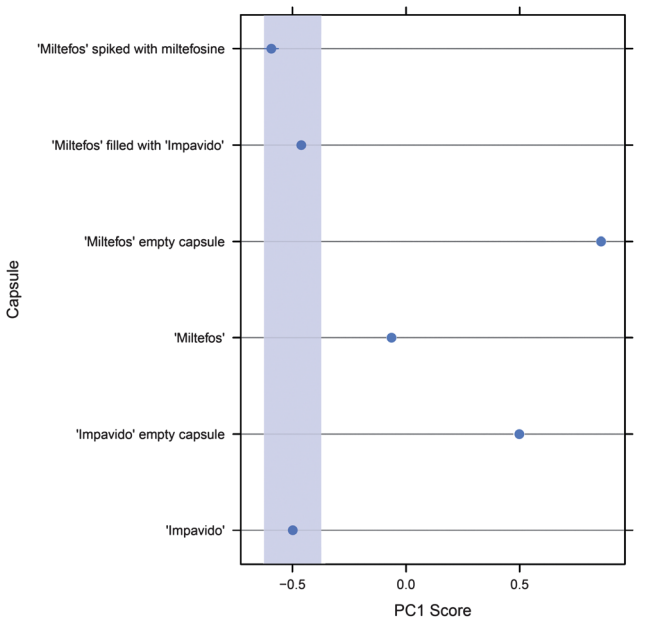

Fig. 7 Principal components analysis (PCA) score plot of the processed spectra taken from the complete intact capsules obtained by near-infrared spectroscopy (NIR). The spectra were taken from the complete capsules without violating the integrity of the capsule. PC1 was calibrated only on the 'Impavido' capsules and the empty 'Impavido' capsules, and based solely on the difference between these two classes, the other spectra were assigned a PC1 score. PC1 scores are depicted as a mean value from 3 capsules. The blue area indicates the range of PC1 scores which are deemed to be not significantly different from the 'Impavido' capsules. Values within this range are thus classified in the same cluster as the genuine 'Impavido' capsules, while values outside the range are classified in a different cluster.

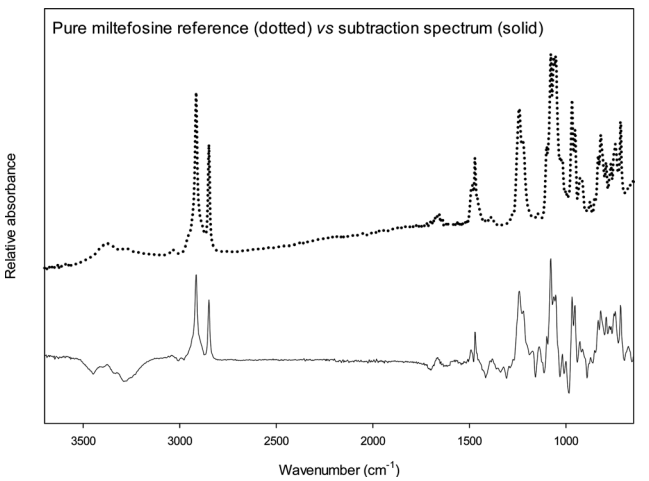

Fig. 8 IR spectrum of the pure miltefosine reference (dotted line) compared to the subtraction spectrum (solid line) resulting from the subtraction of the average spectrum of the 'Impavido' capsule contents minus the average spectrum of the 'Miltefos' capsule contents.

The excipients lactose and microcrystalline cellulose could be separated along the PC2 axis (Fig. 6), which was thus informative about the amount or origin of excipients in the capsule contents. Based on the PC2 scores, 'Impavido' could be differentiated from 'Miltefos 50' spiked with 50 mg miltefosine (Fig. 6), indicating a difference in composition, besides the miltefosine content. More interestingly, 'Miltefos 50' and 'Miltefos 10' could be separated as well along the PC2 axis, which indicates that there is a difference in composition between these two batches of Bangladeshi capsules, although the difference could not be explained by any difference in miltefosine content (similar PC1 values).

NIR was also applied non-invasively, by scanning the complete intact capsule, allowing the integrity of the original

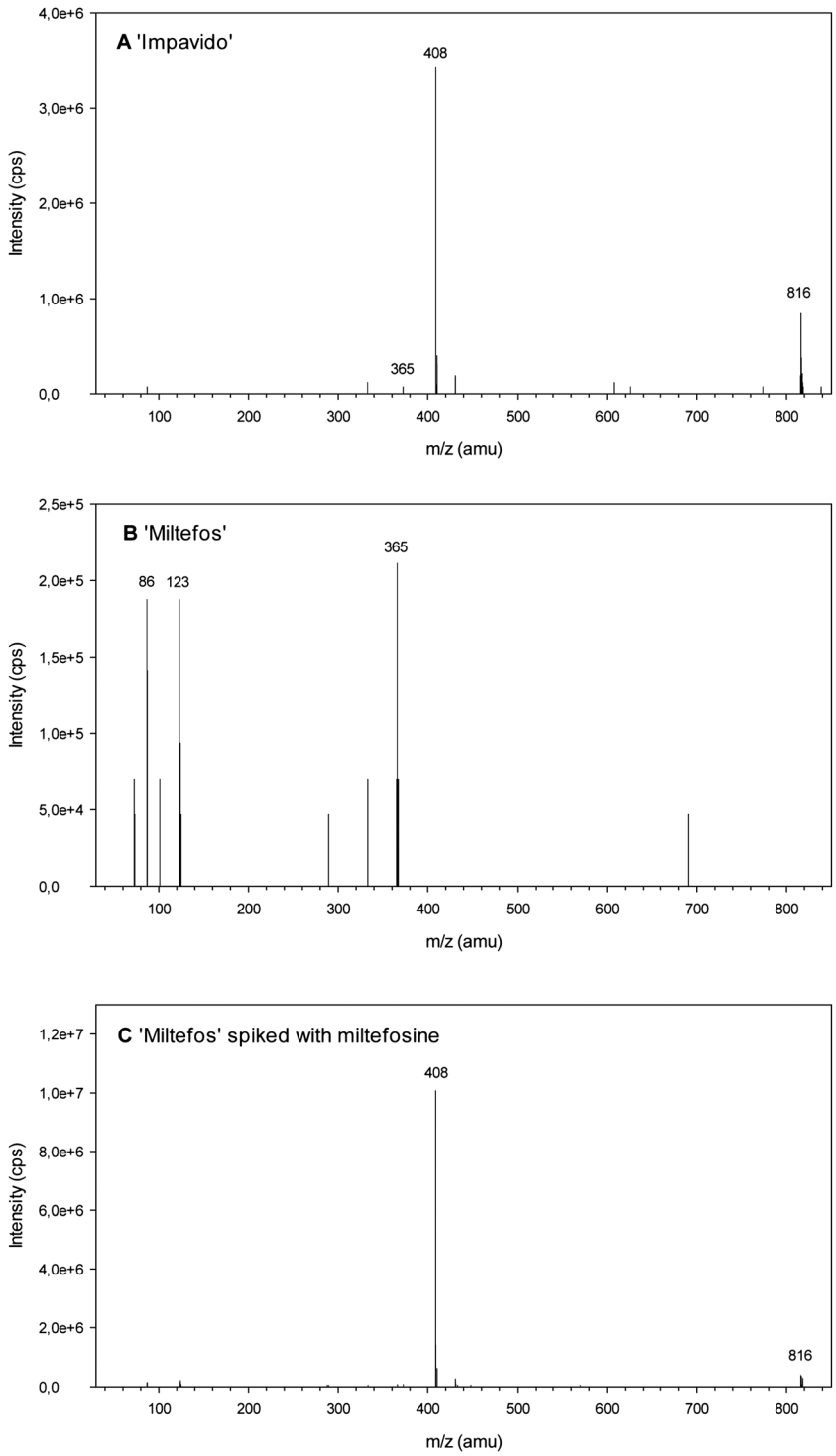

Fig. 9 LC-MS/MS Q1 mass scans (85 cumulative scans, MCA mode) after direct injection of a 10 μL sample, without analytical column; mass range: 30–850 *m*/*z*. The *m*/*z* of 408 corresponds to miltefosine $[M + H]^+$.

capsules. Based solely on the difference between processed spectra of full and empty 'Impavido' capsules, a single PC score (PC1) was calibrated and assigned to other processed spectra of total capsules obtained by NIR (Fig. 7). This yielded a classification which clustered the 'Impavido' capsules together with 'Miltefos 50' capsules spiked with miltefosine and also with 'Miltefos 50' capsules filled with 'Impavido' contents, but more importantly not together with original 'Miltefos 50' capsules and empty 'Miltefos 50' and 'Impavido' capsules (Fig. 7). This clearly indicates that the classification by NIR spectroscopy of the complete capsules was based on the presence or absence of miltefosine, which was again confirmed by agreement between the 'loadings' of the PC1 score per scanned wavelength with the NIR spectrum of miltefosine (data and plot not shown).

3.3 Analysis of the 'Miltefos' capsules

3.3.1 Comparison of the FT-IR spectra. Comparison of the obtained FT-IR spectra to our in-house spectral library of common drugs and excipients revealed that the spectra of the 'Miltefos' capsule contents (Fig. 3C) matched most closely with microcrystalline cellulose (Avicel) and lactose monohydrate (spectra not shown), which both have similar structural components and are often used excipients for the filling of capsules. Comparing these spectra with the spectra obtained from the 'Miltefos' contents revealed no further unexplained peaks. This led to the conclusion that the main ingredients in the capsule contents are either lactose, microcrystalline cellulose or a combination of both. The addition of small amounts of other excipients cannot be excluded on the basis of this analysis. This is further explored by subtraction of the spectra, as described in the following section.

The FT-IR spectra of the dried methanol : water (1 : 1, v/v) extracts of the 'Miltefos' capsules showed clear similarity with the spectrum of lactose (spectrum not shown). Lactose is partly soluble in a methanol : water (1 : 1, v/v) mixture, while microcrystalline cellulose fails to dissolve. During extraction the formation of non-soluble sediment was observed, which indeed could be identified as microcrystalline cellulose with spectral analysis.

3.3.2 Subtraction of the FT-IR spectra. The components of the total capsule contents of the 'Impavido' capsules were compared with those of 'Miltefos' capsules by subtraction of the average FT-IR spectra. The resulting subtraction spectrum (Fig. 8) indicates the differences in chemical bonds of ingredients in the two formulations: peaks indicate absorbing chemical bonds that are absent in the 'Miltefos' capsule contents, but present in the contents of the 'Impavido' capsule; and troughs indicate the inverse. The subtraction spectrum corresponds exactly to the spectrum of the pure miltefosine reference, indicating that the major difference between the two capsules is the absence of miltefosine in the 'Miltefos' capsules.

No other significant, high intensity, identifiable aberrations were observed in the subtraction spectrum. The broad medium troughs located at 3200 and 3300 cm^{-1} (Fig. 8) are probably caused by the higher relative amount of lactose and microcrystalline cellulose in the 'Miltefos' capsules.

3.3.3 LC-MS/MS Q1 mass scans. To characterize the contents of the 'Miltefos' capsules, a Q1 mass spectrum was obtained of the diluted supernatant of their extracts. In Fig. 9, representative Q1 mass spectra are depicted of extracts of (A) 'Impavido', (B) 'Miltefos' and (C) 'Miltefos' spiked with miltefosine. In Fig. 9A and C, the most abundant ion is miltefosine $[M + H]^+$ at *m/z* 408 with a very high intensity, but also visible is a miltefosine dimer $[2M + H]^+$ at *m/z* 815. In the mass spectrum of 'Miltefos' (Fig. 9B) no highly ionized masses appear to be present (all lower than 2.5×10^5 cps). No (either mono- or di-) miltefosine ions could be detected in any of the Q1 mass spectra of the 'Miltefos' samples. The recurrent masses that are recognizable in the spectra of the extracts of the 'Miltefos' capsules are *m/z* 365 (lactose, $[M + Na]^+$), *m/z* 123, *m/z* 86 and *m/z* 72 (all unidentifiable).

4 Conclusion

The diverse set of different analytical chemical techniques presented here was found to be very useful to detect and identify miltefosine, both qualitatively and quantitatively, in pharmaceutical preparations. Using these complementary techniques it was possible to conclude that no miltefosine was present in both batches of 'Miltefos' capsules under investigation, in contrast to the genuine 'Impavido' product. Characterization of the 'Miltefos' capsules by FT-IR, LC-MS/MS and NIR could only reveal the presence of two excipients, *i.e.* lactose and microcrystalline cellulose, and no other active pharmaceutical ingredient could be demonstrated or identified. NIR was applied to differentiate not only between the capsule contents of the two 'Miltefos' batches but also between capsules without and with (added) miltefosine while leaving the integrity of the capsules intact.

The application of a simple, rapid, inexpensive colorimetric test for the demonstration of miltefosine was successfully demonstrated. The test exhibited a linear response over a range of 12.5 to 800 μg mL^{-1} of miltefosine and can thus also be used semi-quantitatively. This simple test is very useful for application in the field without the need for extensive laboratory equipment, which is particularly practical in resource-poor settings.

In conclusion, we present a platform of analytical and chemical techniques that were all able to identify a suspected counterfeit miltefosine product. Most techniques can also be used (semi-)quantitatively, which enables the identification of drugs containing subtherapeutic quantities of miltefosine.

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