Chapter 11

Analytical strategies for the detection of counterfeit erectile dysfunction drugs.

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Abstract:

Among all classes of drugs, the type 5 phosphodiesterase inhibitors (PDE5-i) (sildenafil, vardenafil and tadalafil) are the most counterfeited and copied in industrialized countries. This is why it is very important for regulatory agencies to have a panel of analytical methods to analyse these drugs and to be able to detect counterfeit or substandard medicines.

During this chapter, several analytical techniques will be described and discussed. Among spectroscopic techniques, Raman, NIR, FT-IR and chemical imaging techniques will be envisaged. Their ability to detect counterfeits and predict if a new sample is genuine or not will be presented. Other techniques such as XRay powder diffraction and NMR spectroscopies will be briefly presented.

Chromatographic impurity fingerprints will be considered and described for the analysis of suspect samples of Cialis® and Viagra®. Once again, the possibility to predict the authenticity of a new sample by the mean of this technique is envisaged.

Finally, a MS-compatible UHPLC-UV method for the analysis of the three authorised PDE5-i and some of their analogues will be shortly described. This technique has been validated using spiked placebo samples in a vegetal matrix representing a suspect sample with a complex matrix.

As a conclusion, a generic strategy for the detection and quality evaluation of counterfeit drugs will be presented.

1. Introduction:

The counterfeiting of medicines exists for millennia. At the first century of our era, Pedanius Dioscorides, a Greek physician, already warned about the dangers of adulterated drugs (WHO, 1999). Since then, many crises of falsification of medications have been documented (Newton *et al.*, 2006). Most of those crises implicated falsified herbal medicines and resulted in many deaths due to the lack of efficacy and/or toxicity of adulterated drugs. Categories of adulterated drugs and the risks associated vary according to the region considered.

In developing countries, the most counterfeited class of medicine is the anti-infective class (WHO, 2010). This represents a serious public health problem. Indeed, most of the population buy their drugs in the street at low prices. These drugs generally have less or no therapeutic activity. When treating diseases associated with a high untreated mortality such as malaria, pneumonia, meningitis, AIDS, typhoid and tuberculosis with inefficient drugs, mortality and morbidity increase. Moreover, the use of subtherapeutic amounts of active ingredients increases the risk of developing microbial resistance. In this case, even genuine drugs would be inefficient (Newton *et al.*, 2006).

In industrialized countries, the main counterfeited therapeutic categories are “lifestyle” drugs (weight loss drugs and potency drugs). The risks associated with these drugs are mostly due to the presence of toxic compounds or impurities, too high amounts of active ingredients, presence of unexpected active ingredient or new unknown designer drugs and wrong, missing or inadequate information concerning the use of the drug (RIVM, 2005). Other categories such as antineoplastic drugs or cardiovascular drugs have also been found to be counterfeited (FAMHP, 2010). The fact that counterfeit drugs may be found in the legal market constitutes a major public health risk. Indeed, besides the potential adverse effects encountered by the users, the patients may not trust medicines anymore, even if they are sold in pharmacy. A report of the WHO states that: “*As a consequence of such damaging effects, counterfeit drugs may erode public confidence in health care systems, health care*

professionals, the suppliers and sellers of genuine drugs, the pharmaceutical industry and national Drug Regulatory Authorities (DRAs). Incorrect labeling as to the source can also be detrimental to the reputation and financial standing of the original and/or current manufacturer whose name has been fraudulently used." (WHO, 2009)

2. Phosphodiesterase type 5 inhibitors

In 1998, Pfizer (New York, USA) obtained the marketing authorisation for its new drug Viagra®. This product contains sildenafil citrate as active ingredient. A few years later, Lilly (Indianapolis, USA) launched Cialis® (containing tadalafil) in 2002 followed by Bayer (Leverkusen, Germany) with Levitra® (containing vardenafil hydrochloride) in 2003. These three drugs are the only authorised phosphodiesterase type 5 inhibitors (PDE5-i) for the treatment of erectile dysfunction.

2.1. Pharmacodynamics:

The physiological mechanism responsible for erection of the penis involves the release of nitric oxide (NO) in the corpus cavernosum during sexual stimulation. Nitric oxide then activates the enzyme guanylate cyclase, which results in increased levels of cyclic guanosine monophosphate (cGMP), producing smooth muscle relaxation in the corpus cavernosum and allowing inflow of blood resulting in erection.

Phosphodiesterase type 5 (PDE5) is responsible for degradation of cGMP. When the NO/cGMP pathway is activated during a sexual stimulation, inhibition of PDE5 by the PDE5-i results in increased corpus cavernosum levels of cGMP. These high levels of cGMP induce a sustained erection. Therefore sexual stimulation is required since sildenafil has no direct relaxant effect on isolated human corpus cavernosum but potently enhances the relaxant effect of NO on this tissue (Pfizer, 2010; Schoen *et al.*, 2009).

Actually, eleven families of phosphodiesterase have been identified. Each of these families exerts its role in specific localizations. Sildenafil, tadalafil and vardenafil have a relative selectivity towards PDE 5 and they may inhibit other PDE families in function of their local

concentrations. These inhibitions explain some of their undesirable effects such as the visual disturbances (inhibition of PDE6), inhibition of platelet aggregation and increased heart rate (inhibition of PDE3) or dyspepsia (inhibition of oesophageal PDE5) (Bischoff *et al*., 2004).

2.2. History of PDE5-i counterfeiting

Viagra® is one of the most counterfeited drugs in industrialized countries. This may be explained by the high prices and by the embarrassment caused by the medical consultation for an erectile dysfunction problem. Internet is an easy, fast and anonymous way to obtain these kinds of drugs.

Only eighteen months after the approval of the genuine Viagra®, counterfeit tablets containing sildenafil appear. Tadalafil appeared in Viagra® counterfeits one month before the approval of Cialis® and one year after appeared the first counterfeits of Cialis®. In the Netherlands, in 2004, Viagra® counterfeits represented 98% of the PDE5-i illegal market and Cialis® the last two percent. In 2006, Viagra® represented only 69% of the illegal PDE5-i market while Cialis® (25%) and Levitra® (6%) have become more prevalent (RIVM, 2007).

Besides these three approved molecules, numerous analogues exist. Most of them have been found as adulterants of herbal dietary supplement (RIVM, 2007). These analogues also show a relative selectivity towards the PDE5 (see table 1). However, their inhibition potency might be very different of the one of sildenafil and are rarely taken into account for their dosage in illegal preparations. Furthermore, the differences in their chemical structures lead to differences in their pharmacokinetic parameters such as their onset of action, blood levels, half-lives, brain penetration and metabolism. All these parameters are unknown for the analogues. This represents a huge toxicological risk linked to their intake especially when associated with wrong precautions of use (RIVM, 2007; Medsafe, 2009).

Table 1 PDE5 *in-vitro* pharmacological potencies of the three approved PDE5-i and some of their analogues (data from (RIVM, 2007; Medsafe, 2009))

Compound	*Potency relative to the inhibition of PDE 5 by sildenafil*
sildenafil	1
tadalafil	1,4
vardenafil	10,1
piperidino-sildenafil	0,62
acetildenafil	0,9
homosildenafil	1,9
hydroxyhomosildenafil	2,1
morpholinosildenafil	3,9
benzamidenafil	3,9
thiosildenafil	11,63

2.3. Definitions and classification

The WHO defines a counterfeit medicine as:

- "One that is deliberately and fraudulently mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products. Counterfeit products may include products with the correct ingredients or with the wrong ingredients, without active ingredients, with insufficient (inadequate quantities of) active ingredient(s) or with fake packaging (WHO, 2009a)."

The substandard medicines definition is the non-deliberate and genuine side of the counterfeit medicines definition:

- "Substandard medicines (also called out of specification (OOS) products) are genuine medicines produced by manufacturers authorized by the NMRA (National Medical Regulatory Authority) which do not meet quality specifications set for them by national standards (WHO, 2009a)."

Practically, illegal samples seized at the customs may be divided in two main groups (counterfeit or imitations) according their physical appearance. These two main groups are themselves subdivided in function of the chemical composition of the tablets. This classification, proposed by the Dutch National Institute for Public Health and the Environment (RIVM, 2007), is showed in table 2.

Table 2: Classification of illegal medicines proposed by the Dutch National Institute for Public Health and the Environment (adapted from RIVM, 2007).

Main category	Subcategory	Inclusion and exclusion criteria
Counterfeit	Accurate	Appearance in conformity with genuine medicine; Content of correct API within 90 - 110 % of declared value; No other APIs; not genuine medicine.
	Non-Accurate	Appearance in conformity with genuine medicine; Content of correct API outside 90 - 110 % of declared value; No other APIs.
	Mixed	Appearance in conformity with genuine medicine; Contains correct API and another, known API
	Fraudulent	Appearance in conformity with genuine medicine; Contains a different, known API.
	Analog	Appearance in conformity with genuine medicine, Contains other, unapproved API
	Placebo	Appearance in conformity with genuine medicine; Does not contain APIs.
Imitation And Food Supplements	Accurate	Appearance not in conformity with genuine medicine; Content of correct API within 90 - 110 % of declared value; No other APIs.
	Non-Accurate	Appearance not in conformity with genuine medicine; Content of declared API outside 90 - 110 % of declared value; No other APIs.
	Mixed	Appearance not in conformity with genuine medicine; Contains declared API and another API.
	Fraudulent	Appearance not in conformity with genuine medicine; Contains an undeclared API.
	Analog	Appearance not in conformity with genuine medicine; Contains other, unapproved API
	Placebo	Appearance not in conformity with genuine medicine; Does not contain APIs.

3. Detection of counterfeit PDE5-i drugs:

Several analytical techniques have already been used for the detection of counterfeit PDE5-i most of them are fingerprinting methods. These techniques are separated in two main groups: spectroscopic and chromatographic techniques.

3.1. spectroscopic techniques

Spectroscopic techniques study the interaction between an electromagnetic radiation and the matter. These techniques are classified according to the region of the electromagnetic spectrum measured.

Techniques such as Raman and Near Infrared (NIR) spectroscopy are becoming more and more used for the detection of counterfeit medicines. This is explained by their rapidity and easiness of use. Moreover only a little sample preparation or no preparation at all is needed and these methods are non destructive (Rodionova *et al.*, 2010). For the detection of counterfeit drugs, the signals obtained with spectroscopic techniques must be pre-treated and analysed by chemometric clustering tools such as principal component analysis (PCA) to extract understandable and useful information from the spectra. The data analysis is the only way to come to unambiguous and objective conclusions.

Raman spectroscopy

Raman spectroscopy studies the radiation scattered by a sample when irradiated with a monochromatic radiation. It may be used in the elucidation of a molecular structure and is a complementary technique to mid-IR and NIR spectroscopies. Indeed, a vibrational mode is Raman active when there is a change in polarisability during the vibration whether it is mid-IR and NIR active when there is a change in the molecular dipole moment during the vibration. Thus, a vibrational mode that is highly Raman active will be weakly mid-IR active and vice versa (Bugay *et al.*, 2004).

de Veij *et al.* (2008) recorded the Raman spectra (between 700-1800 cm^{-1}) of eighteen suspect samples and one genuine sample. They were able to check the presence of sildenafil and some of the excipients by visual inspection of the Raman spectra. This visual inspection permitted the discrimination of two groups among the counterfeits but not to distinguish them from the genuine one. Therefore, hierarchical cluster analysis (HCA) and PCA analysis were performed. The combination of these two techniques allowed the complete discrimination between genuine and counterfeit tablets. Raman spectroscopy has also been used to analyse Cialis® counterfeits in association with ^{1}H NMR and 2D Diffusion Ordered Spectroscopy (DOSY) ^{1}H NMR (Trefi *et al*., 2008). Seven counterfeits and one genuine tablet were analysed. The presence of characteristic bands in the Raman spectra

corresponding to unusual excipients confirmed that the analysed samples were counterfeited. The identity of these excipients was confirmed by the NMR spectra providing a "signature" of the manufacturer which could be useful in sourcing studies.

Near infrared (NIR) spectroscopy

The NIR region of the electromagnetic spectrum is comprised between 12500 and 4000 cm^{-1} (800-2500 nm). While the mid-IR absorbances correspond mainly to fundamental vibrations, the NIR absorbances correspond to overtones (excitation of a vibration to a double or higher frequency) and combinations of molecular vibrations. This is why the interpretation of NIR spectra is more difficult than mid-IR spectra.

NIR spectra are essentially used for qualitative analysis because of their fingerprint nature and the fact that NIR spectra contain both chemical and physical informations. (Jee, 2004).

The analysis by NIR spectroscopy of 103 samples containing sildenafil citrate (in the range of 12000-3000 cm^{-1}) allowed the detection of counterfeit samples based on the wavelength correlation applied on spectra without pre-treatment (threshold value of 0.998). Analysts were also able to predict the presence of sildenafil citrate in 98% of the samples (Vredenbregt *et al.*,2006).

Fourier transform mid-infrared (FT-IR) spectroscopy

When irradiating a sample with a mid-IR radiation (wavenumbers of 4000-400 cm^{-1}), a part of the energy is absorbed by the molecules. If the energy is sufficient, the molecular bonds go up to an excited state of energy. The amount of energy needed to excite a molecular bond depends on the atoms involved and the strength of the bond. This is very useful to detect the existence of functional groups in a molecule. Indeed, a specific wavelength radiation will excite specific bonds. This opportunity to identify functional groups in a molecule is only possible within the 4000-1800cm^{-1} region. Indeed, in the 1800-400 cm^{-1} region (*fingerprint region*), too many peaks are present and are therefore hardly assigned to specific functional

groups. It may, on the other hand, be used as a specific fingerprint of the analysed molecule for the identification of pure compounds or the identification of specific mix of compounds (such as tablets).

The fingerprint region of the FT-IR spectrum has been successfully used for the detection of counterfeit Viagra® and Cialis® tablets (Sacré *et al.*, 2010). Transmittance spectra of 55 Viagra-like samples and 9 genuine Viagra® were measured using KBr disks. Then, Partial Least Square (PLS) analysis has been performed on the normalized spectra. The same procedure has been applied on 39 Cialis-like samples and 4 genuine Cialis®. A good discrimination between genuine and illegal samples has been obtained in both cases.

Combination of infrared spectroscopic methods

Sacré *et al.* (2010) investigated which technique (FT-IR, NIR or Raman spectroscopy) or combination of these techniques was the best to (1) detect counterfeit Viagra® and counterfeit Cialis® and (2) to make clusters in illegal medicines. They found that the combination of FT-IR (1800-400 cm^{-1}) and NIR (700-4000 cm^{-1}) spectroscopies provides the best results for the analysis of illegal drugs containing sildenafil citrate while the combination of NIR (700-4000 cm^{-1}) and Raman (1400-1190 cm^{-1}) spectroscopies was the best for the analysis of medicines containing tadalafil as active pharmaceutical ingredient (API).

However, these conclusions were obtained by visual inspection of the PLS factors plots. Therefore, Classification And Regression Trees (CART) algorithm was applied on the infrared datasets in order to discriminate between genuine and counterfeit drug samples and to classify counterfeit samples in different classes following the RIVM classification system (Deconinck *et al.*, 2012). The different models were validated for their descriptive and predictive properties.

For the Viagra-like samples, the best results were obtained with two comparable models based on the FT-IR and the NIR spectra respectively. Both models returned a 100% correct classification rate (CCR) for the discrimination between genuine and counterfeit tablets during both internal and external validation. The models have cross validation errors of

11.6% and 14.4% respectively and equal misclassification rates of 2/12 after external validation for the classification of the samples in their respective RIVM class. The combination of the different types of spectroscopic data did not result in better models compared to the ones obtained with only FT-IR or NIR data.

For the Cialis-like samples, the best model has been obtained combining NIR and Raman spectroscopy. This model gives 77,5% CCR during internal validation but 100% CCR during external validation.

The results obtained with PLS are comparable to the results of the CART models. However, CART also allows a clear discrimination of the counterfeit samples in different classes. This possibility of classifying new unknown samples in their respective RIVM class allows a fast, easy and reliable overview of their risk for public health (Sacré *et al.*, 2010).

Raman Microspectroscopy imaging

Chemical imaging is a powerful tool that combines physico-chemical information and spatial information of the sample. Raman microspectroscopy allows a complete mapping of a limited area of a tablet with a limited sample preparation. The maps of 26 counterfeits and imitations of Viagra® tablets and 8 genuine tablets of Viagra® were recorded (Sacré *et al.*, 2011a). The different maps were pre-processed to allow multivariate analysis.

Three different analyses were performed:

- discrimination between genuine and illegal samples based on the whole Raman spectrum (200-1800cm^{-1}).
- discrimination between genuine and illegal samples based on the presence of lactose in the core of the tablets (830-880 cm^{-1}). Lactose is not present in the core of genuine Viagra® tablets but is a common and cheap filler probably used by counterfeiters.
- discrimination between genuine and illegal samples based on the distribution of sildenafil among the core of the tablets (1200-1290 cm^{-1}). It is therefore expected that the spatial distribution of sildenafil is less homogenous in illegal tablets than in genuine ones.

PCA analysis performed between the 200-1800cm^{-1} and the 830-880 cm^{-1} spectral range (Figures 1 and 2) show a clear discrimination between genuine and illegal tablets. In the 830-880 cm^{-1} region, genuine tablets shows no peak because lactose is not present in the core of genuine Viagra® tablets whereas illegal samples show two peaks at 851 and 876 cm^{-1}. These two peaks are attributed to lactose (de Veij *et al.*, 2008; Degelder *et al.*, 2007).

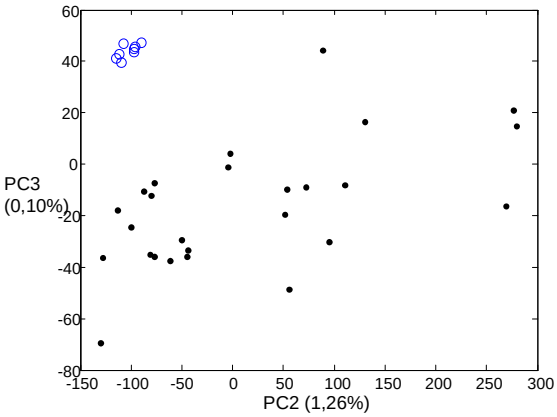


Figure 1: PCA plots of the Raman microspectroscopy data between the 200-1800cm^{-1} spectral range dataset. Black dots are illegal samples and blue circles are genuine Viagra® samples.

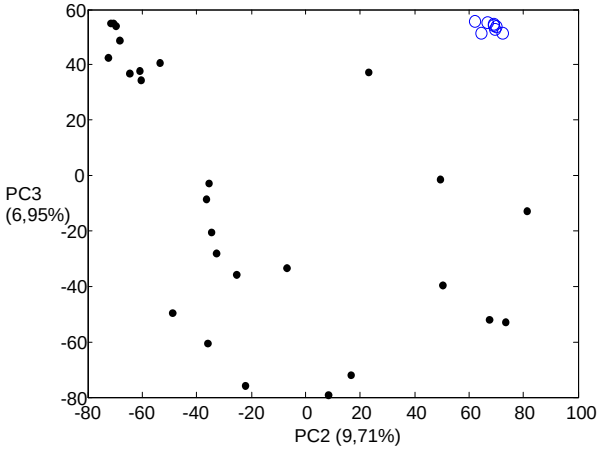


Figure 2: PCA plots of the Raman microspectroscopy data between the 830-880cm^{-1} spectral range dataset. This spectral region corresponds to lactose peaks. Black dots are illegal samples and blue circles are genuine Viagra® samples.

No discrimination has been obtained by the multivariate analysis of the spectral region corresponding to the peak of sildenafil revealing that the spatial distribution of sildenafil between illegal and genuine samples is not sufficiently different.

k-Nearest Neighbour (k-NN) algorithm has been applied on the spectral regions of 200-1800 cm^{-1} dataset. 100% correct classification rate has been obtained during both internal and external validation confirming the ability of Raman microspectroscopy to detect counterfeit Viagra samples.

X-Ray (XR) and Nuclear Magnetic Resonance (NMR)

X-Ray powder diffraction (XRD) allowed Maurin *et al.* (2007) to identify sildenafil and the two most concentrated excipients (microcrystalline cellulose and anhydrous calcium hydrogen phosphate) in suspect Viagra® samples. The discrimination between fake and original tablets can be easily done, even visually, by comparison of diffraction patterns. The diffraction patterns may be used as fingerprints of a given pharmaceutical (legal or not) since it is a superposition of the patterns from all crystalline components of this product. XRD does not require any sample preparation, is relatively fast (~45 min) and do not require advanced statistical treatment of the data to obtain useful information.

Recently, Ortiz *et al.* (2012) analysed imitations and counterfeit sildenafil citrate and tadalafil tablets by X-Ray Fluorescence spectrometry (XRF). XRF is mainly used in the pharmaceutical industry to characterize the presence of metals. This technique may be applied to a wide variety of samples and has many advantages among which its high precision, good detectivity, short analysis times and its non destructive character. The authors were able to perform semi-quantitative determination of the active ingredient (sildenafil is detected by the presence of sulphur) and excipients such as calcium phosphate, titanium oxide and iron oxide (Ca, P, Ti, Fe). Applying PCA and HCA on the data allowed the distinction of seven groups of samples mainly due to differences in their Ca and Ti content.

Trefi *et al.* (2009) used 2D DOSY and 3D DOSY-COrrelation SpectroscopY (COSY) ^{1}H NMR to analyse counterfeit Viagra® samples. They were able to identify different active ingredients and various excipients in a single experiment. The biggest advantage of this technique is the amount of information available while the duration of measurement remains reasonable

(~2h). However, mineral components are not detected, it can only analyse compounds soluble in the NMR solvents and it requires highly trained analysts to interpret the spectra. Another disadvantage, common to both NMR and X-Ray diffraction is the cost of equipments which can, therefore, not be present in the majority of control laboratories.

Visible light based techniques

The visible light range of the electromagnetic spectrum (380-730 nm) may also be used for detecting counterfeit drugs. Rodomonte *et al.* (2010) investigated the possibility to use the secondary packaging or the tablet colour as discriminating parameter. They analysed ten to twenty batches for each dosage of genuine Viagra®, Cialis® and Levitra®. The reflectance visible spectrum was measured on the less carved side of the tablets and on the more distinctive colour of secondary packages (green for Cialis®, violet for Levitra® and blue for Viagra®). The method was validated (ruggedness, repeatability and inter-operator and inter-day precision). The built model allowed the authors to detect counterfeit samples. This colorimetric method may be used as very first step during the visual inspection of samples as it is very fast, inexpensive portable and easy to use.

Photographs of Viagra® and Cialis® tablets have been used to detect counterfeit tablets (Jung *et al.*, 2012). Photographs of 19 genuine and 24 counterfeit tablets of Viagra® and 20 genuine and 53 counterfeit tablets of Cialis® were obtained using Video Spectral Comparator (VSC) in order to acquire images with the same conditions (illumination, lens, …) Images were then segmented and transformed in a statistical model based on the RGB (Red-Green-Blue) colour component. The similarity of the statistical distributions has been measured and compared between all samples and a threshold value has been calculated. Validation of the model resulted in 100% classification accuracy for Viagra® samples while Cialis® samples had False Positive and False Negative rates below 2%.

These two techniques based on visible light have limitations. The colour of both tablets and secondary packaging may be altered (even for genuine drugs) by conservation conditions. This may lead to a higher False Positive rate. The biggest problem is that if counterfeiters produce packaging or coatings of very high quality, their products may be recognised as genuine tablets no matter their physico-chemical quality. Therefore, the results obtained with these techniques should imperatively be confirmed by another technique analysing the content of the tablet.

3.2. Chromatographic techniques

Chromatography enables the separation, identification and quantification of chemical compounds. In the field of counterfeit PDE5-i analysis, reversed phase high performance liquid chromatography (RP-HPLC) is the most used technique to identify new analogues and to quantify API in suspect tablets. Generally, the mobile phase is a combination of acidic aqueous phase and acetonitrile as organic modifier while octadecylsilane (C_{18}) is employed as stationary phase (Singh *et al.*, 2009). A range of detectors can be used, where mass spectrometers and diode array detectors (DAD) are the preferred ones.

Reference draft methods have been published in Pharmeuropa for both sildenafil citrate and tadalafil (EDQM, 2010; EDQM, 2011). The sildenafil citrate assay method is performed on a HPLC system with an endcapped C_{18} stationary phase column of 150 x 3,9 mm and 5 µm particle size. Mobile phase is constituted by a mixture of 17 volumes acetonitrile, 25 volumes methanol and 58 volumes of a 0,7 % (V/V) solution of triethylamine adjusted at pH 3,0 (+/- 0,1) with phosphoric acid. The analysis is performed at 30°C with a flow rate of 1mL/min. An aliquot of 20 µL of a solution containing 20 µg/mL of sildenafil base (~ 28 µg/mL sildenafil citrate) is injected and the detection is performed at 290nm.

The tadalafil assay method is performed on a HPLC system with a sterically protected diisopropyl-C_8 stationary phase column of 250 x 4,6 mm and 5 µm particle size. Mobile phase is constituted by a mixture of 45 volumes acetonitrile and 55 volumes of a 0,1% (V/V) solution of trifluoroacetic acid. The analysis is performed at 40°C with a flow rate of 1,5

mL/min. An aliquot of 20 µL of a solution containing 100 µg/mL of tadalafil is injected and the detection is performed at 285 nm.

Chromatographic impurity fingerprints

The treatment of chromatographic data as fingerprints is widely used in the field of pharmacognosy for quality control of plants (Xu *et al.*, 2006). Chromatographic fingerprints allow a reliable evaluation of the quality and the identity of plant extracts even if neither the identity nor the quantity of the constituents are known.

In the frame of counterfeit drug detection, chromatographic impurity fingerprints is interesting. Indeed, a major hazard of counterfeit products is the presence of toxic impurities in unknown amount. Therefore, as it is the case for herbal medicines, the fingerprint approach allows the discrimination of tablets according to their chromatographic profiles without knowing *a priori* neither the identity nor the quantity of the constituents. Furthermore, it provides a general overview of the chemical composition of the drug with a possibility of identification of the active compounds or impurities and the simultaneous quantification of the active compounds. However, to obtain good and reliable results, a pre-processing step is crucial. This step is mainly composed of signal enhancement, warping and mixture analysis.

A chromatogram contains three major components:

- Noise (highest frequency component)
- Signal (intermediate frequency component)
- Background (lowest frequency component)

If needed, noise and background components are eliminated to enhance the signal. But the biggest challenge is due to the shift in retention times that appears when performing several chromatographic analyses. This shift is mainly due to stationary phase degradation, temperature variations and fluctuations in mobile phase composition. Several alignment

techniques exist (Daszykowski *et al.*, 2006) and each of them should be tested in order to obtain the best results.

Sacré et al. (2011a) have analysed 73 Viagra-like samples and 44 Cialis-like samples on a HPLC system with dual UV detector. The chromatograms were obtained on the same HPLC-UV system with the same mobile phase (Figure 3).

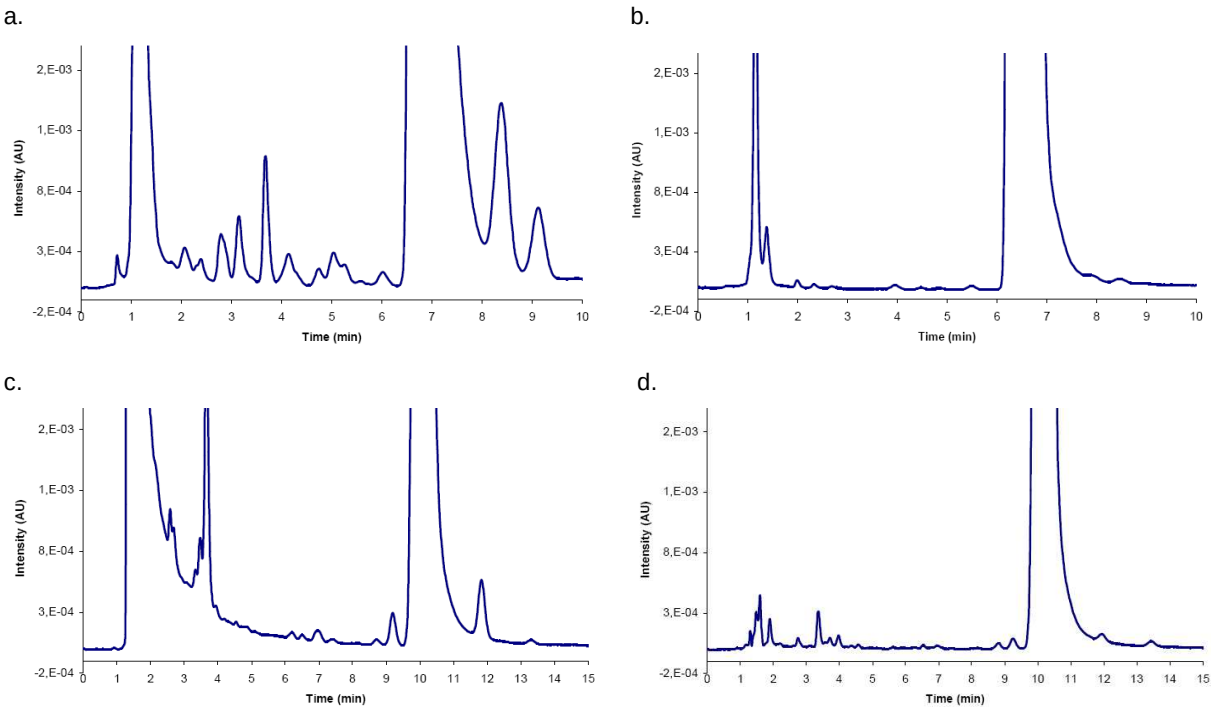
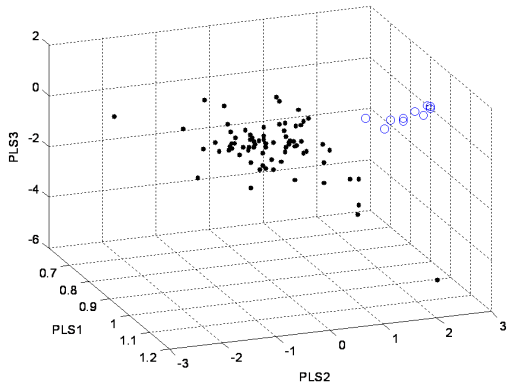


Figure 3:

a. Impurity profile of a counterfeit tablet of Viagra®
b. Impurity profile of a genuine tablet of Viagra®
c. Impurity profile of a coloured imitation tablet of Cialis®
d. Impurity profile of a genuine tablet of Cialis®.

However, a shift in retention times was present and the chromatograms needed an alignment prior any multivariate analysis. For each speciality, chromatograms were aligned in function of the major (largest) peak. Once aligned, the chromatograms underwent logarithmic transformation in order to reduce the influence of remaining slight differences and baseline perturbations. After pre-treatment, PLS was applied on each dataset. The score plots showed a good separation of genuine samples from illegal ones (Figure 4).

a.



b.

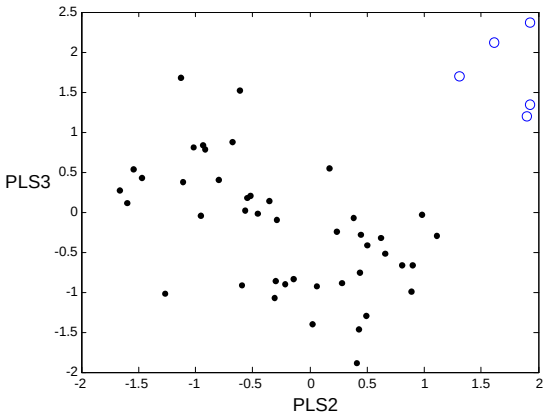


Figure 4:

a. PLS three dimensional plot of the log transformed impurity profiles of the Viagra® dataset. Black points are the illegal samples and blue circles are the genuine ones.
b. PLS2-PLS3 plot of the log transformed impurity profiles of the Cialis® dataset. Black points are the illegal samples and blue circles are the genuine ones.

Predictive models were built using the k-nearest neighbour algorithm (k-NN) and validated. For the Viagra dataset, k-NN classifier allows 100% of correct classification rate in both internal and external validation whether for the Cialis dataset, it returns 92,3% and 100% during internal and external validation respectively.

The results showed that impurity fingerprints can be an interesting approach for the detection of counterfeit drugs.

UHPLC-UV method

Liquid chromatography remains the gold standard technique to separate active compounds and to quantify them in pharmaceutical preparations. If fingerprinting methods have classified a suspicious sample as counterfeit, one needs to confirm the identity of the API and to quantify it to know to which RIVM class (and therefore the public health risk associated) it belongs. The same is true to classify a new sample and to detect new analogues. It is therefore interesting to have a generic method able to detect and quantify the three authorised PDE5-i and their analogues.

Sacré *et al.* (2011b) developed and validated a UHPLC method able to analyse sildenafil, vardenafil, tadalafil and six of their analogues (Figure 5).

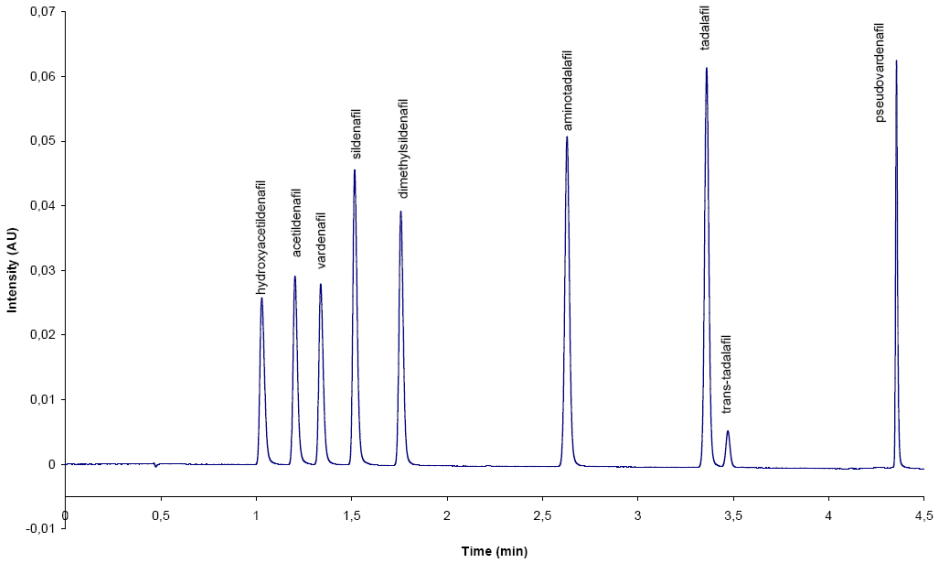


Figure 5: Chromatogram obtained by applying the gradient conditions for the UHPLC method developed by Sacré *et al.* (2011b)

The UHPLC gradient was performed on an Acquity^{TM} BEH Shield RP18 (100 mm x 2.1 mm, 1.7 µm particle size) column. Mobile phase A consisted of a 10 mM ammonium formate buffer (pH 3.5) and mobile phase B was acetonitrile. The gradient conditions are presented in table 3.

Table 3 : Gradient conditions (Sacré *et al.*, 2011b)

Time (min)	Flow rate (ml/min)	% A	% B
0	0.55	75	25
2.5	0.55	65	35
3.5	0.55	55	45
3.8	0.55	30	70
4.5	0.55	30	70
5.0	0.55	75	25

injection volume: 1.5µl

Column temperature: 40°C

The analogues of the registered API's were chosen according to their availability and their structural differences. The diastereoisomer of tadalafil, (-)-trans-tadalafil, was produced in the laboratory starting from tadalafil as no standard was commercially available.

Therefore, it was not quantified but its separation from the other peaks is important since it is frequently present in illegal preparations as an impurity.

The method has been validated using the total error approach (Hubert *et al.*, 2004; Hubert *et al.*, 2007a; Hubert *et al.*, 2007b; Hubert *et al.*, 2008). Spiked herbal matrix placebos were used as validation samples. This is justified by the fact that the method could be applied to a great variety of matrixes (among them, vegetal matrixes are the most complex) and that analogues are essentially found in vegetal alimentary complements. The validation acceptance limits were set at +/- 5% as for pharmaceutical specialities. Once developed and validated, the method has been compared to the reference draft method published in Pharmeuropa (EDQM, 2011). This comparison showed that both methods gave comparable results. The elucidation of structures and the confirmation of identity may be performed by LC-MS systems since the mobile phase is compatible. The method has already been applied to real samples and showed no interference with common other substances present as yohimbine (retention time of 0.77 min) and caffeine (retention time of 0.57 min).

4. Conclusion

Counterfeit medicines become more and more sophisticated as well the packaging (i.e. holograms etc.) as the tablet itself. Customs must therefore use more and more sophisticated analytical techniques. Among them a difference must be made between the expensive and complicated ones available in industrialized countries and the less efficient and cheaper ones available in developing countries while these countries are the most affected by the counterfeiting problem. As counterfeit erectile dysfunction drugs are mainly a problem of industrialized countries, the developed methods use apparatus available in control laboratories of these countries.

These results obtained with the different techniques described in this chapter indicate that control laboratories can, in function of their equipment, implement fast, easy and reliable detection of illegal preparations. However, the reliability of the results is dependent of the database upon which the model is based.

The bigger and diversified the database is, the more reliable are the results.

Therefore, before starting to analyse new samples, laboratories must analyse a minimum number of genuine and illegal preparations. As time goes by, each new analysed sample could be included in the database and the models rebuilt. Each laboratory could then have a powerful model of classification adapted to the kind of medication analysed.

To conclude, a generic approach to detect counterfeit drugs containing PDE5-i is proposed (see Figure 6).

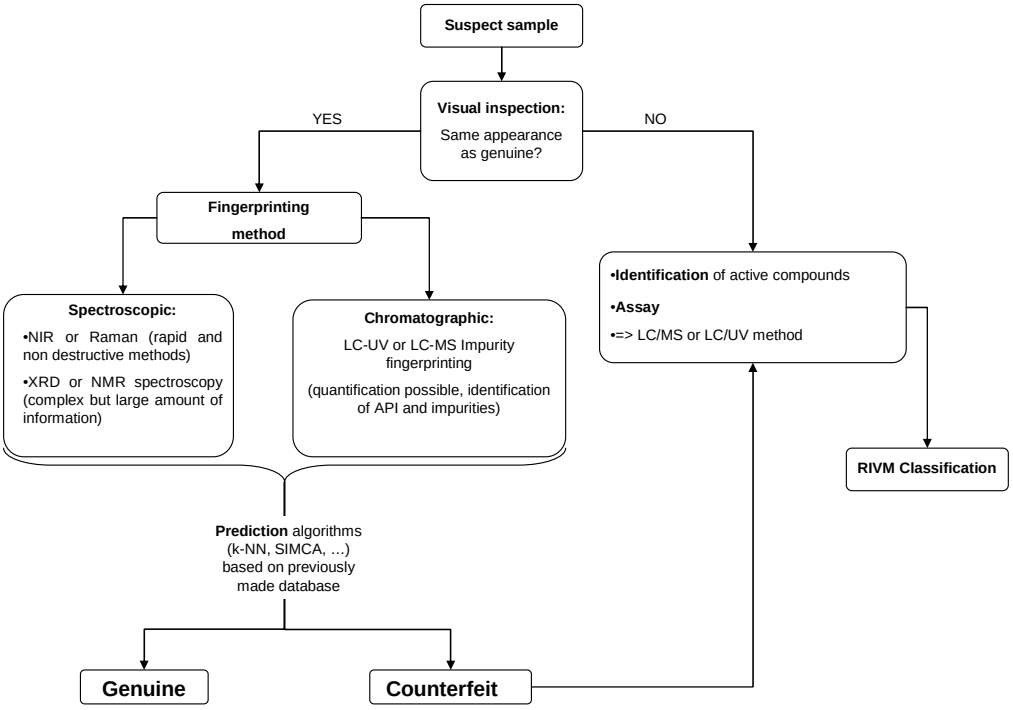


Figure 6 : General strategy for the detection and classification (RIVM) of samples suspected to contain PDE5-i.

When a new sample arrives in the laboratory for analysis, the visual inspection is the first analysis performed (eventually helped with colorimetry analysis). If the samples do not look like genuine drug or if it is a dietary supplement, the only analysis to perform is a qualitative and quantitative analysis using UHPLC-UV and/or LC-MS method. Indeed, no fingerprinting method is necessary since it is evident that it is not a genuine drug. Using the obtained information, it is therefore possible to classify the sample following the RIVM classification (see table 3).

If the new sample has the same appearance as genuine Viagra® or Cialis®, a fingerprinting method is the first recommended analysis. Prior to any fingerprinting analysis, the control laboratory must have developed and validated a predictive model based on the fingerprints of a sufficient number of genuine and illegal samples. Thereafter, each new measured sample will be added to the database and the predictive model rebuilt and revalidated. As time goes by, the model will be more and more accurate as its database grows.

Two kind of fingerprinting approaches are available: spectroscopic and chromatographic fingerprinting.

Spectroscopic fingerprinting is quick, non destructive and reproducible. However, not every laboratory has a NIR or a Raman spectrometer and the apparatus are quite expensive.

On the other hand, chromatographic fingerprinting allows chemical analysis of the tablets and the identification of potential toxic impurities while simultaneously quantifying the active ingredient(s). Another advantage is that these fingerprints are obtained on classical HPLC-UV systems present in every control laboratory. However, the main drawbacks are the fact that it is a time consuming method and that a lot of parameters may change (mobile phase composition, different analytical columns, ageing of stationary phase, different HPLC system, etc.). All these parameters introduce changes in chromatograms, complicating data alignment and pre treatment. In conclusion, we recommend the use of spectroscopic fingerprinting methods. If the tablet has been declared counterfeit, it undergoes quantitative and qualitative analysis with UHPLC-UV and/or LC-MS method and is classified following the RIVM classification.

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