# New Approach for Determination of the Degradation Products of Fenspiride Hydrochloride Found in Oral Liquid Formulations

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**Abstract**

Fenspiride hydrochloride (FNS) is used in treating chronic inflammatory diseases, most commonly as a liquid oral solution. FNS produces degradation products along with fenspiride *N*-oxide (FNO) and 1-phenylethyl-4-hydroxy-4-aminomethyl piperidine hydrochloride (PHAP). We aimed to develop and validate a chromatographic method in order to identify the main degradation products in the presence of other compounds from a liquid preparation. The method used a dual gradient using two buffer solutions; first with pH 4.5 (buffer 1 (pH 4.5)); MeOH 90%:10% (*v/v*)) and the second with pH 2.9 (buffer 2 (pH 2.9); acetronitrile:methanol (65%:15%:10% *v/v/v*). As mentioned, there was a modification of the organic mixture, starting with 10% methanol and ending with a mixture of acetonitrile:methanol (15%:10% *v/v*). The flow-rate was 1.5 mL/min. According to the elution program, experimental conditions started with 100% of solution S1, which decreased to 0% and, simultaneously, solution S2 increased to 100% during the first 10 min and was maintained for a further 5 min. After 15 min, initial conditions were re-established. Linearity interval was 0.5–2 μg $mL^{-1}$ and minimum correlation coefficient was 0.999. Recovery factor was 100.47–103.17% and the limit of quantification was 0.19–0.332 μg/mL. Intra-day maximum precision was 4.08% for FNS and 2.65% for PHAP. This double-gradient mobile phase produced good specificity in relation to the degradation products of FNS and other constituents of the oral liquid formulation. Forced degradation studies revealed other related substances that were confirmed in mass balance analyses. Degradation products were confirmed in acidic, basic and oxidative media.

**Keywords**: Fenspiride hydrochloride; Dual gradient; HPLC; Reversed-phase; Degradation media.

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## Introduction

Fenspiride hydrochloride {8-(2-phenylethyl)-1-oxa-3,8-diazaspiro[4.5]decan-2-one.HCl} (FNS) is used for the treatment of chronic inflammatory diseases of the otolaryngologic system, respiratory tract (Sweetman, 2009) and in therapy for bronchial asthma, allergic rhinitis and other allergenic symptoms.

FNS can contain several impurities. Fenspiride *N*-oxide (FNO) (Arnone *et al*., 1998) is an impurity of FNS resulting from the reaction of a tertiary amine with perfluoro *cis*-2,3 dialkyl aziridines and is produced at the carbon-carbon double bond or at the nitrogen atom under protic conditions. Two other impurities have been identified and isolated as metabolic products: 3-hydroxy fenspiride and 4-hydroxy fenspiride (Megel *et al.*, 1973). Other impurities developed in preclinical studies have been identified with respect to FNS metabolism (Dumasia *et al*., 2002).

Several methods have been developed for determination of FNS impurities. For the screening of metabolites, the usual methods are liquid chromatography–mass spectrometry (LC–MS) (Pidpruzhnykov *et al.*, 2013) and gas chromatography–mass spectrometry (Dumasia *et al*., 2002). LC–MS includes the use of mobile phases suitable for positive-mode ionization, employing aqueous solutions formic acid with concentrations 0.1–0.2% and acetonitrile. Other methods include substitution of acetonitrile with mixtures of propylene carbonate and ethanol (Tachea *et al.*, 2013). For a limited number of compounds (e.g., hydroxyl metabolites), simple reversed-phase chromatographic methods coupled with mass spectrometry were employed (Medvedovici *et al.*, 2011). Other approaches include electrochemical detection for analyses of biological samples (e.g., plasma, urine) (Sauveur *et al.*, 1989).

The most common formulations are liquids, tablets and capsules administered *via* the oral route. Specificity is the greatest challenge when using a chromatographic method for liquid formulations, which contain a large number of compounds that may interfere with the main analytes. In the case of the syrups, compounds such as preservatives, excipients and flavoring agents can interfere with chromatographic separation. Oral liquid formulations may contain dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilizing and stabilizing substances as well as different flavorings that can interfere considerably with chromatographic separation (Council of Europe, 2014). In the ultraviolet range fenspiride hydrochloride produces a signal at 210 nm, which is specific to most organic compounds (Hale *et al*., 1973).

Guidelines set by the International Committee on Harmonization (ICH) for new drug products include rules for reporting known and unknown impurities based on detector responses. The identification of the main compounds and impurities will be made in a single correlation based on mass balance analyses (1).

High-performance liquid chromatography (HPLC) can be used to assess the loss in mass of the main molecule and increases in degradation products during a study. The aim of this paper was to develop and validate a suitable chromatographic method that has good specificity for the identification of the main degradation products in the presence of other compounds from a liquid preparation. This method had to be applicable in the pharmaceutical field.

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## Experimental

*Reagents and Materials*

FNS, FNO and 1-phenylethyl-4-hydroxy-4-aminomethyl piperidine hydrochloride (PHAP) were supplied by Erregierre (San Paolo d'Argon, Italy). Monosodium hydrogen phosphate, acetonitrile and methanol were purchased from Merck Millipore (Billerica, MA, USA). Purified water (HPLC grade) was produced using a purification system supplied by Elga (Lückenweg, Germany). The experimental oral liquid formulation contained: propylene glycol and anhydrous citric acid (BASF, Ludwigshafen, Germany); sorbitol (Roquette, Lestrem, France); sodium benzoate, potassium sorbate, and edetate disodium (Merck, Whitehouse Station, NJ, USA); purified water, pharmaceutical sugar (Nordic Sugar, Copenhagen, Denmark); flavorings and orange dye E110 (George Rutz, Schlieren, Switzerland).

Hydrochloric acid, sodium hydroxide and hydrogen peroxide were produced by Sigma–Aldrich (Saint Louis, MO, USA). UV degradation was initiated at a wavelength of 365 nm with a radiation exposure of 200 $W/m^2$.

*Equipment and Methods*

The chromatographic method used an Ultimate 3000 chromatographic system with a quaternary pump (600 bar), online degasser, autosampler, column thermostat and diode array detector produced by Thermo Scientific (Waltham, MA, USA). The chromatographic column was a Hypersil Gold™ aQ C18 (length, 250 mm; internal diameter, 4.6 mm; particle dimension, 5 µm; Thermo Scientific). The mobile phase consisted in a quaternary mixture with solution S1 (0.05 M monosodium hydrogen phosphate, pH 4.5), acetonitrile, methanol and solution S2 (0.05 M monosodium hydrogen phosphate, pH 2.9). The flow rate was 1.5 mL/min and the gradient elution was carried out as follows (table 1).

**Table 1.** Mobile phase gradient program.

[Insert Table 1]

Injection volume was 20 µL, column-oven temperature was 25°C and the detection was performed at 210 nm. Data analyses were undertaken on Chromeleon v7.2 (Thermo Scientific). Statistical analyses were carried out on Statistica v10 (Statsoft, Tulsa, OK, USA).

*Standard Solutions and Analytical Solutions*

Stock solutions of FNS, FNO and PHAP were prepared using a mixture of water and acetonitrile at 1 mg $mL^{-1}$. Several dilutions were carried out for validation of linearity and other parameters. Working solutions for quality control were prepared at high, low, and medium concentrations.

Analytical samples consisted in oral solutions of FNS; placebo samples of FNS were also prepared. Solutions were prepared in a mixture of S1 and acetonitrile (50%:50% *v/v*). Concentration of analytical solutions was 0.5 mg $mL^{-1}$. FNS and a reference solution of 0.1% were prepared by successive dilutions from the analytical solution. Final concentration was 0.5 µg $mL^{-1}$ for FNO and FNS, and 1 µg $mL^{-1}$ for PHAP. Identical solutions were prepared for blanks using the placebo without FNS and related impurities.

*Method Validation*

Method validation was undertaken according to ICH guidelines (2). Briefly, the method was validated in terms of linearity, specificity, limit of quantification (LOQ), precision (within-run and between run), recovery and accuracy.

The linearity range was determined by plotting minimum five calibration points and three replicates for each point. The analytical response was determined over the concentration range of 1.25–0.5 µg $mL^{-1}$ for FNO and FNS, and 2.5–1 µg $mL^{-1}$ for PHAP. Accuracy and recovery

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were determined for three concentrations of spiked samples in the placebo product in the range of 0.5, 0.75 and 1 μg $mL^{-1}$ for FNO and FNS, as well as 1, 1.5, and 2.5 μg $mL^{-1}$ for PHAP. Along with recovery factors, the percentage error was calculated.

Specificity testing involved blank samples, samples containing placebo and samples containing the liquid product. Peak retention, peak areas and secondary signals were evaluated from the corresponding chromatograms.

The LOQ was determined using solutions prepared with placebo as well as solutions of FNS and FNO at a nominal concentration of 0.5 μg $mL^{-1}$ and 1 μg $mL^{-1}$ for PHAP. Corresponding peaks were analyzed with respect to the height and signal-to-noise ratio. The LOQ was determined by using the factor 10 and the relative standard deviation (RSD) of the signal for five consecutive determinations.

Within-run and between-run determinations for precision were considered using the RSD. Differences between runs using different analysts or different days were determined using analysis of variance (ANOVA).

Stability of the samples was determined in degradation media at various time intervals. The interval was 24 h for hydrochloric acid (0.5 N), sodium hydroxide (10%) and hydrogen peroxide (10%) at 60°C. Samples were exposed to a temperature of 80°C for 72 h. Samples were exposed to UV light at a wavelength of 365 nm for 24 h. The chromatograms were recorded and signals were associated with unknown impurities. Samples containing placebo were used to validate secondary signals against other compounds in the matrix.

**Results**

The proposed method involved determination of the optimal conditions for specificity in relation to various secondary compounds (e.g., sodium benzoate, potassium sorbate, fragrances) present in a pharmaceutical product. The method validation involved optimization of the concentration in relation to the quantity of product used for determination of impurities present in FNS.

*Method Development*

Our HPLC method involved the use of a gradient for the organic solvents, but also a linear variation of pH from 4.5 to 2.9. The pH gradient was needed because of insufficient resolution between the fenspiride hydrochloride peak and secondary signals. A similar approach is used in the *European Pharmacopoeia* (Council of Europe, 2014). The method development started with the separately use of the two pH systems: 4.5 and 2.9. First, solutions containing buffer pH-4.5 and acetonitrile in 85%:15% (*v/v*) proportion were used. Buffer with pH- 4.5 enabled elution of most compounds in range for retention time from 8 to 13 min. In the same conditions, pH-2.9 increased the retention time to 15 min.

Optimization of specificity continued with the use of a mobile phase containing buffer solution 1 (pH 4.5), acetonitrile and methanol with a proportion (75%:15%:10% (v/v/v)). Main resolution factor was 3.12, and the total chromatographic dramatically decreased to 4.88 min. Lowering the pH to 2.9 using also acetonitrile and methanol (75%:15%:10% (v/v/v)) increased the total chromatographic time to 13.3 min, but the median resolution was 8.26, which showed a higher distribution of signals (which could result in a loss of specificity).

To choose the right chromatographic system, a full factorial design was used for the optimization of our model. The design had 2 factors, 4 runs and 1 block. Factors were coded as –1 and +1. First factor was associated with a variation in pH. We have used pH 4.5 as (–1) and the gradient of the pH from 4.5 to 2.9 (β=0.66) as (+1). Second factor was acetonitrile variation (ACN) in the mobile phase. A change in the pH and also acetonitrile produced important modifications in terms of resolution and separation efficiency. Factor code (–1) was associated with an isocratic percentage of 10% for acetonitrile in the mobile phase, and (+1)

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was associated with a gradient from 0% to 15% during 10 min chromatographic time and maintained at 15% for an additional 5 min (Table 2).

**Table 2**. Factor codes and factors used in the central composite design.
[Insert Table 2]

In the design of experiment we have chosen another complementary pair of factors, namely the slope with 0.66 value for pH from 4.5 to 2.9 and also flow-rate.
The full factorial design used a multilinear regression model and evaluated the final response (terms of chromatographic response function) as a function of the experimental modifications and estimated effects upon the factors (Gamst *et al.*, 2008).
In this case, the equation had the following distribution:

$$Y = 71.17 + 31.36pH + 32.44ACN + 13.15pH * ACN \quad \text{Eq. (1)}$$

A high interaction was produced mostly upon pH modification as well as the acetonitrile variation (13.15). The values of individual factors elicited important effects on the performance. Equation 1 shows that the greatest influence was produced upon pH modification (31.36) and variation of acetonitrile (32.44).

Parameters in the model were: final chromatographic time, resolution between each peak and the number of peaks. These parameters were included in the chromatographic response function (CRF) for evaluation of the performance of our method (Poole, 2003).
According to the method (Gamst *et al*., 2008), the CRF is expressed as:

$$CRF = \sum R_i + n^a - b(t_x - t_{01}) - c(t_{02} - t_1) \quad \text{Eq. (2)}$$

Where *Ri* is the resolution obtained for the width peak pair, *n* is the total number of detected peaks, $t_{01}$ is the minimum desired time for the first eluted peak, $t_1$ is the observed retention time for the first eluted peak, $t_x$ is the maximum desired chromatography time, $t_{02}$ is the retention time of the final eluted peak, and *a*, *b* and *c* are adjustable weighting factors. Factors *a*, *b* and *c* are equal to 2 to increase the selection of criteria at high significance.
Under these conditions, the CRF had maximum values when using a linear gradient for the pH and linear variation of the organic modifier (acetonitrile). Furthermore, a higher resolution factor was noted between the peak corresponding to FNS and adjacent signals, which could enable identification of the degradation products.
The model was validated by undertaking an experiment in the predicted conditions. Distribution of the desired function (CRF) was calculated according to the monitoring parameters (pH gradient and organic-modifier gradient) and is presented in figure 1.
Finally, in the chromatogram of the test solution we identified signals for FNO, FNS, PHAP, sodium benzoate, potassium sorbate and three signals corresponding to fragrances.

[Insert Figure 1]
**Figure 1**. 3D plot for CRF as overall function of method performance in relation with gradient pH (from pH -4.5 to 2.9 in 10 minutes, slope 0.66) and acetonitrile variation (0% to 15% during 10 minutes of linear gradient, as slope 1.00).

*Method Validation*
*Method Specificity*

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Analytical samples of fenspiride and specified impurities were prepared and evaluated using the developed method. Chromatograms confirmed that all the compounds were determined with high accuracy and the purity was ≥ 98.
Chromatograms corresponding to analytical samples after degradation revealed a high profile of impurities, including unspecified impurities. These impurities showed no interference with the main target compounds. Sample chromatograms are presented in Figure 2. Our results show a method with higher selectivity capacity being able to produce a better separation between a greater number of compounds than other methods reported in the literature (Sauveur *et al.*, 1989), thus we can easily separate, the main substance, two known impurities, three unknown impurities revealed in the stability and 4 specific compounds from matrix which give intensive signals at the wavelength of 210 nm. Also, the method is superior to other reported approaches that used the GC-MS because the peaks were revealed by direct identification, none of supplementary derivatization procedure needed to expose the characteristic signals (Dumasia *et al*., 2002).

[Insert Figure 2]
**Figure 2.** Chromatograms a) placebo samples; b) spiked placebo with 0,5 µg/ml FNO, FNS and 1 µg/ml PHAP; c) standard of 0,5 µg/ml FNO, FNS and 1 µg/ml PHAP; d) Sample under oxidation conditions; e) Sample under basic media conditions (NaOH 1M).

*Linearity*
Linearity was evaluated in the range of 0.5–1.25 µg $mL^{-1}$ for FNO and FNS, and 1–2.5 µg $mL^{-1}$ for PHAP. The difference between the FNO, FNS and PHAP is determined by the fact that FNO was limited to 0.1% and PHAP to 0.2%. Unspecified impurities were limited to 0.1%.
Calibration curves were generated from standards prepared in mobile phase. A partial linear regression equation with concentration as a function of area was evaluated. The mean values for the regression coefficient were $R^2$=0.997 for FNO, 0.996 for FNS, and 0.997 for PHAP.
ANOVA statistics on the regression parameter showed that was a direct relation between the concentration and peak area with a confidence level higher than 95% ($p<0.05$). The calculated *t* value is higher than the statistical critical of 2.16 so the model explains the dependency and the null hypothesis is rejected (3).

*LOQ*
LOQ was determined using three independent standards and blank samples. The signal-to-noise ratio was calculated for confirmation of LOQ. Using the standard error of slope, the LOQ (in µg $mL^{-1}$) was 0.19 for FNSO, 0.195 for FNS and 0.33 for PHAP, therefore the detection limits show comparative values with those which are reported in the literature (Sauveur *et al.*, 1989).

*Accuracy*
Accuracy was evaluated by calculation of the recovery factors determined by spiking placebo samples with the concentrations that showed linearity. Three model intervals were used for the evaluation of recovery at low, medium, and high concentrations (Poole, 2003).
Mean recovery was 103.18% for FNO, 100.47% for FNS and 100.46% for PHAP. Percent deviations that explained the maximum skewness of regression were ≤5%. The maximum value was 4.8% for FNO, 3.78% for FNS and 4.7% for PHAP.

*Precision*
Precision was evaluated according to signal stability using repeated determinations on 10 replicates undertaken on the same day and in different days by different analysts. One-way

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ANOVA with replicate tests was used for within-day evaluation. Stability of the retention time and peak area were evaluated. For FNO with a retention time of 4.49 min, the RSD was 0.31 %, for PHAP it was 0.99 % and for FNS it was 0.13 %. With regard to peak area, a low concentration range produced a higher dispersion of area. RSD showed higher values (2.65–4.08%) for target analytes. Within-run precision showed no significant difference between groups for retention time ($p<0.05$) but a significant difference was noted for variation in peak area.
A summary for the method validation is shown in Table 3.

**Table 3**. Method validation parameters.
[Insert Table 3]

*Stability Studies*
Analytical samples were exposed to hydrochloric acid, sodium hydroxide, hydrogen peroxide, temperature and UV light. Concentration of analytical solutions was 0.5 mg $mL^{-1}$. Mass balance was evaluated by analyses against the FNS standard. Known impurities were quantified using the linear slope. Unknown impurities were evaluated by assessment of the area to the slope of FNS.
Considerable degradations were produced in oxidative media. The FNS signal decreased and the concentration was shown to be 0.4 mg $mL^{-1}$. FNO and PHAP were determined in oxidative media. A new impurity ("impurity 1"), with a relative retention time of 0.55, and "impurity 2", with a relative retention time of 1.28, were recorded.
Acidic media produced impurity 1. Basic media produced another impurity with a relative retention time of 2.22 ("impurity 3"). To confirm the source of impurities associated with fenspiride, placebo samples were included: these signals were not present (figure 2).
The mass balance study verified the correlation between the decrease in the FNS signal and the presence of known and unknown impurities. A mass balance evaluation has been produced for monitoring of possible changes related to direct degradation of active substances [16]. The mass balance for all stressed samples (% analyte + % known impurities + % unknown impurities) was >98% (Table 4). In all cases, the purity of the signals was not affected by the presence of other substances and degradation products.

**Table 4**. Mass balance analysis used in the stability study performed on the acidic, basic, oxidant, UV and temperature degradation media.
[Insert Table 4]

*Robustness*
Robustness was evaluated by modification of certain parameters (composition of organic mixture in solution S2; temperature; starting value of pH) all at a variation of ±10%. Monitored responses were resolved between all compounds through the CRF. Modifications were produced according to a three-factor central composite design that included two replicates of a standard point. Factors were coded and regression analyses were performed for CRF, as plotted in table 5. Using a 5% significance level, the calculated value of the Pearson coefficient was not considered to significantly affect the response of the factors to pH or final composition of the solution ($p<0.05$).

Three-dimensional plots against two factors showed that the pH produced the highest level of modification of responses. However, variations in temperature and flow rate produced a linear modification in terms of total chromatographic time (figure 3).

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**Table 5.** Effect Estimates. Var.: RS (m); R-sqr =0.9987; Adj: 0.99089 3 factors at two levels; MS Residual = 0.1826485, DV: RS(m).
[Insert Table 5]

[Insert Figure 3]
**Figure 3.** 3D plot for medium resolution variation (CRF) variation at modification with ±10% of a) – (pH –solution b composition), b) (temperature (°C)-pH), and c) solution b composition-pH.

## Conclusions

A novel, simple and robust method for determination of FNS and its impurities was developed. This method is accurate and presents sufficient sensitivity in order to enable the detection of impurities at low concentrations.
The method used a dual gradient. The first gradient was for pH (4.5 to 2.9). The second gradient was variation of the organic mixture, starting with 10% methanol and ending with a mixture of acetonitrile:methanol (15%:10% *v/v*) with the flow of 1.5 mL/min. The method used solution S1 in buffer 1 (pH 4.5; MeOH 90%:10% (*v/v*)) and solution S2 in buffer 2 (pH 2.9; acetronitrile:methanol (65%:15%:10% *v/v/v*). According to the elution program, experimental conditions started with 100% of solution S1 which decreased to 0% and simultaneously, solution S2 was increased to 100% during first 10 min and maintained for a further 5 min. After 15 min, initial conditions were re-established.
These conditions were necessary to establish a good selectivity in relation with several degradation products which were revealed in the stability studies. Also, the method identified a greater number of compounds, all the signals which were revealed at the wavelength of 210 nm were completely separated and those we could identify all the compounds from the pharmaceutical formulation based on their ability to give signals in uvvis detection. Also, the method has low detection limits which gives the opportunity to evaluate the structure of possible toxic degradation products al low levels of concentration.
The most important parameters that affected the specificity of the proposed method were pH, temperature and organic components. The robustness of our method was not affected by variation in these parameters within a limit of ±10%. This method is suitable for pharmaceutical agents and active substances that require HPLC determination.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding publication of this article.

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**Table 1**. Mobile phase gradient program.

Elution of solution S1 from 90% to 0% in 10 minute. Methanol constant at 10% for 18 minutes. Acetonitrile from 0 to 15% and solution S2 to 75% in 10 minutes. From 10 minutes to 15 minutes constant at acetonitrile:methanol:solution S2 (15:10:75). From 15 to 16 minutes initialization of initial conditions and reechilibration for 2 minutes.

| Time (min) | Solution S1 (%) | Acetonitrile (%) | Methanol (%) | Solution S2 (%) |
|---|---|---|---|---|
| 0 | 90 | - | 10 | - |
| 10 | - | 15 | 10 | 75 |
| 15 | - | 15 | 10 | 75 |
| 16 | 90 | - | 10 | - |
| 18 | 90 | - | 10 | - |

**Table 2.** Factor codes and factors used in the central composite design.

| Factor codes | | Factors | | Total chromatographic time | Median resolution | Number of peaks | CRF |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 0.66 | 1.5 | 13.3 | 8.26 | 7 | 48.75 |
| -1 | 1 | 0 | 1.5 | 8.10 | 3.12 | 6 | 19.55 |
| -1 | -1 | 0 | 1 | 10.5 | 3.50 | 6 | 42.39 |
| 1 | -1 | 0.66 | 1 | 14.9 | 7.18 | 7 | 17.61 |

**Table 3.** Method validation parameters.

| Parameter | | FNO | FNS | PHAP |
|---|---|---|---|---|
| Repeatability | Retention time | 0.31 | 0.13 | 0.99 |
| Within day Precision, RSD% | | 2.38 | 1.46 | 0.69 |
| Intra day precision, RSD% | | 3.05 | 4.08 | 2.65 |
| Rezolution | | 15.05 | 8.92 | 11.7 |
| Theoretical plates | | 10189 | 25801 | 33457 |
| Peak purity | | 0.997 | 0.996 | 0.997 |
| Liniarity | Error of slope | 0.933 | 0.894 | 0.793 |
| | Correlation coefficient | 0.998 | 0.995 | 0.996 |
| Accuracy | Average recovery | 103.18 | 100.47 | 100.46 |
| | Skewness $O_x$% | 0.8587 | 0.3164 | 0.4222 |
| Limit of qualification, µg/ml | | 0.190 | 0.195 | 0.332 |
| Confidence level of slope, 95% | | 21.49 26.33 | 22.45 – 26.45 | 26.396 - 31.443 |

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**Table 4.** Mass balance analysis used in the stability study performed on the acidic, basic, oxidant, UV and temperature degradation media.

| | Imp 1 | FNO | FNS | PHAP | Imp2 | Imp3 | TOTAL |
|---|---|---|---|---|---|---|---|
| Acidic media | 0.65 | | 97.56 | | | | 98.21 |
| Basic media | | | 97.75 | | | 0.78 | 98.52 |
| Oxidant media | 3.83 | 4.19 | 75.52 | 3.50 | 13.22 | | 100.2 |
| UV exposure | | | 98.57 | | | | 98.57 |
| Temperature | | | 98.78 | | | | 98.78 |

**Table 5.** Effect Estimates. Var.: RS (m); R-sqr =0.9987; Adj: 0.99089 3 factors at two levels; MS Residual = 0.1826485, DV: RS(m).

| | **Effect** | **Std.Err.** | **t(1)** | **p** | **-95.% - Cnf.Limt** | **+95.% - Cnf.Limt** |
|---|---|---|---|---|---|---|
| **Mean/Interc.** | 10.401 | 0.153 | 67.786 | 0.009 | 8.452 | 12.351 |
| **(1)pH** | 5.068 | 0.307 | 16.514 | 0.039 | 1.168 | 8.967 |
| **(2)Flow** | -3.628 | 0.342 | -10.605 | 0.060 | -7.974 | 0.719 |
| **(3)Temp** | -5.184 | 0.304 | -17.050 | 0.037 | -9.047 | -1.321 |
| **1 by 2** | -1.101 | 0.342 | -3.217 | 0.192 | -5.447 | 3.246 |
| **1 by 3** | -1.254 | 0.304 | -4.126 | 0.151 | -5.118 | 2.609 |
| **2 by 3** | 1.087 | 0.334 | 3.256 | 0.190 | -3.154 | 5.327 |

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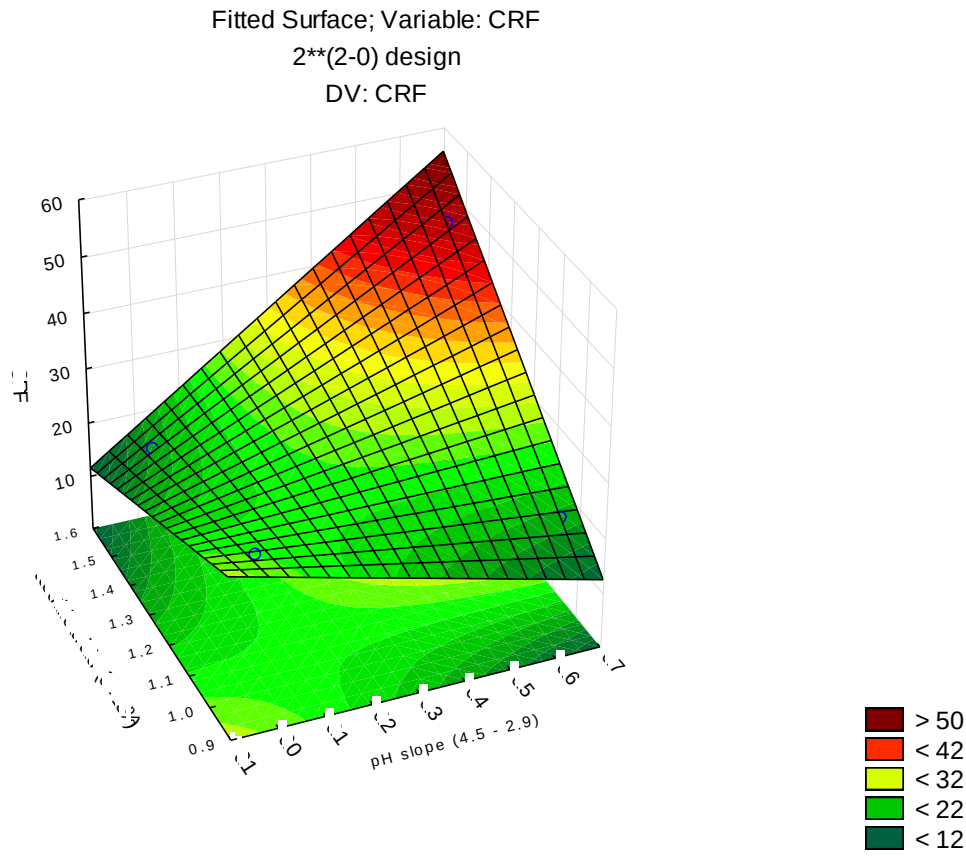


**Figure 1.** Three dimensional plot for CRF as overall function of method performance in relation with gradient pH (from pH - 4.5 to 2.9 in 10 minutes, slope 0.66) and acetonitrile variation (0% to 15% during 10 minutes of linear gradient, as slope 1.00).

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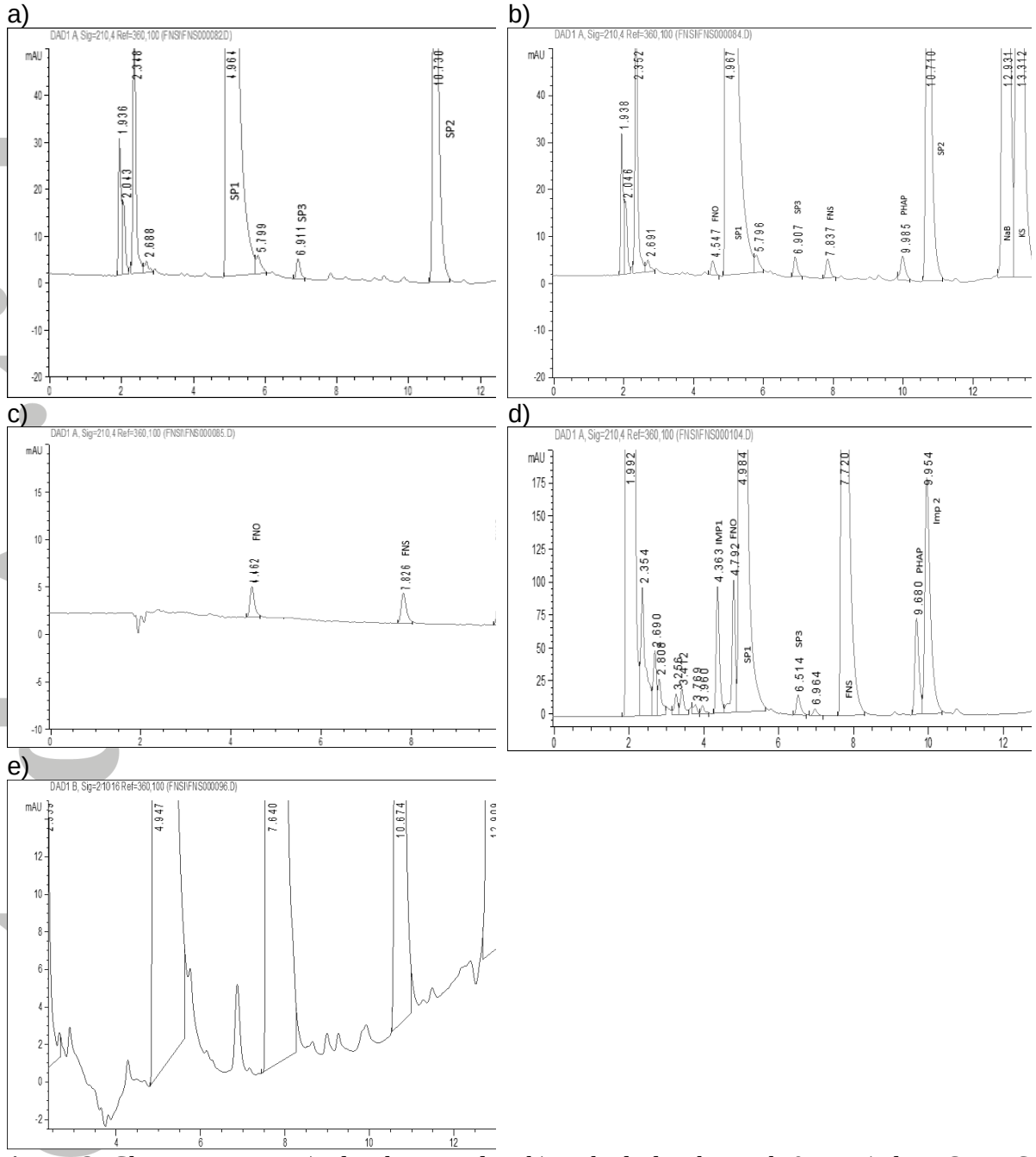


**Figure 2.** Chromatograms a) placebo samples; b) spiked placebo with 0,5 µg/ml FNO, FNS and 1 µg/ml PHAP; c) standard of 0,5 µg/ml FNO, FNS and 1 µg/ml PHAP; d) Sample under oxidation conditions; e) Sample under basic media conditions (NaOH 1M).

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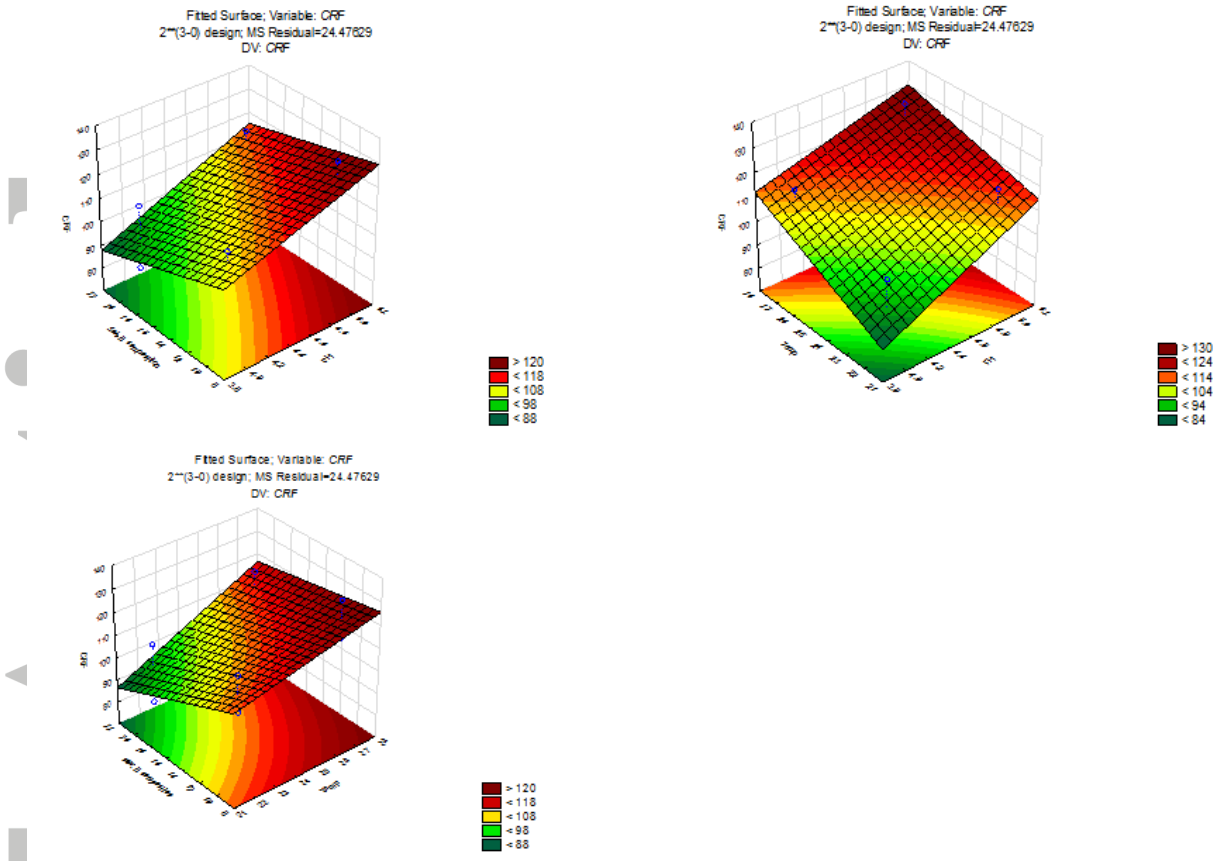


**Figure 3.** Three dimensional plot for medium resolution variation (CRF) variation at modification with ±10% of a) – (pH –solution b composition), b) (temperature (°C) - pH) , and c) solution b composition - pH.

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