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# DETERMINATION OF BIOGENIC AMINES IN WINES BY HPLC-UV AND LC-ESI-MS: A COMPARATIVE STUDY

*This study deals with the comparison between two different methods, high pressure liquid chromatography with UV detection (HPLC-UV) and liquid chromatographic-electrospray ion trap mass spectrometry (LC-ESI-MS), for the determination of eleven biogenic amines in wine samples.*

Bioactive amines may be both essential and detrimental to health [1]. When originating from the natural metabolic pathway they are called "natural polyamines" and are indispensable compounds for cells to either grow or function in an optimal manner [2, 3]. When they are formed by microbial decarboxilation of the corresponding amino acids, they are designated "biogenic" and are known to induce various negative pharmacological reactions [1]. In wine the amount and composition of these bioactive compounds depend on three factors: composition of the grape clusters in natural polyamines and amino acids, fermentation process and pH [4, 6]. During fermentation processes (alcoholic and malolactic) wine acquires its alcohol content and enhances its body and flavour persistence. However, if the microflora possesses decarboxylation enzymes, other substrates, such as amino acids, can be metabolized into biogenic amines. It is well known that high pH values of the culture medium can positively affect the microflora's growth. Consequently, when wine's pH is high more biogenic amines are produced, thus explaining why red wines which have higher pH values show a higher biogenic amines concentration compared to white wines. The main biogenic amines found in wine as a result of amino acids decarboxilation are tyramine, histamine, putrescine and phenylethylamine. Tyramine and histamine are the most effective. They show severe

adverse effects on the central nervous and vascular systems. Tyramine alone, or with phenylethylamine can cause headache due to their vasoconstrictise properties [7]. Histamine has been related to many food poisoning symptoms such as nausea, red rush, cold sweat, palpitations, high or low blood pressure, respiratory distress [8]. These pharmacological effects depend on individual sensitivity and on the simultaneous presence of co-factors (ethanol, drugs, other amines). Therefore, the determination of biogenic amines in wine is of primary importance from a security point of view.

The biogenic amines determination is not simple because of their structure and because they are usually present at low concentrations in wine.

Several methods have been reported for the analysis of biogenic amines. They include HPLC [9-13], gas chromatography [14-16], capillary electrophoresis [17-23], micellar electrokinetic capillary chromatography [24, 25] and micellar liquid chromatography [26]. Among these HPLC is the most used due to its high resolution and sensitivity. As biogenic amines do not show satisfactory absorption in the visible and ultraviolet range nor do they show fluorescence, pre- or post-column chemical derivatization is considered a necessary analytical step. For this purpose, *o*-phthaldialdehyde, fluorenylmethylchloroformate and dansyl or dabsyl chlorides are the most utilized labelling reagents [27-31]. The derivatization step increases the sensitivity of the method but the drawbacks associated with derivatization process, such as low recovery (analyte loss), contamination and time consuming analysis, can also be considered.

HPLC coupled with MS spectrometry is compatible with the detection of raw (underivatized) amines [32] although most of the methods reported in the literature include a derivatization step in order to facilitate the separation and to improve the sensitivity [33]. Once again, drawbacks like side reaction amine products and the presence of additional components may cause a poor resolution of the chromatographic peaks.

The aim of the present work is the determination of 11 bioactive amines, methylamine (Mea), ethylamine (Eta), tryptamine (Trp), β-phenylethylamine (β-Pea), putrescine (Put), cadaverine (Cad), histamine (His), serotonin (Ser), tyramine (Tyr), spermidine (Spd) and spermine (Spm), in 22 red and white commercial wines using HPLC-UV and dansyl chloride as derivatizating agent and LC-ESI-MS without derivatization. A critical comparison between the two different methods has also been presented in order to clearly highlight the advantages and disadvantages of both techniques and facilitate the choice of the most proper method.

## Materials and methods

Methylamine (MEA), ethylamine (ETA), tryptamine (TRP), β-phenylethylamine (β-PEA), putrescine (PUT), cadaverine (CAD), histamine (HIS), serotonine (SER), tyramine (TYR), spermidine (SPD), spermine (SPM), dansyl chloride, heptafluorobutiric acid (HFBA) and 1,7-diaminoheptane, used as internal standard, were purchased by Sigma Chemical (St. Louis, USA). $HClO_4$ and methanol of chromatographic grade were obtained from Carlo Erba (Milan, Italy).

For chromatographic analysis acetonitrile of HPLC grade Merck (Darmstadt, Germany) and purified water Millipore (Bedford, MA, USA) were used throughout.

The wine samples, 12 white wines and 10 red wines, produced in different regions of Italy, were purchased from local supermarkets.

For the preparation of the amine standard solutions, an individual standard solution of 1 mg/ml of each amine were prepared in purified water and stored in darkness at 4±1 °C, while a standard solution containing all the amines (Mix 11) was obtained with 1 ml of each water solution diluted to 25 ml with purified water. Different aliquots of the standard solution were used to obtain the necessary final amine concentrations to construct calibration curves and to perform recovery experiments. For HPLC-UV analysis the standard solutions were added with $HClO_4$ 10.3 M in such a manner to obtain a final acid concentration of 0.2 M. The final amine concentration injected were 0.1, 0.4, 0.8, 4.0, 8.0 and 16.0 mg $l^{-1}$. All solutions contained the internal standard at the same concentration of 0.8 mg $l^{-1}$.

For LC-ESI-MS analysis the amine standard solutions were acidified with HFBA to obtain a final acid concentration of 5 mM and ranged from 0.1 to 16 mg $l^{-1}$ with 0.8 mg $l^{-1}$ of internal standard, as for standard solutions used in HPLC-UV.

For HPLC-UV the derivatization of the standard solutions was necessary before analysis while for LC-ESI-MS no derivatization was carried. The dansylated derivatives of the amines were formed by adding to a 1 ml standard solution Mix 11, 200 µl of NaOH 2N, 300 µl of satured $NaHCO_3$ solution and 2 ml of dansyl chloride solution (15 mg/ml in acetone). Fresh dansyl chloride solutions were prepared each time just before use. After shaking, samples were left in the dark at room temperature for 20 min [34]. To stop the reaction 100 ml of $NH_4OH$ 25% v/v were added and the final volume was adjusted to 5 ml with acetonitrile. After filtration, a volume aliquot of 50 µl was injected for the HPLC-UV analysis.

As for the wine sample preparation, wine samples were initially filtered through a 0.20 µm membrane Millipore filter. Then, for HPLC-UV analysis, 25 ml of the filtered wine samples were added with $HClO_4$ 10.3 M to obtain the final acid concentration of 0.2 M. 1 ml of the acidified wine was successively derivatized as previously described. After a second filtration, a volume aliquot of 50 µl was injected (loop 50 µl) for the HPLC analysis. For LC-ESI-MS analysis 25 ml of the filtered wine samples were added with HFBA to obtain a final acid concentration of 10 mM. After a second filtration, a volume aliquot of 50 µl was injected in the chromatographic column.

All measurements in the HPLC-UV analysis were performed with a liquid chromatograph Shimadzu (Tokyo, Japan) LC-10 ATVP, equipped with a UV-VIS detector SP-10 AVP (Shimadzu) operating at λ=254 nm and a loop of 50 µl. The analytical column was Supelcosil LC-18 (250 mm x 4.6 mm x 5 µm) with a Supelguard LC-18 (Supelco Inc., Bellefonte) pre-column. The analysis were performed to a fixed temperature

Tab. 1 - Data acquisition parameters used in LC-ESI-MS for the detection of biogenic amine (SIM conditions)

| Biogenic amines | MW | Channel, *m/z* (relative aboundance) | Cone voltage (V) | Retention window (min) |
|---|---|---|---|---|
| Tyramine | 137.2 | 121.2(30), 138.3 (100) | 30 | 0-12.85 |
| β-phenylethylamine | 121.2 | 105.1 (10), 122.3 (100) | 30 | 12.85-16.00 |
| Putrescine | 88.2 | 89.3 (100) | 40 | 0-12.85 |
| Cadaverine | 102.2 | 86.2 (10), 103.3 (100) | 30 | 0-12.85 |
| Histamine | 111.1 | 95.2 (30), 112.1 (100) | 40 | 0-12.85 |
| Serotonin | 176.2 | 160.3 (10), 177.2 (100) | 40 | 0-12.85 |
| Tryptamine | 160.2 | 144.3 (40), 161.2 (100) | 30 | 12.85-16.00 |
| Spermidine | 145.2 | 112.3 (10), 129.2 (10), 146.3 (100) | 40 | 12.85-16.00 |
| Spermine | 202.3 | 129.2 (20), 112.3 (10), 203.4 (100) | 40 | 12.85-16.00 |

of 25 °C. A homogenizer Universal Laboratory Aid MPW-309 and a centrifuge ALC 4236 were also employed.

All measurements in the LC-ESI-MS analysis were achieved using a Thermoquest (Manchester, UK) model P2000 with an Alltima (Alltech, IL, USA) C18 reverse-phase column (250 x 4.6 mm i.d., particle size 5 µm). Mass spectrometric analysis was carried out on a Finnigan AQA benchtop single-quadrupole mass spectrometer (Thermoquest). The ESI unit operated at 4.0 kV, the capillary was heated at 200 °C and nitrogen was used as desolvation and nebulizer gas at a flow rate 300 and 50 L/hour, respectively. The ESI-MS system operated in the positive ionization mode (PI). Diagnostic fragment ions were obtained by in-source collisop-induced dissociation (CID) of the protonated molecule $[M+H]^+$ after optimization of the voltage of the skimmer cone. Selected ion monitoring (SIM) was applied for the time-scheduled recording of the analytes.

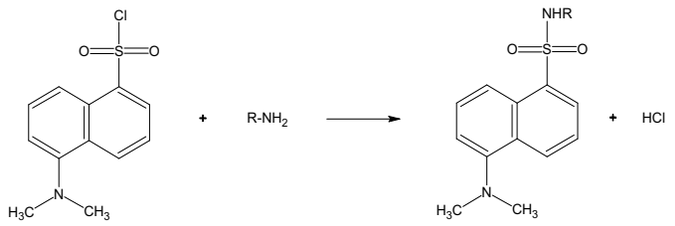
Data acquisition parameters are reported in Tab. 1.

Instrument control, data acquisition and processing were carried out with Mass Lab (version 2.22) from Thermoquest Finnigan (Manchester, UK).

The chromatographic conditions were different in the two methods.

For the HPLC-UV analysis, two solvent reservoirs containing (A) purified water and (B) acetonitrile were used to separate all the amines with an HPLC elution programme which began with 3 min of isocratic programme A-B 50:50 (v/v) reaching after 20 min A-B 10:90 (v/v). Then 3 min of isocratic elution were carried out and 4 min further were necessary to restore again the starting conditions (A-B 50:50, v/v). Flow was kept constant at 1.2 ml/min, for a total analysis time of 30 min and a time interval of 10 min between two injections was applied.

Scheme 1




The injected volume was 50 µL. For the LC-ESI-MS analysis, the mobile phase solvents A and B were methanol (10 mM heptafluorobutiric acid) and water (10 mM heptafluorobutiric acid) respectively, at a flow rate of 1 ml/min. The column was maintained at room temperature and analytes were eluted using an initial linear gradient program from 10% of solvent A to 85% in 15 min, then passing from 85% of solvent A to 100% in 1 min, followed by an isocratic elution of 100% of A for 3 min. An additional 10 min was added to reach the initial conditions.

The injected volume was 50 µL.

## Results and discussion

### *Optimization of derivatization conditions for the HPLC-UV analysis*

In order to optimize the derivatization conditions the following parameters were studied: pH, temperature and reaction time.

The dansylation reaction between dansyl chloride and a primary amine is shown in Scheme 1. HCl is formed during the reaction and therefore pH can affect the equilibrium of the reaction. The influence of pH on

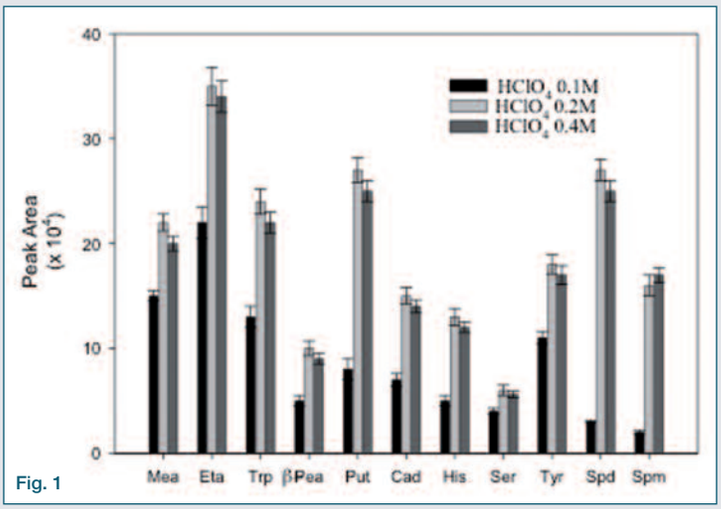



Fig. 1

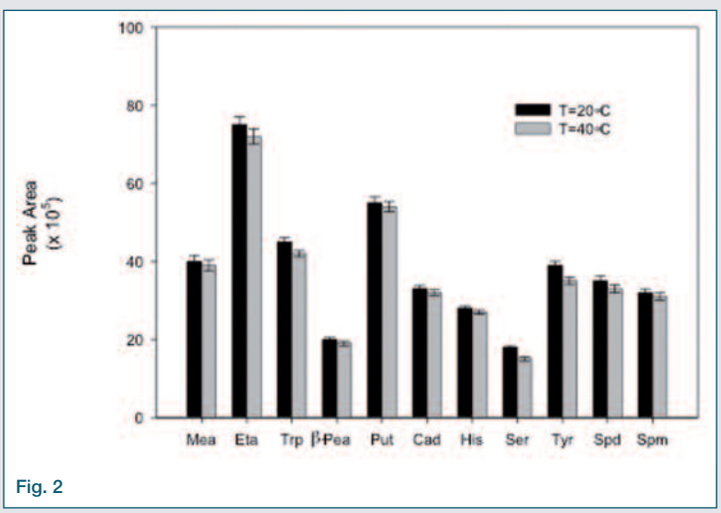



Fig. 2

the derivatization reaction of standard amine solutions was studied in terms of $HClO_4$ concentration added to the solution, as reported in Fig. 1. The effect of $HClO_4$ concentration was tested in the range from 0.1 to 0.4 M. It was found that peak areas of the derivatives reached the largest values with a concentration of $HClO_4$ of 0.2 M, which was chosen as the optimal acid concentration for derivatization. Reaction temperature was also studied: the dansylation reaction was carried out at two different temperatures: 20 °C and 40 °C. As shown in Fig. 2 no appreciable difference was observed when the temperature was increased at 40 °C and therefore 20 °C was selected as the optimum temperature, being close to the room temperature.

Additionally, the reaction time was studied in the range 20÷180 min. At increasing time, an increase of the peak areas of about 2-3% was observed during the first 60 min of reaction for all amine solutions studied, while no further increase was registered in the following time. Therefore 20 min was chosen as the optimal time for further experiments.

### *Optimization of the LC-ESI-MS conditions*

In order to investigate the fragmentation behaviour of these analytes, single amine standard solutions were injected without any column and analyzed in the full scan mode. These analytes have low relative molecular mass resulting in very small number of fragments. The MS conditions optimized to obtain maximum fragments are summarized in Tab. 1. For quantitative determination in select ion monitoring, the quasi molecular ion $[M+H]^+$ was selected for all compounds. Nevertheless for specificity reasons, the detection of two or three confirming ions was carried out. In particular, the quasi molecular ion which has lost a $NH_3$ group and for spermidine and spermine the quasi molecular ion without two $NH_3$ molecules were selected. In the case of putrescine its low molecular weight allowed the monitoring of the quasi molecular ion only.

Successively a reverse phase C18 column was installed to achieve amine separation. Biogenic amines are organic bases without any large hydrophobic side-chains; as a consequence reverse phase chromatography is ineffective, eluting them with the dead volume. To overcome this problem, underivatized amines can be separated by ion-pair reversed phase liquid chromatography. The choice of the ion-pairing reagent has to fit two conditions: the first is to permit sufficient retention for good chromatographic separation and the second, most important, is that this reagent has to be volatile with minimum signal suppression. The additive has to allow at the same time optimum separation and recovery of amines and optimum detection by LC-ESI-MS. The addition of an acid to the mobile phase increases the retention times of the different analytes. This effect was due to the interactions between the negative charges on the inner column surface provided by the acid and the positive charges of the amines.

Among the acid ion-pairing agents HFBA has demonstrated to work well in LC-ESI-MS. Moreover, a low pH (2<pH<3) improves the analyte's ionization efficiency and analytical sensitivity due to the capacity of HFBA to facilitate nebulization and desolvation in the electrospray ionization source. Therefore, the use of HFBA allowed to obtain a longer total run time for best amine separation and the elution of other components present in the matrix that could co-elute with the analytes. The concentration of the ion-pairing reagent HFBA was studied as it is recommended to use a concentration as low as possible to avoid any signal suppression of the analytes. Some standard amine solutions were studied at four different HFBA concentrations (in the range from 1mM to 10 mM) of the mobile phase. Fig. 3 shows that increasing amounts of HFBA up to 5 mM result in an increase of the signal/noise ratios but at 10 mM the signal is strongly suppressed. For this reason, 5 mM was chosen as the optimal HFBA concentration for further experiments.

The volume of sample injected in the column was also optimized. Fig. 4 shows the relative signal response and signal/noise ratio obtained for various injected volumes of a standard solution of cadaverine in HFBA 5 mM. The best signal/noise ratio was obtained with a volume injected of 50 mL and this volume was chosen for further experiments.

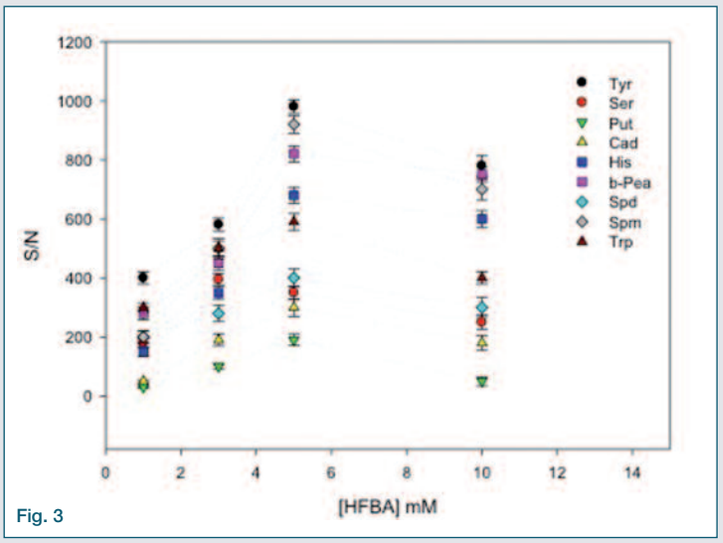


Fig. 3

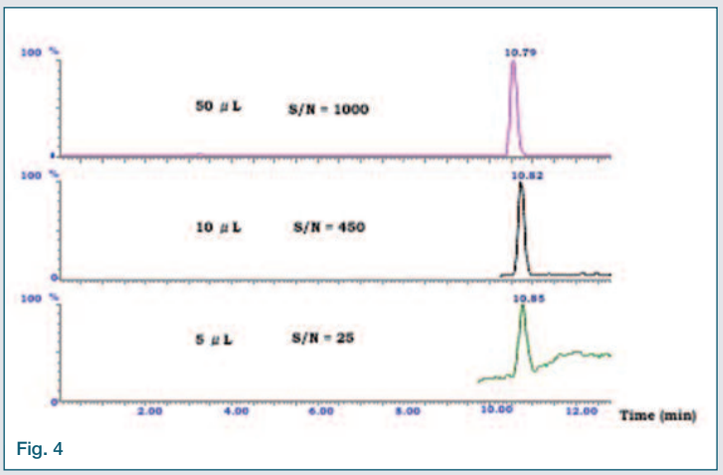


Fig. 4

Tab. 2 - HPLC-UV method performances. $R_t$: retention time; $R^2$: square of regression coefficient; LIN: linearity on-line; AS: analytical sensitivity; LOD: detection limit

| Biogenic amines | $R_t$ (min) | Conc. Range (mg $l^{-1}$) | $R^2$ | LIN % | AS (µg $l^{-1}$) | LOD (µg l-1) |
|---|---|---|---|---|---|---|
| Methylamine | 6.9 | 0.1 - 16 | 0.998 | 99.26 | 29.0 | 54.2 |
| Ethylamine | 9.0 | 0.1 - 16 | 0.999 | 99.10 | 25.7 | 58.0 |
| Tryptamine | 15.6 | 0.1 - 16 | 0.999 | 98.99 | 23.1 | 42.8 |
| β-phenylethilamine | 16.1 | 0.1 - 16 | 0.998 | 99.20 | 37.3 | 64.2 |
| Putrescine | 17.6 | 0.1 - 16 | 1.000 | 99.70 | 3.3 | 8.0 |
| Cadaverine | 18.8 | 0.1 - 16 | 0.999 | 99.25 | 8.2 | 17.1 |
| Histamine | 19.2 | 0.1 - 16 | 0.999 | 99.85 | 27.1 | 50.4 |
| Serotonin | 22.0 | 0.1 - 16 | 0.990 | 98.75 | 37.7 | 66.9 |
| Tyramine | 23.9 | 0.1 - 16 | 0.999 | 99.10 | 30.3 | 61.2 |
| Spermidine | 24.9 | 0.1 - 16 | 1.000 | 99.97 | 11.1 | 20.4 |
| Spermine | 29.2 | 0.1 - 16 | 0.999 | 98.96 | 14.7 | 27.0 |

Tab. 3 - LC-MS method performances. $R^2$: square of regression coefficient; LIN: linearity on-line; AS: analytical sensitivity; LOD: detection limit

| Biogenic amines | $R_t$ (min) | Conc. Range (mg $l^{-1}$) | $R^2$ | LIN % | AS (µg $l^{-1}$) | LOD (µg l-1) |
|---|---|---|---|---|---|---|
| Methylamine | 6.9 | 0.1 - 16 | 0.998 | 99.26 | 29.0 | 54.2 |
| Ethylamine | 9.0 | 0.1 - 16 | 0.999 | 99.10 | 25.7 | 58.0 |
| Tryptamine | 15.6 | 0.1 - 16 | 0.999 | 98.99 | 23.1 | 42.8 |
| β-phenylethilamine | 16.1 | 0.1 - 16 | 0.998 | 99.20 | 37.3 | 64.2 |
| Putrescine | 17.6 | 0.1 - 16 | 1.000 | 99.70 | 3.3 | 8.0 |
| Cadaverine | 18.8 | 0.1 - 16 | 0.999 | 99.25 | 8.2 | 17.1 |
| Histamine | 19.2 | 0.1 - 16 | 0.999 | 99.85 | 27.1 | 50.4 |
| Serotonin | 22.0 | 0.1 - 16 | 0.990 | 98.75 | 37.7 | 66.9 |
| Tyramine | 23.9 | 0.1 - 16 | 0.999 | 99.10 | 30.3 | 61.2 |
| Spermidine | 24.9 | 0.1 - 16 | 1.000 | 99.97 | 11.1 | 20.4 |
| Spermine | 29.2 | 0.1 - 16 | 0.999 | 98.96 | 14.7 | 27.0 |

## *Performance characteristics of the HPLC-UV method*

Tab. 2 shows the performance characteristics of the method. Calibration graphs were constructed by plotting the amine to the internal standard peak areas ratios (A) against the amine concentrations (C). Data for calibration curves were collected for six amine standard concentrations and each measure was repeated four times. Linearity was observed in the tabulated concentration range for each biogenic amine with a good regression coefficient. The linearity "on-line" is defined as LIN%=100(1-$S_b$) [35] where $S_b$ is the slope standard deviation and indicates the greater or lesser dispersion of the data around the calibration line. The analytical sensitivity (AS) was calculated as follows: AS=$S_{A,C}$/b where $S_{A,C}$ is the regression standard deviation of A versus C and b is the slope of the regression line. Good values are obtained for all amines. The limits of detection (LODs) were calculated as the concentration of amines required to give a signal-to-noise ratio of 3 and found between 8.0 µg $l^{-1}$ for putrescine and 66.9 µg $l^{-1}$ for serotonin.

## *Performance characteristics of the LC-ESI-MS method*

Linearity was tested using standard solutions of amines in acidified water (5 mM HFBA). Tab. 3 summarizes the results obtained. The response was linear in the range 0.1-16 µg $l^{-1}$ and the correlation coefficients ($R^2$) were above 0.98, with the only exception of putrescine. The linearity "on line" (LIN) and the analytical sensitivity (AS) were calculated as reported above. The limits of detection were calculated according to the criterion of S/N=3, resulting in the range between 6.2 µg $l^{-1}$ for tryptamine and 105.5 µg $l^{-1}$ for putrescine.

The LOD values obtained with the LC-ESI-MS method (Tab. 3) result-

Tab. 4 - HPLC-UV concentration values (mg $l^{-1}$) and relative standard deviation (RSD %) of 11 biogenic amines in 22 Italian wine samples (12 white wines and 10 red wines)

| Sample | Mea | | Eta | | Trp | | ß-Pea | | Put | | Cad | | His | | Ser | | Tyr | | Spd | | Spm | | Total Amines |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | |
| Wine 1$^{w}$ | 0.40 | 1.8 | 0.93 | 2.9 | 0.72 | 4.2 | 0.13 | 2.8 | 0.90 | 2.9 | 1.54 | 2.7 | 2.61 | 3.1 | 0.55 | 2.0 | ND | - | 1.03 | 2.7 | 0.94 | 3.4 | 9.74 |
| Wine 2$^{w}$ | ND | - | 0.56 | 3.4 | ND | - | ND | - | 1.17 | 2.8 | 1.79 | 3.7 | ND | - | 1.78 | 5.1 | 0.57 | 2.8 | 0.62 | 3.2 | ND | - | 6.49 |
| Wine 3$^{w}$ | ND | - | 0.88 | 2.0 | 0.23 | 2.7 | 0.16 | 1.9 | 1.83 | 2.1 | 2.76 | 3.3 | 1.52 | 2.0 | 0.96 | 2.5 | 2.81 | 2.8 | ND | - | 0.41 | 3.8 | 11.59 |
| Wine 4$^{w}$ | 3.10 | 0.9 | 2.66 | 2.6 | D | - | 0.24 | 2.0 | ND | - | 0.51 | 3.5 | ND | - | 0.34 | 2.3 | ND | - | 0.52 | 1.8 | ND | - | 7.37 |
| Wine 5$^{w}$ | ND | - | 0.44 | 1.8 | 0.16 | 3.7 | 3.22 | 2.2 | 1.40 | 3.4 | 3.97 | 1.7 | 4.42 | 2.4 | ND | - | 0.32 | 3.5 | 0.42 | 4.3 | 0.29 | 5.2 | 14.44 |
| Wine 6$^{w}$ | 0.77 | 2.1 | ND | - | ND | - | ND | - | 1.70 | 3.0 | 2.73 | 1.7 | 0.14 | 3.6 | 1.05 | 3.1 | 0.26 | 4.0 | ND | - | 0.22 | 2.3 | 6.87 |
| Wine 7$^{w}$ | 1.06 | 1.2 | 1.12 | 2.7 | 0.83 | 4.2 | ND | - | 0.89 | 1.8 | ND | - | ND | - | 0.95 | 1.6 | 0.10 | 2.8 | 0.21 | 2.8 | ND | - | 5.16 |
| Wine 8$^{w}$ | 0.27 | 1.4 | 0.68 | 2.7 | 0.89 | 3.7 | 0.85 | 1.8 | 0.85 | 4.1 | 2.36 | 2.8 | 0.51 | 1.4 | 1.37 | 4.1 | 0.67 | 1.8 | ND | - | 0.58 | 5.5 | 9.03 |
| Wine 9$^{w}$ | ND | - | 0.52 | 2.3 | ND | - | 0.92 | 3.2 | 2.57 | 1.6 | ND | - | 2.28 | 3.0 | 1.37 | 3.4 | 0.24 | 1.1 | 0.26 | 3.2 | 1.63 | 3.5 | 9.79 |
| Wine 10$^{w}$ | 1.26 | 2.5 | ND | - | 1.07 | 1.6 | 0.66 | 3.5 | 0.96 | 2.0 | 1.80 | 1.1 | ND | - | ND | - | 0.21 | 2.8 | ND | - | 0.80 | 1.2 | 6.76 |
| Wine 11$^{w}$ | ND | - | 0.60 | 1.4 | ND | - | 0.88 | 2.4 | 0.65 | 2.0 | ND | - | 1.49 | 2.0 | ND | - | 0.88 | 1.5 | 0.52 | 2.9 | ND | - | 5.02 |
| Wine 12$^{w}$ | 0.14 | 2.9 | 0.84 | 0.9 | ND | - | 0.27 | 2.6 | 2.95 | 1.7 | ND | - | 1.10 | 1.8 | 0.88 | 3.1 | 1.04 | 1.8 | 0.35 | 3.5 | ND | - | 7.57 |
| Wine 1$^{r}$ | 0.63 | 2.2 | 0.54 | 1.8 | 1.28 | 1.9 | 1.58 | 2.1 | 2.09 | 1.9 | ND | - | 1.85 | 2.4 | 2.41 | 1.9 | 1.37 | 1.2 | ND | - | 0.51 | 1.8 | 12.26 |
| Wine 2$^{r}$ | ND | - | 0.99 | 2.5 | 0.77 | 2.4 | 2.75 | 1.8 | 2.76 | 2.0 | 4.22 | 3.1 | 3.25 | 2.5 | ND | - | 3.71 | 1.9 | 0.20 | 3.1 | 0.17 | 2.6 | 18.82 |
| Wine 3$^{r}$ | 0.35 | 3.1 | 1.60 | 2.1 | ND | - | ND | - | 1.57 | 1.8 | 1.75 | 2.9 | 0.51 | 3.8 | 1.51 | 3.1 | 0.38 | 1.7 | 0.33 | 2.6 | ND | - | 8.00 |
| Wine 4$^{r}$ | 0.95 | 1.2 | 2.28 | 3.2 | ND | - | 2.68 | 2.5 | 3.39 | 2.9 | ND | - | 6.51 | 1.8 | 0.80 | 2.1 | 6.59 | 4.1 | 0.72 | 2.6 | 0.39 | 3.1 | 24.31 |
| Wine 5$^{r}$ | 0.29 | 2.2 | 0.77 | 2.7 | ND | - | ND | - | 1.65 | 2.7 | 1.90 | 1.7 | 1.23 | 2.3 | 2.57 | 1.9 | ND | - | ND | - | ND | - | 8.41 |
| Wine 6$^{r}$ | 0.25 | 1.9 | 1.33 | 1.0 | 0.47 | 3.1 | 0.29 | 1.5 | 2.47 | 3.4 | 1.84 | 2.6 | ND | - | 3.80 | 2.7 | 2.68 | 3.3 | 0.42 | 1.8 | 0.56 | 1.8 | 14.11 |
| Wine 7$^{r}$ | 0.59 | 2.8 | 2.91 | 1,9 | 1.41 | 2.6 | 3.75 | 2.7 | 7.59 | 2.1 | 2.91 | 1.4 | 3.10 | 4.5 | ND | - | 1.99 | 3.0 | ND | - | ND | - | 24.25 |
| Wine 8$^{r}$ | 0.82 | 2.3 | 0.46 | 2.2 | 1.83 | 1.5 | ND | - | 1.52 | 2.4 | 2.25 | 2.2 | 1.52 | 3.0 | 2.66 | 2.9 | 1.20 | 3.8 | 0.58 | 2.3 | 0.30 | 2.2 | 13.14 |
| Wine 9$^{r}$ | 0.27 | 2.5 | ND | - | ND | - | 0.35 | 3.1 | <LOD | - | 2.80 | 1.8 | ND | - | 0.84 | 3.1 | 2.22 | 1.9 | 0.55 | 3.2 | 1.56 | 4.7 | 7.51 |
| Wine 10$^{r}$ | 0.63 | 1.1 | 0.53 | 2.1 | 0.80 | 2.4 | ND | - | 1.96 | 2.0 | 3.37 | 1.5 | 3.61 | 3.1 | 0.33 | 4.3 | 0.86 | 2.1 | ND | - | ND | - | 12.09 |

* mean of four replicates; $^{w}$white wines; $^{r}$ red wines; ND not detected

Tab. 5 - LC-ESI-MS concentration values (mg $l^{-1}$) and relative standard deviation (RSD %) of 9 biogenic amines in 22 Italian wine samples (12 white wines and 10 red wines)

| Sample | Trp | | ß-Pea | | Put | | Cad | | His | | Ser | | Tyr | | Spd | | Spm | | Total Amines |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean* | RSD | Mean | RSD | |
| Wine 1[w] | 0.72 | 4.2 | ND | - | ND | - | 1.20 | 2.2 | 2.85 | 2.8 | 0.50 | 2.0 | ND | - | 1.05 | 1.5 | 0.51 | 2.0 | 6.83 |
| Wine 2[w] | ND | - | ND | - | 0.21 | 2.7 | 1.82 | 3.6 | ND | - | 1.80 | 3.1 | 0.58 | 2.4 | 0.48 | 4.1 | ND | - | 4.89 |
| Wine 3[w] | 0.05 | 2.5 | 0.09 | 2.0 | 0.42 | 2.6 | 3.55 | 2.6 | 1.51 | 2.3 | 1.07 | 2.8 | 2.65 | 2.8 | ND | - | 0.35 | 2.0 | 9.69 |
| Wine 4[w] | ND | - | 0.18 | 2.0 | ND | - | 0.78 | 1.5 | ND | - | 0.52 | 1.9 | ND | - | 0.44 | 3.0 | ND | - | 3.84 |
| Wine 5[w] | 0.22 | 1.8 | 3.67 | 1.7 | 0.42 | 3.4 | 4.91 | 2.2 | 4.15 | 2.3 | ND | - | 0.28 | 3.0 | 0.35 | 4.0 | 0.10 | 2.6 | 14.10 |
| Wine 6[w] | 0.21 | 3.0 | ND | - | 1.00 | 3.5 | 2.55 | 2.5 | 0.22 | 3.2 | 1.24 | 4.1 | 0.28 | 2.3 | ND | - | 0.15 | 1.5 | 5.65 |
| Wine 7[w] | 0.98 | 1.6 | 0.12 | 1.9 | ND | - | ND | - | ND | - | 1.22 | 2.7 | 0.08 | 2.9 | 0.11 | 2.8 | ND | - | 3.49 |
| Wine 8[w] | 0.68 | 3.1 | 0.75 | 2.2 | ND | - | 2.10 | 2.0 | 0.58 | 1.5 | 1.35 | 3.8 | 0.73 | 2.1 | ND | - | 0.39 | 2.5 | 6.58 |
| Wine 9[w] | ND | - | 0.85 | 2.2 | 0.52 | 2.3 | 0.08 | 3.1 | 2.65 | 2.0 | 1.08 | 3.6 | ND | - | 0.30 | 1.6 | 1.52 | 4.2 | 7.00 |
| Wine 10[w] | 1.18 | 2.4 | 0.55 | 2.5 | 0.25 | 3.2 | 2.45 | 1.5 | 0.10 | 1.5 | 0.18 | 2.0 | 0.24 | 1.6 | ND | - | 0.93 | 2.7 | 5.88 |
| Wine 11[w] | ND | - | 0.87 | 2.6 | ND | - | 0.22 | 2.2 | 1.65 | 1.8 | ND | - | 1.05 | 1.9 | 0.46 | 2.6 | ND | - | 4.25 |
| Wine 12[w] | 0.68 | 2.3 | 0.17 | - | 0.62 | 1.4 | ND | - | 1.40 | 2.2 | 1.03 | 1.8 | 1.10 | 1.7 | 0.32 | 1.5 | ND | - | 5.32 |
| Wine 1[r] | 1.31 | 2.5 | 1.38 | 1.8 | 0.75 | 1.9 | 0.15 | 2.7 | 1.85 | 2.4 | 2.48 | 1.9 | 1.47 | 1.2 | ND | - | 0.64 | 3.4 | 10.03 |
| Wine 2[r] | 0.77 | 2.7 | 2.85 | 2.3 | 0.55 | 2.0 | 4.40 | 3.7 | 2.95 | 2.8 | ND | - | 4.01 | 1.4 | 0.18 | 4.2 | 0.14 | 5.3 | 15.85 |
| Wine 3[r] | ND | - | ND | - | 0.33 | 3.2 | 2.00 | 1.9 | 0.44 | 2.7 | 1.70 | 2.5 | 0.41 | 2.1 | 0.23 | 1.6 | ND | - | 5.11 |
| Wine 4[r] | 0.55 | 1.5 | 2.99 | 1.8 | 1.11 | 2.0 | ND | - | 6.01 | 1.4 | 0.92 | 1.8 | 6.75 | 1.4 | 0.65 | 1.9 | 0.30 | 2.9 | 19.28 |
| Wine 5[r] | 0.05 | 2.5 | ND | - | ND | - | 1.55 | 1.9 | 1.09 | 3.3 | 2.38 | 1.5 | 0.15 | 2.6 | ND | - | ND | - | 5.22 |
| Wine 6[r] | 0.38 | 4.2 | 0.15 | 2.4 | 1.20 | 2.4 | 2.10 | 1.5 | ND | - | 3.55 | 3.7 | 2.88 | 3.3 | 0.32 | 2.2 | 0.39 | 2.3 | 10.97 |
| Wine 7[r] | 1.20 | 3.0 | 4.05 | 1.9 | 2.47 | 2.5 | 2.75 | 2.2 | 3.35 | 1.9 | ND | - | 1.78 | 2.4 | ND | - | ND | - | 15.60 |
| Wine 8[r] | 1.95 | 2.5 | ND | - | 0.26 | 3.5 | 2.43 | 1.9 | 1.68 | 3.0 | 2.55 | 2.5 | 1.40 | 1.3 | 0.65 | 2.3 | 0.25 | 1.5 | 11.17 |
| Wine 9[r] | ND | - | 0.35 | 1.8 | ND | 2.0 | 3.15 | 3.0 | ND | - | 0.96 | 3.0 | 1.95 | 2.2 | 0.38 | 2.4 | 1.43 | 1.8 | 8.22 |
| Wine 10[r] | 1.05 | 1.8 | 0.20 | 2.5 | 0.42 | 3.1 | 3.51 | 1.6 | 3.85 | 2.5 | 0.25 | 1.3 | 0.90 | 2.2 | ND | - | ND | - | 19.63 |

* mean of four replicates; [w] white wines; [r] red wines; ND not detected.

ed to be quite lower than those obtained with the HPLC-UV method (Tab. 2), with the exception of putrescine which shows a LOD value more than 10 times higher, probably due to signal suppression.

## *Determination of biogenic amines in wine samples*

22 wine samples (12 white and 10 red) were analyzed using both HPLC-UV and LC-ESI-MS methods under the selected experimental conditions. Four replicates for each determination were performed.

HPLC-UV chromatograms of the amines standard solution (A) and of a red wine sample (B) are reported in Fig. 5, as an example.

Fig. 6 shows the MS spectra of 9 of the 11 amines studied. Methylamine and ethylamine were not detectable because of their low molecular weight. The complete results obtained with HPLC-UV and with LC-ESI-MS for all wine samples studied are reported in Tab. 4 and 5, respectively. Each column refers to a specific biogenic amine. In the last column is reported

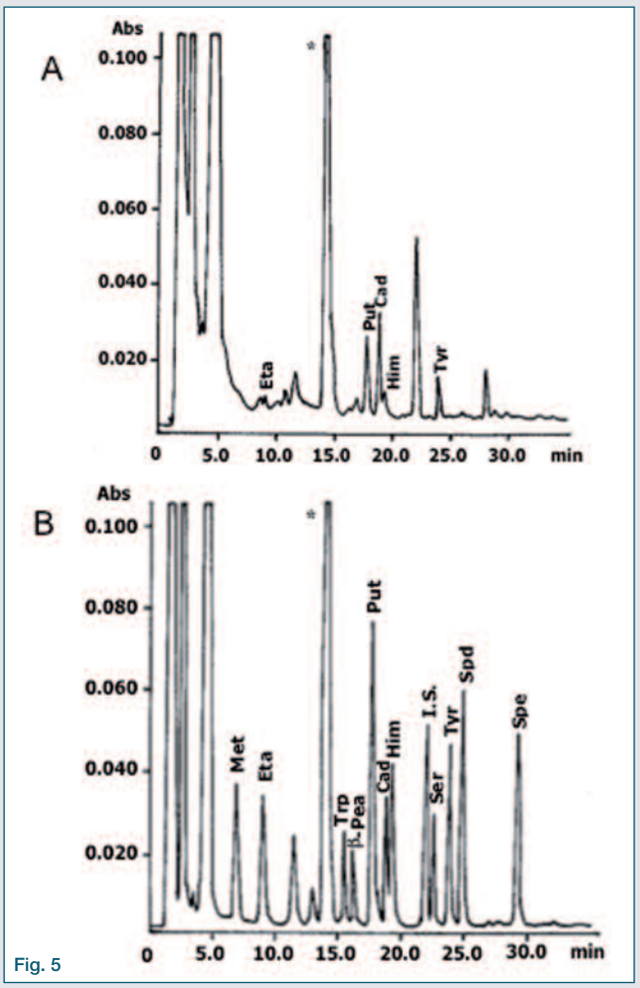



Fig. 5

the total amine amount, calculated for each wine sample.

The values of total amines calculated for both techniques resulted in good agreement, taking into account the fact that with the LC-ESI-MS the volatile amines, methylamine and ethylamine, are not detectable and that the values obtained for putrescine are definitely lower compared to those obtained with the HPLC-UV method.

By comparing the values reported in the last columns of Tab. 4 and 5 it can be also easily noted that the total amine concentrations are much higher in red wines compared to white wines.

This result can be explained by the fact that red wines are generally less acidic than white wines and it is known in literature that at high pH biogenic amines are produced in high amounts [38]. The higher the pH, the more complex the bacterial microflora. An easier total growth and a greater bacterial diversity is observed in red wines which, therefore, show a larger amine content.

The significant differences observed in the values reported for the wine samples in the various rows of Tab. 4 and 5 are probably due to the fact that the biogenic amine amount in wines is strongly dependent on different variables such as pH, wine aging and wine producing area. pH is the most important factor determining not only the biological activity of bacteria in wine but also their variety, as reported above. As for wine aging and production area, the literature reports that old wines contain significantly higher

amounts of biogenic amines than young wines [36] and that in some producing areas biogenic amines are found in higher levels than in others [38]. This is related in part to the type of winemaking and whether it involves malolactic fermentation or not.

Tab. 6 clearly shows the amine concentration range found for each amine with both techniques.

The most abundant amines determined with the HPLC-UV method resulted to be putrescine, histamine and tyramine. In particular putrescine was found to be the highest value in red wine samples (7.59 mg $l^{-1}$) while in white wine samples histamine was found at the highest concentration values (4.42 mg $l^{-1}$). The correlation between putrescine and histamine and tyramine has already been noted by Bauza *et al.* [36], especially in red wines where these amines are present in greater quantities. This fact could be a consequence of malolactic fermentation which is required after alcoholic fermentation for nearly all red wines. The concentration of these amines is low after alcoholic fermentation and increases in most wines during malolactic fermentation to a very variable extent [37].

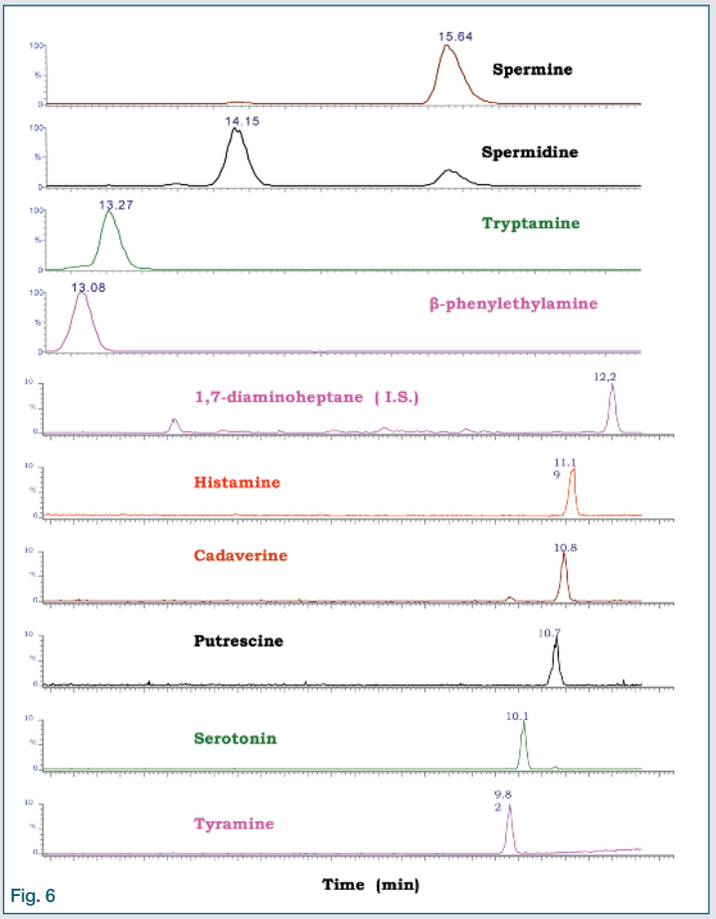


Fig. 6

With the LC-ESI-MS method the most prevalent amines resulted to be histamine in white wines (4.15 mg $l^{-1}$) and tyramine in red wines (6.75 mg $l^{-1}$). Putrescine concentrations were much lower than the values normally encountered in wines [28, 33], with levels slightly higher in red wines (up to 2.47 mg $l^{-1}$) than in white wines (up to 1.00 mg $l^{-1}$). This result can be ascribed to the quite low molecular weight of putrescine (Tab. 1), which is, in fact, at the limit of lower mass calibration of the instrument. Therefore, the signal relative to putrescine suffers from high background noise and no significant increase in the S/N ratio was obtained due to instrumental limitations.

Spermine and spermidine were found in the lowest amount in red wine samples while in white wine samples spermidine and tryptamine showed the lowest concentration values, which is in accordance with Soufleros *et al.* [28]. These results are relative to the 9 amines determined with both techniques. It is interesting to observe that the results obtained with the two different methods resulted in good agreement with each other for all amines examined with the only exception of putrescine, for the reasons mentioned above. The accuracy of both methods was calculated by means of a spiking and recovery study on both white and red wines. The recovery was calculated as mean spiked concentration minus the mean original sample concentration divided by the spiked concentration. The spiked levels were 0.2 mg $l^{-1}$ and 1.0 mg $l^{-1}$. Tab. 7 shows good recoveries with both methods. The HPLC-UV method resulted to be quite accurate with a recovery range between 86.9% for spermine at 0.2 mg $l^{-1}$ and 108.6% for methylamine at 0.2 mg $l^{-1}$. The precision of the method is satisfactory with a RSD<5.1%. The accuracy of the LC-ESI-MS method was also quite good with recovery values between 94.5% for serotonin at 1.0 mg $l^{-1}$ and 104.4% for spermine at 0.2 mg $l^{-1}$, with the exception of putrescine which shows recovery values ranging between 68.9% and 79.1%. The precision is quite satisfactory with a RSD<5%, except for putrescine with RSD values between 5.8 and 11.1%. A comparison of the recoveries of the LC-ESI-MS method with respect to HPLC-UV method, shows a general improvement for all amines studied (with the exception of putrescine).

## Conclusions

Both HPLC-UV and LC-ESI-MS methods demonstrated to be effective methods for the determination of the 11 biogenic amines in wine samples. The HPLC-UV method resulted to be more versatile and

Tab. 6 - Amine concentration range detected with HPLC-UV method and LC-ESI-MS method - not detected

| Amine biogenic (mg $l^{-1}$) | HPLC-UV | | LC-ESI-MS | |
|---|---|---|---|---|
| | White | Red | White | Red |
| Methylamine | 0.14 ÷ 3.10 | 0.25 ÷ 0.95 | - | - |
| Ethylamine | 0.44 ÷ 2.66 | 0.46 ÷ 2.91 | - | - |
| Tryptamine | 0.16 ÷ 1.07 | 0.47 ÷ 1.83 | 0.05 ÷ 1.18 | 0.05 ÷ 1.95 |
| β-phenylethilamine | 0.13 ÷ 3.22 | 0.29 ÷ 3.75 | 0.09 ÷ 3.67 | 0.15 ÷ 4.05 |
| Putrescine | 0.65 ÷ 2.95 | 1.52 ÷ 7.59 | 0.21 ÷ 1.00 | 0.26 ÷ 2.47 |
| Cadaverine | 0.51 ÷ 3.97 | 1.75 ÷ 4.22 | 0.22 ÷ 3.55 | 0.15 ÷ 4.40 |
| Histamine | 0.14 ÷ 4.42 | 0.51 ÷ 6.51 | 0.10 ÷ 4.15 | 0.44 ÷ 6.01 |
| Serotonin | 0.34 ÷ 1.78 | 0.33 ÷ 3.80 | 0.50 ÷ 1.80 | 0.25÷ 3.55 |
| Tyramine | 0.10 ÷ 2.81 | 0.38 ÷ 6.59 | 0.08 ÷ 2.65 | 0.15 ÷ 6.75 |
| Spermidine | 0.21 ÷ 1.03 | 0.20 ÷ 0.72 | 0.11 ÷ 1.05 | 0.18 ÷ 0.65 |
| Spermine | 0.22 ÷ 1.63 | 0.17 ÷ 1.56 | 0.10 ÷ 1.52 | 0.14 ÷ 1.43 |

Table 7 - Recovery study on white and red wine samples (n=4)

| Biogenic amine | HPLC-UV | | | | LC-ESI-MS | | | |
|---|---|---|---|---|---|---|---|---|
| | White wine | | Red wine | | White wine | | Red wine | |
| | Recovery % | RSD % | Recovery % | RSD % | Recovery % | RSD % | Recovery % | RSD % |
| | 0.2 mg $l^{-1}$ | 1.0 mg $l^{-1}$ | 0.2 mg $l^{-1}$ | 1.0 mg $l^{-1}$ | 0.2 mg $l^{-1}$ | 1.0 mg $l^{-1}$ | 0.2 mg $l^{-1}$ | 1.0 mg $l^{-1}$ |
| Methylamine | 108.6 (5.1) | 89.0 (3.3) | 92.8 (4.1) | 105.2 (3.2) | - | - | - | - |
| Ethylamine | 93.5 (4.8) | 106.2 (4.0) | 103.2 (1.3) | 97.5 (2.1) | - | - | - | - |
| Tryptamine | 100.8 (3.9) | 95.8 (2.0) | 91.0 (2.0) | 106.3 (1.9) | 98.4 (4.1) | 101.9 (4.3) | 95.6 (5.0) | 103.7 (3.5) |
| β-phenylethilamine | 95.2 (2.9) | 106.3 (2.3) | 98.2 (2.5) | 99.2 (3.2) | 103.9 (5.0) | 95.2 (3.3) | 104.2 (2.8) | 98.6 (4.2) |
| Putrescine | 105.4 (2.9) | 98.9 (3.8) | 95.3 (2.1) | 102.2 (3.1) | 75.2 (11.1) | 68.9 (7.8) | 73.4 (9.9) | 79.1 (5.8) |
| Cadaverine | 96.9 (4.2) | 100.4 (1.9) | 106.5 (3.0) | 95.5 (3.0) | 103.1 (4.4) | 98.5 (2.0) | 99.9 (1.8) | 103.3 (2.0) |
| Histamine | 107.0 (1.9) | 96.6 (2.3) | 94.9 (4.0) | 92.1 (2.0) | 97.9 (2.3) | 97.7 (3.0) | 103.3 (2.9) | 96.8 (1.7) |
| Serotonin | 91.7 (2.3) | 95.3 (1.2) | 100.0 (2.2) | 98.0 (3.2) | 95.8 (1.6) | 94.5 (4.9) | 96.0 (3.5) | 99.9 (4.1) |
| Tyramine | 98.5 (1.8) | 107.0 (2.9) | 101.2 (3.0) | 105.2 (2.2) | 97.9 (3.4) | 103.4 (5.0) | 99.3 (2.7) | 97.6 (1.1) |
| Spermidine | 107.1 (2.1) | 93.1 (2.0) | 95.3 (3.1) | 102.4 (2.7) | 103.0 (2.5) | 96.5 (3.5) | 101.0 (4.0) | 98.4 (3.3) |
| Spermine | 87.4 (2.2) | 107.3 (3.1) | 86.9 (1.8) | 92.4 (4.1) | 104.4 (3.0) | 98.4 (4.2) | 98.0 (2.0) | 103.1 (1.8) |

cheaper compared to the LC-ESI-MS method as it allowed, using a less expensive apparatus, the determination of all the 11 amines studied while with the MS method the volatile amines, such as methylamine and ethylamine, were not detectable. The most significant improvement of LC-ESI-MS method is the possibility of avoiding the tedious and time-consuming derivatization step, necessary in the HPLC technique, thus decreasing the time of the analysis, being the MS technique compatible with the detection of raw amines. Besides, no sample clean-up or pre-concentration procedures are required. This fact is of particular relevance in respect to the green chemistry.

Moreover, LC-ESI-MS method contributed to improve significantly the sensitivity with LODs values definitely lower than those obtained with HPLC-UV and better recoveries. As a conculsion, HPLC-UV detection can be successfully used for preliminary experiments as both volatile and not volatile amines can be easily detected, but the LC-ESI-MS method, which does not require any sample clean-up or pre-treatment, demonstrated to give definitely better results in terms of time of analysis, cost, sensitivity and accuracy.

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RIASSUNTO

***Determinazione delle ammine biogene nei vini mediante HPLC-UV e LC-ESI-MS: un confronto tra i due metodi***

*Il lavoro presenta un confronto dettagliato tra due differenti metodi analitici, la cromatografia liquida ad alta pressione con rivelatore UV (HPLC-UV) e la cromatografia liquida accoppiata alla spettrometria di massa con ionizzazione elettrospray (LC-ESI-MS), per la determinazione di undici ammine biogene in campioni di vino.*