False Positives I – Naphthylamines identification towards HPLC-DAD-MS analysis with ISO 17234-1 technique

Dr. Manila De Cicco, Gustavo Adrián Defeo F.S.L.T.C.

Ars Tinctoria SRL – Via del Bosco 125 (IT-56029) Santa Croce sull'Arno (PI) Italy +39 0571 35110 info@arstinctoria.it

Abstract

The restriction of certain dangerous substances according to REACH (Registration, Evaluation, Authorisation of Chemicals as well as theRestricted Substances Lists (RSL) requirements promoted by various renowned brands is bringing new analytical challenges to our industry. This fact obliges tanneries to everyday more numerous analysis with undesired conflictual situations on false positive tests results. Current official method ISO 17234-1: 2015 leave space to doubt on the amines derived from azo dyestuffs: 4-aminodiphenyl (CAS N. 92-67-1) and 2-naphthylamine (CAS N. 91-59-8). The norm states on 13e the following phrase: *"In the case of levels of 4-aminodiphenyl and/or 2- naphthylamine > 30 mg/kg:Use of this analytical method has detected 4-aminodiphenyl and/or 2-naphthylamine. According to the current state of knowledge it cannot be unequivocally confirmed without additional information that azo colorants which release amines were used."* This uncertainty is source of numerous false positive results. Our paper illustrates one case of a 2-naphtylamine fail result as stated by fourwell-known laboratories. After a detailed evaluation of the dyes included in this process, we have considered their molecular structures, eventual hydrolytic decomposition, and the research of products with similar molecular weight in the NIST databank (National Institute of Standards and Technology). With this information, we have concluded that the incriminated product was its isomer, 1-naphthylamine (CAS N. 134-32-7), by means of an HPLC-DAD-MS analysis.

Keywords: analysis**,** 2-naphtylamine, 4-aminodiphenyl.

1 - Introduction

The new analytical challenge for Leather industry will be to understand the chemical-leather interaction, forecasting the involuntary generation of substances present in SL / MRSL specifications. Our lab begun a series of mass and ionic chromatography research to verify stabilities and interactions of industrial chemicals under normal application scenarios, as well as to prove eventual dangerous substances generations and false positive tests. Every day we receive new of chemical specifications for leather articles with sometimes limits overpassing the requests from cosmetics and even food industry, and in some cases far below the state of art analytical limits.

In this condition false positive results are everyday more frequent. The scope of this paper is to consider the instrumental variables available to avoid false positive results.

2 - Chemical tests for the determination of certain azo colorants in dyed leathers

The profusion of chemicals specifications is bringing, in many cases, wrong concepts to our industry. Many times, I read expressions in certain requests such as "azo dyes free".

"Azo dyes" are most of the colorants used, not only for leather, but also for most industrial applications.

The title of ISO 17234-1:2015 "Chemical tests for the determination of certain azo colorants in dyed leathers" is very clear in concept in

spite it does not specify the fact that the "certain amines" are obtained by dithionite cleavage.This concept is very important considering the additional tests on aromatic amines derived from azo colorants we should run regularly:

- Free aromatic amines according to REACH: The European regulations considers a limit of "free substances" as per Annex XVII to Regulation (EC) No 1907/2006 regarding "Restrictions on the manufacture, placing on the market and use of certain dangerous substances, mixtures and articles". In this case, the method applied is without reductive cleavage.
- Aromatic amines on dyes as perIUC 21: aromatic amines analysis with reductive cleavage, which serves to foresee eventual aromatic amines generation after dyeing.
- For other industrial applications, such as "food packaging and inks" the request is for free aromatic amines on the said materials. This request was recently extended by certain first quality brands to their leather and textiles articles' packaging.

Far from our leather application,ISO 17234.1:2010 was proposed in a research done by ISS (IstitutoSuperioredellaSanità) for Tattoo inks monitoring.

The current method was developed three decades ago to understand which dyes were based on certain amines, and for that purpose a detection limit of 0,1% was enough. Initial qualitative tests were done by thin layer chromatography (TLC). IUC 20 (later ISO 17234.1) suggests a detection limits down to 30 mg/kg. Actually certain brands requests 5 mg/kg, which makes challenging the correct validation of this method according to uncertainty parameters. Regarding the number of amines, it is destined to continue growing with the increasing knowledge of the ecological and toxicological properties of substances. This fact will led to the unavoidable review of ISO 17234.1:2015, in particular reductive cleavage technique, to adapt it to an extended range of substances.

The state-of-art method leaves space to doubt particularly on two aromatic amines: 4-aminodiphenyl and 2-naphtylamine stating:
"In the case of levels of 4-aminodiphenyl and/or 2-naphthylamine > 30 mg/kg:
Use of this analytical method has detected 4-aminodiphenyl and / or 2-naphthylamine. According to the current state of knowledge it cannot be unequivocally confirmed without additional information that azo colorants which release amines were used." ISO 17234.1:2015 (13.g pg.7)".

Although this phrase is normally written in most reports, the suspected presence of 2-naphthylamine and / or 4-aminodiphenyl is considered a "de facto" confirmation by most laboratories.

Uncertain results on isomers identification (as in 2,4 and 2,6 Xylidine), can be solved by an accurate observation of the difference and sameness of analytical parameters, and by internal standard addition.

3 – Reductive cleavage

After sampling according to ISO 2418, and grinded as per ISO 4044, Leather samples are degreased, treated in a citrate buffer solution and heated to 70ºC. A dithionite solution is added to perform the reduction of the dyes amines as per the following reaction scheme (Fig. 1):

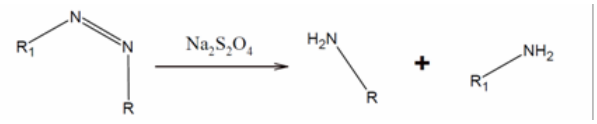


Fig. 1 Dithionite cleavage of a generic dye.

Azo (N=N) bonds are broken into aromatic amines. A liquid- liquid separation in t-butyl methyl ether is performed, extract concentrated with rotary vacuum evaporator, dried with N2, then dissolved in methanol and taken to 2 ml. volume for instrumental analysis.

4 Instrumental methods

ISO 17234.1:2015 identifies different instrumental methods for qualitative and quantitative purposes:

HPLC-DAD (Qualitative & quantitative), HPLC-MS (Qualitative), GC-MS (Qualitative & quantitative), CE-DAD (Qualitative), HPTLC / TLC / Qualitative.

For more accurate results in our internal methods, we opted for GC-MS-MS (triple quadrupole gas chromatographer) and UHPLC-DAD-MS-MS (triple quadrupole ultra-high performance liquid

chromatographer). In both cases, these instruments are not yet officially adopted for the ISO method. It is possible to work in single quadrupole mode (MS) to emulate the approved instruments, or in MS- MS-MRM mode (Multiple Reaction Monitoring) allowing an alternative accurate mass quantification.

By means of GC-MS analysis (Fig.2), we will get from the instrument the following information: Retention time (needed time to exit from the separation column), nominal molecular mass, product ion distribution (formerly called daughter ion) which shows the molecular weight distribution of the generated fragments. Additionally working in MS-MS and in MRM mode, selected reaction monitoring to multiple product ions from one or more precursor ions can be chosen to achieve an accurate mass quantification.

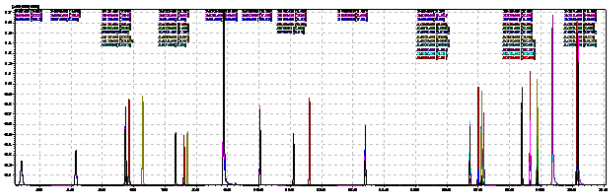


Fig. 2 - Retention times for 25 aromatic amines standard in GC-MS

In HPLC-DAD-MS analysis (Fig. 3) the information we get is retention time, DAD (Diode Array Detection, showing a complete UV-Vis spectrum), a nominal mass, product ion distribution, as well as eventual adducts (depending the eluent and its pH). Working in MS-MS-MRM as in GC-MS-MS it is possible to achieve a reasonable mass based quantitative.

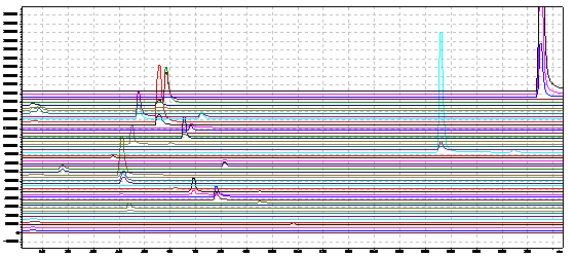
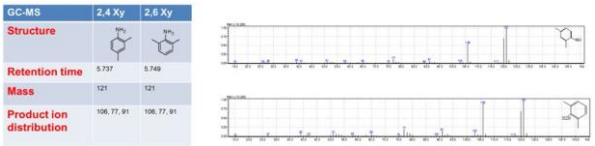


Fig. 3 - Mass ranges scanning for 25 aromatic amines standard in UHPLC-DAD-MS-MS

The method suggest that confirmation should be done by means of at least two instrumental methods, normally one qualitative and another quantitative: "*If any amine is detected by one chromatographic method, then confirmation shall be made using one or more alternative methods*."

5 – Isomers identification

Isomers being "twin substances" regarding mass point of view may have a very similar behaviour in instrumental analysis, considering that their differences may just be the position of a substituent or thedifferent spatial arrangement as in metal complex dyes. We took the recently added to ISO 17234- 1:2015 :2,4 and 2,6 Xylidine as an example of difficult to identify isomers (Fig. 4).

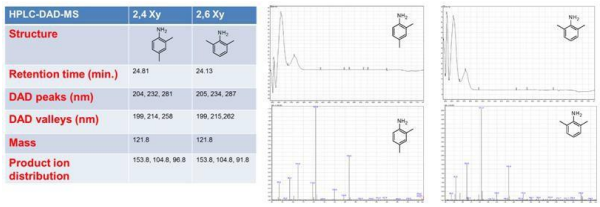


GC-MS	2,4 Xy	2,6 Xy
Structure		
Retention time	5.737	5.749
Mass	121	121
Product ion distribution	106, 77, 91	106, 77, 91

Fig. 4 – GC-MS mass spectra of 2,4 and 2,6 Xylidine and typical parameters (NIST databank).

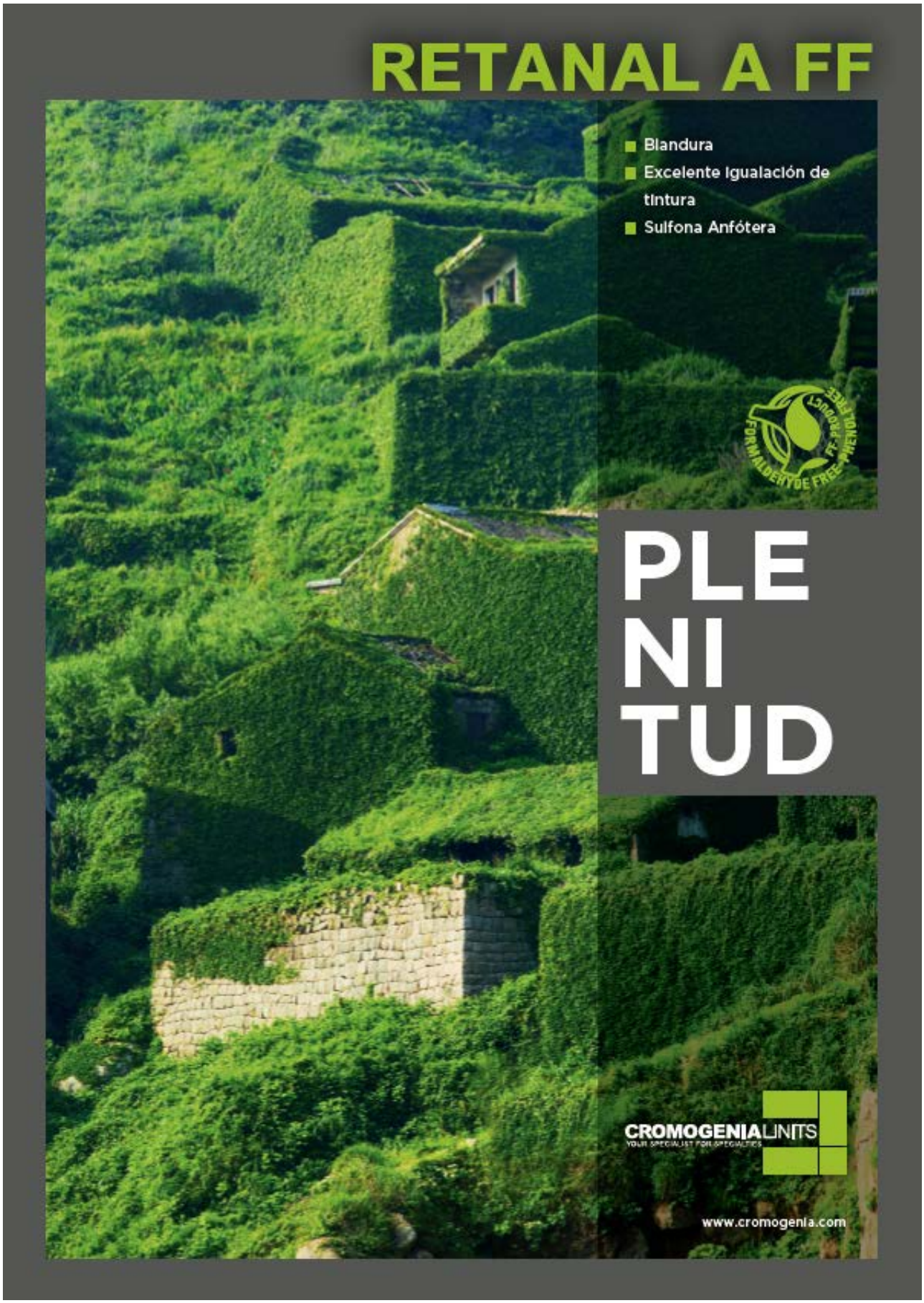
GC-MS analysis shows very similar retention times (just some seconds difference), same nominal masses, as well as the three more abundant fragments of product ions. The addition of internal standard may define which isomer is present.

HPLC-DAD-MS retention times differs half a minute ca. each other, DAD peaks and valleys differs of few nanometers, nominal masses are the same, while product ion distribution differs from the third more abundant ion on (Fig. 5). The addition of internal standard may define the isomer more accurately considering the retention time difference.



HPLC-DAD-MS	2,4 Xy	2,6 Xy
Structure		
Retention time (min.)	24.81	24.13
DAD peaks (nm)	204, 232, 281	205, 234, 287
DAD valleys (nm)	199, 214, 258	199, 215,262
Mass	121.8	121.8
Product ion distribution	153.8, 104.8, 96.8	153.8, 104.8, 91.8

Fig. 5- HPLC-DAD-MSof2,4 and 2,6 Xylidine parameters and DAD / MS results.


RETANAL A FF
Blandura
Excelente igualación de tintura
Sulfona Anfótera
FORMALDEHYDE FREE PRODUCT
PLE
NI
TUD
CROMOGENIA UNITS
YOUR SPECIALIST FOR SPECIALTIES
www.cromogenia.com

6 – A 2-naphthylamine false positive case

One tannery claimed that a dye developed on leather ca. 150 mg/kg 2-Naphthylamine. Two local Italian and two well-known European laboratories' reports supported the claim, confirming the presence of this substance. The dye used for this dyeing process was C.I. Acid Black 24.

Reductive cleavage with dithionite of this dye (Fig. 6) will generate three main products and other residues will be extracted from the ethereal phase, while most of the -SO3- substituted products should be retained in the absorbent during liquid – liquid separation. Considering the 1, 4 substituted naphthalene in the ethereal phase and the relative positions of the remaining products (all naphthalene derivatives),we suspected a false positive 2-Naphthylamine case. A parallel test was done on the powder dye using IUC 21 test method.eventual residues and contaminants: 1,4 naphthalenediamine as well as other eventual lipophilic

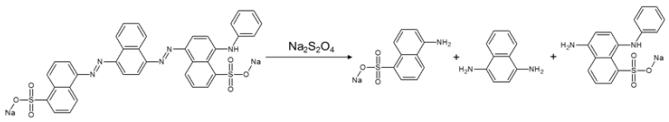


Fig. 6 - Reductive cleavage of Acid Black 24

Both extracts were analysed instrumentally in HPLC-DAD-MS: Analysis of Acid Black 24 dyed on leather (ISO 17234.1:2015) compared to the dye (IUC 21). Leather analysis shown 150 mg/kg suspected 2-naphthylamine and 240 mg/kg aniline not present in the powder dye (Fig. 7).

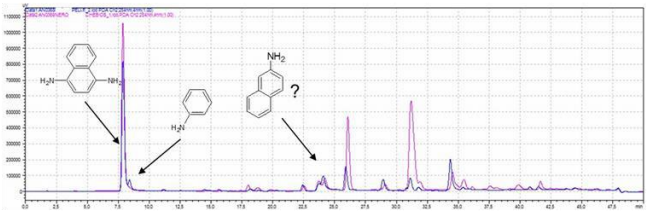


Fig. 7 - Aromatic amines HPLC Chromatogram of C.I. Acid Black 24 with ISO 17234.1:2015 on leather (blue) and IUC 21 on powder dye (red).

Comparing dyed leather sample injected against aromatic amines standard (Fig. 8) we can observe a slight running time difference: a substance with RT=24 min. and molecular weight 143.7 while 2-naphthylamine standard flows at RT=25 min. in the same analytical conditions.

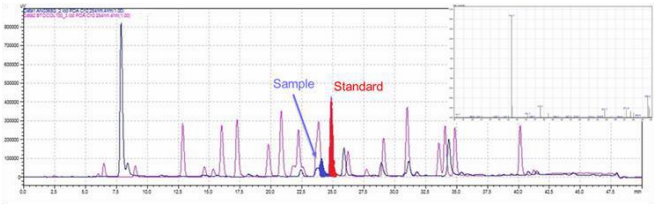


Fig. 8 - Aromatic amines HPLC Chromatogram of C.I. Acid Black 24 (blue) with ISO 17234.1:2015 against standard (red) and mass spectrum (upper right corner).

Comparison among DAD spectra showed very different results. Considering that both sample and standard have exactly the same molecular weight and very close retention time, spectral peaks and valleys occur at different wavelength. The fact that chromatographic eluents are prepared to keep a stable pH, the huge difference found cannot be attributed to a bathochromic effect but to substances with different molecular structure.

Considering the 1,4 substituted naphthalene, the most probable contaminant is 1-nafthylamine.

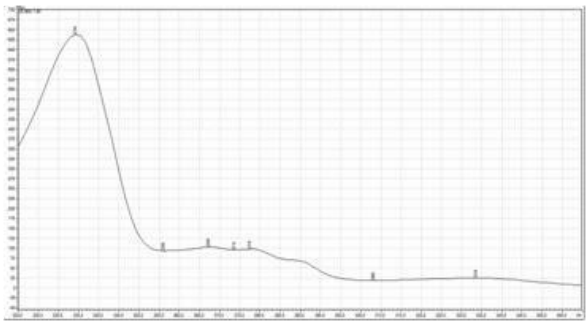


Fig. 9 - DAD spectra sample at RT= 24 min.

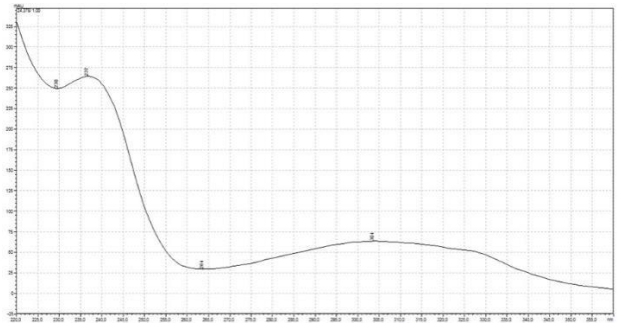
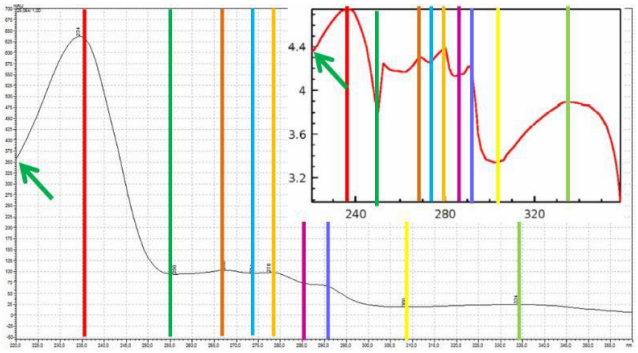


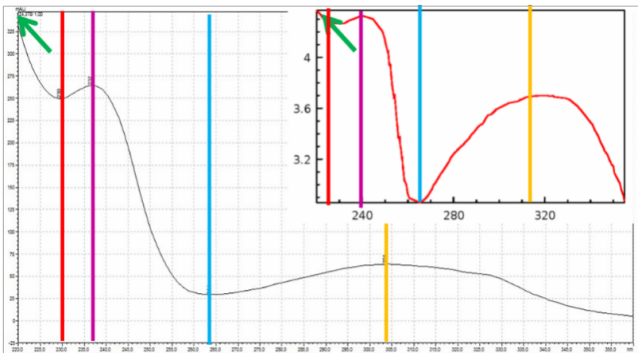
Fig. 10 - DAD spectra2-naphtylamine RT= 25 min.

We searched in the NIST spectral databank, (National Institute of Standards and Technology) both 1 and 2-naphtylamine UV spectral profile and compared them with the spectra obtained by HPLC- DAD. This analysis on 1-naphtylamine(Fig. 11) shown 10 points of similarity:



Increment from 220 nm.
Peak @ 234 nm.
Valley @ 256 nm.
Peak @ 266 nm.
Valley @ 274 nm.
Peak @ 278 nm.
Valley @ 286 nm.
Peak @ 290 nm.
Valley @ 306 nm.
Peak @ 334 nm.

Fig. 11 - Sample extracted compared with NIST's databank 1-naphthylamine



Decrement until 229 nm.
Valley @ 229 nm.
Peak @ 236 nm.
Valley @ 263 nm.
Peak @ 304 nm.

Fig. 12 - Standard compared with NIST's databank2-naphthylamine

In this case there were 5 points of coincidence: Decrement until 229 nm, a valley at 229 nm, a peak at 236 nm. a valley at 263 nm, and a peak at 304 nm.

In the case of 2-naphtylamine DAD profile was enough confirmation to define the false positive without need of internal standard addition. Clearly depending the set of instruments chosen for ISO 17234.1:2015 (for example GC-MS / HPLC-MS) this result would not have been confirmed.

7 – Conclusions

Extreme care must be taken in the selection of a correct technique and instruments. Certain instruments combinations (GC-MS + HPLC-MS) may lead to false positive results, showing us the importance of an accurate analysis of the data available from analysis (retention time, DAD spectrum, product ion distribution, etc.). In cases like this, it may be the difference among "acceptable" and "fail" results.

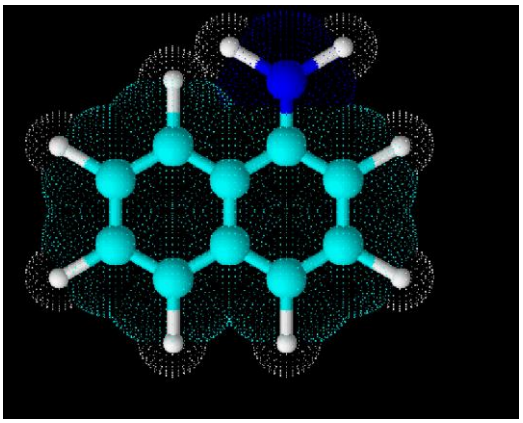


Fig. 13 - 1-naphtylamine

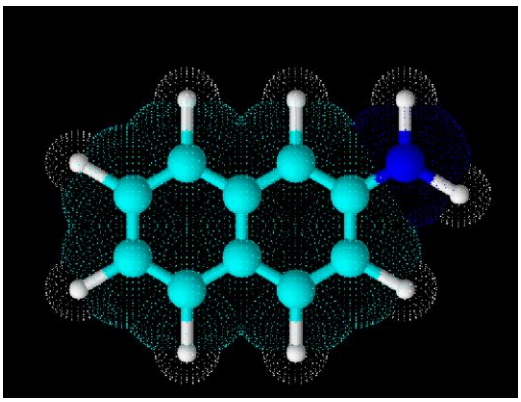


Fig. 14 - 2- naphtylamine (REACH Annex XVII)

Regarding 2-naphthylamine is included in REACH Annex XVII. This substance is restricted as free amine to 0,1%, while the limit for leather is 30 mg/kg (ISO 17234.1:2015) and textiles 20 mg/kg (ISO 14362.1). Both leather and textile tests are done with reductive cleavage. 1-naphthylamine is not included in annex XVII, but present as suspected carcinogen

in OSHA protocols.

8 – References

1. (Official Journal of the European Union, L 164/7, 26.6.2009), COMMISSION REGULATION (EC) No 552/2009 of 22 June 2009 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annex XVII.
2. (M.Fontana, M. Fenoglietto, M. Agnello 2014) Regione Piemonte, ARPA (Agenzia Regionale di Protezione Ambientale),cosmetici e rischi emergenti in campo estetico vigilanza consolidata e problemi emergenti.
3. ISO 17234-1:2015 10 pg. 6.