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# Quantitative relations among measurands of molecular isotopologies of halogenated pharmaceutics – stochastic dynamic mass spectrometric approach

Chaired by **Dr. Alfredo Berzal-Herranz** and **Prof. Dr. Maria Emília Sousa**
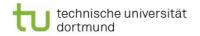
*pharmaceuticals*  MDPI

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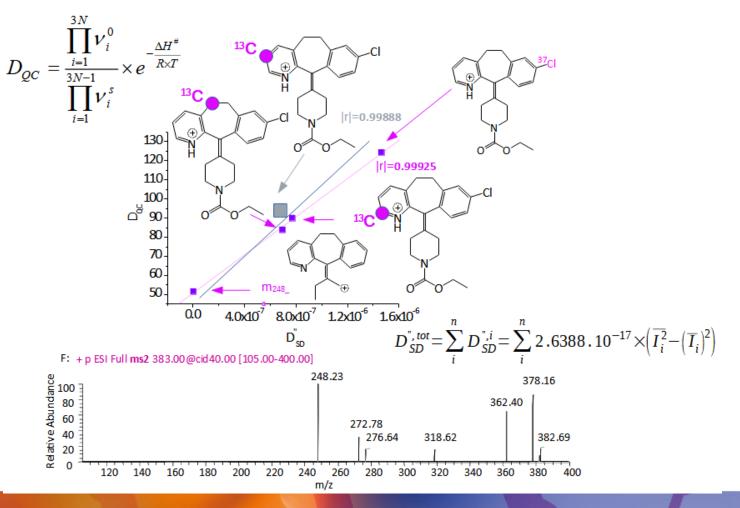* Corresponding author: [bojidarka.ivanova@yahoo.com]; [b_ivanova@web.de]

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**Quantitative relations among measurands of molecular isotopologues of halogenated pharmaceutics – stochastic dynamic mass spectrometric approach**

**Graphical Abstract**



$$D_{QC} = \frac{\prod_{i=1}^{3N} v_i^0}{\prod_{i=1}^{3N-1} v_i^s} \times e^{-\frac{\Delta H^{\#}}{R \times T}}$$

$|r|=0.99888$

$|r|=0.99925$

$m_{248\_}$

$$D_{SD}^{",tot} = \sum_i^n D_{SD}^{",i} = \sum_i^n 2.6388 \cdot 10^{-17} \times \left( \overline{I_i^2} - \left( \overline{I_i} \right)^2 \right)$$

F:  + p ESI Full ms2 383.00@cid40.00 [105.00-400.00]

## Abstract:

The paper serves two goals. Its first aim is to approach *mass spectrometric* measurands such as mass-to-charge (m/z) and peak intensity variables of *molecular isotopologies* of halogenated pharmaceutics diclofenac (1) and loratadine (2) with respect to conditions of measurements, involving collision energy and concentration of presented formic acid; if any, *via* our stochastic dynamic model equation [**equation 2**]. In addition, it is tested its most recently derivative formulas [**equation 4**] and [**equation 5**], connecting among experimental measurands with respect to experimental conditions, particularly, accounting for collision energy and concentration of formic acid. So far, the latter equations have been tested on two molecular models of labetalol and acetaminophen. Secondly, the first shown equation is used to determine 3D molecular and electronic structures of analytes, mass spectrometrically. The task is carried out *via* its complementary application to Arrhenius's equation. Those two domains constitutes fundamental background of the *analytical mass spectrometry,* consisting in analyte quantitative and 3D structural analyses, which are approaches only using one and the same stochastic dynamic equation. There are utilized ultra-high resolution electrospray ionization mass spectrometric data in addition to high accuracy quantum chemical static methods and molecular dynamics. Tests of *chemometrics* are used, as well.

**Keywords:** Mass spectrometry ; stochastic dynamics ; molecular isotoplogies ; pharmaceutics

$$D_{SD}^{tot} = \sum_i^n D_{SD}^i = \sum_i^n 1.3194.10^{-17} \times A^i \times \frac{\overline{I_i^2} - \left(\overline{I_i}\right)^2}{\left(I_i - \overline{I_i}\right)^2}$$

**Equation (1)**

$$D_{SD}^{",tot} = \sum_i^n D_{SD}^{",i} = \sum_i^n 2.6388.10^{-17} \times \left(\overline{I_i^2} - \left(\overline{I_i}\right)^2\right)$$

**Equation (2)**

$$D_{QC} = \frac{\prod_{i=1}^{3N} v_i^0}{\prod_{i=1}^{3N-1} v_i^s} \times e^{-\frac{\Delta H^{\#}}{R \times T}}$$

**Equation (3)**

$$\overline{I^{TOT,q}} = \frac{1}{2} \times \frac{A_I^q}{A_D^q} \times D_{SD}^{",q}$$

**Equation (4)**

$$D_{SD,l}^{"} + D_{SD,m}^{"} = \left| r_{l,m} \right| \times \sqrt{\overline{I_{l,q}^2} - \left(\overline{I_{l,q}}\right)^2} \times \sqrt{\overline{I_{m,q}^2} - \left(\overline{I_{m,q}}\right)^2}$$

**Equation (5)**

# Introduction

Various -omics-methods (*lipidomics; metabolomics; proteomics, including (neuro)-proteomics; foodomics; steroidomics; pesticide analysis and control; glycomics; genomics and DNA adductomics; lignomics; genomics, transcriptomics, interactomics; clinical trans-omics; doping control; petrolomics; isotomics*; and more) have widespread application to *mass spectrometry* (**MS**) [1–3]. They have gained importance in studying chemistry of biological systems. *Bioinformatics is* mainly governed, in fact, by MS based *bioanalytical approaches [3]. Metabolomics,* is based on hyphenated instrumentation of LC-MS$^n$ [4,5]. Its databases can be complex and chemically heterogeneous; thus, causing for a challenge to determine reliably structurally similar metabolites; enantiomers; isomers; or molecular *isotopologies*, respectively. Identification of LMWs is associated with significant error. M*etabolomics* databases often show relatively low reproducibility of measurands among instrumentation and laboratories; also, a great variability of data-quality. Therefore, structural elucidation of LMWs and reliability of both quantitative and structural analyses are challenging research tasks; strictly speaking - errors occur. The *molecular annotation* is carried out *via* tasks involving: (i) Conversion of measurands; (ii) peak detection and (iii) alignment; (iv) correction of retention time; (v) detection of isotopes, including molecular isotopologies; (vi) annotation of MS adducts; if any; (vii) annotation of peaks, and (viii) data-processing of measurands [4–7]. Molecular structural information is gained *via* tandem operation MS$^n$ mode. However, under term *annotation* there is understood a putative association of a molecule to its structure [4], or a rather deduction of analyte molecular structure . It is very far from chemical term *3D molecular structure* [8]. It assumes determination of 3D molecular geometry parameters and electronic structures of analytes.

Further: What there might be termed *molecular identification* is focused on confirmation of annotated molecular structure with metabolic standard; if any [4]. Often, this is a drawback to -omics-methods. Even in cases, when there are standard reference spectra of analytes, we shall turn to comment on made above that neither annotated nor identified 2D molecular structure provide crucial for the chemistry 3D molecular geometries and electronic structures. Arguments for maintaining the later position has been shown [8].

Furthermore, we could make point in that task of *molecular annotation* is frequently a manual process. Selection of best candidate among a set of possible molecular structures which is significantly time and labour consuming research effort [4–7].

Despite, for deduction of 2D molecular structure of analyte among a large number of metabolites of biological or environmental samples application of -omics-methods is sufficient as a first analytical stage. However, detail understanding on chemistry of molecules, particularly examining chiral compounds, in addition to their chemical reactivity, the second stage of 3D structural determination of molecular conformation and electron density distribution of atoms in molecules is crucial task. At this point, the readers shall be recalled that we have returned their attention on issue of methodological development of exact MS methods for quantitative and 3D structural analyses. A central issue is whether there is approach capable of serving at both quantitative and structural research tasks of *analytical mass spectrometry*. A seemingly very plausible strategy of solving the latter problem appears equations (1) and (2), quantifying *fluctuations* of MS measurands *per* short spans of scan time [9–15]. As we saw in contributions devoted to the issue, so far, equation (2) which is derived from equation (1) appears exact model for quantifying analytes mass spectrometrically.

In practice, complementary employment in equation (2) and Arrhenius's equation (3) provides exact 3D molecular and electronic structures; thus, allowing us not only to determine analyte quantitatively, but also 3D structurally, using formula (2). *Chemometrics* of correlation of data on equations (2) and (3) has shown r=1–0.9833 [13,16]. Equation (2) is capable of assigning subtle electronic effects of tautomers, showing |r|=1 [13,14]. It is applicable to complex environmental and foodstuff samples in addition to biological fluids [8,14].

Equation (2) has been tested on soft ionization MS methods and technique such as ESI, APCI, MALDI, CID, HCD, and UVPD [8–18], quantifying results from $MS^2$–$MS^7$ operation modes under positive and negative polarities [15,17]. Equation (2) has been justified correlating MS and chromatographic data on steroids. The chromatographic analysis shows $|r|=0.9991_4$, while MS quantitation according to equation (2) leads to $|r|$-$0.9999_4$ [12]. Equation (2) processes measurands from hard ionization MS methods, as well [19,20]. An assessing on its advantages has revealed a problem, however. It is an exact MS law obeyed by temporal distribution of measurand intensity of MS peak *per* any span of scan time, however, is applicable to a certain set of experimental conditions. In solving this problem we have further derived equations (4) and (5) from equations (1) and (2), thus writing innovative models capable of quantifying measurands of any two set of experimental conditions of measurements accounting for experimental factors such as collision energy, infusion volumes, and more [14,15,17].

Perhaps, there is created impression that equations (1), (2), (4) and (5) have already practical bearing, but that it is far from being the case. The capability of equations (1) and (2) for exact quantitative and structural analyses has been tested on a number of molecules and *chemometric* data have been reviewer [16,20]. Despite, many important for the field of chemistry research problems are still not addressed. An example is determination of *molecular isotopologies*. Furthermore, equations (4) and (5) have been tested, so far, on only two molecular objects [8,15], showing $|r|=0.9997_2$–0.99999 of CID-$MS^7$ spectra of labetalol [15]. However, a lack of a statistically representative set of tested molecules unable us to asses risk to which those formulas may be regarded as universally applicable ones to purposes of *analytical mass spectrometry*. Thus, most striking empirical justification of statement that as equation (2), formulas (4) and (5) are also universal MS laws may be provided by a systematic examining of a set of molecular objects and samples, having complex matrix effects. Part of our effort in addressing the issue is this study, dealing with quantitation of isotopologies of diclofenac (1) and loratadine (2) *via* formulas (1), (2), (4) and (5) in addition to determine their 3D molecular and electronic structures using complementarily equations (2) and (3). Pharmaceutics (1) and (2) exhibit distinctive isotope patterns of ions, due to chlorine atom(s), causing for MS isotope shape components exhibiting difference of m/z values $\Delta(m/z)=$ 1 and 2 of $^{13}C$ and $^{37}C$-isotopes. The study refers to determine isotope specific positions. New empirical proofs of great capability of formula (2) to quantify exactly isotope specific positions of $^{13}C$-isotopes are vigorously deployed, since, as can be expected looking at chemical diagrams of analytes (1) and (2) there is little affect on molecular properties from positions of $^{13}C$-isotope of aromatic fragments. The claim about distinctive capability of equation (2) of determining subtle electronic effects and isotope specific positions examining MS measurands and high accuracy quantum chemical data on electronic structures of analytes which is proven empirically in this study, thereby, extends capability of our model formulas, and experimental mass spectrometry as a robust analytical method for not only exact determination of analytes, but also their 3D conformational and electronic structural analytses.

There are issues in the study which play crucial role in research devoted to *analytical mass spectrometry*. The method has a many applications, ranging from environmental quality control monitoring; food control; clinical diagnostics; and medicine, respectively. The most important of these issues is that the innovative method presented, herein, provides exact quantification of the *fluctuations* of experimental variable intensity of analyte MS peak; furthermore, accounting for sub-peaks of isotope patterns. We shall draw on important most recent application of isotope ratio to quantitative mass spectrometry. The determination of isotope ratio of chlorine adducts of sialic acid of N-linked glycans has been used to develop MS based method for determination of content of sialic acid. Changes in sialylation of glycans as a tumor marker are associated with various types of cancer, in addition to cardiovascular disease and genetic disorders [21]. The [13]C- and ^{15}N-isotope ratio is used to develop MS method for determining smokeless powder in improvised explosive devices for purposes of forensic science and anti-terrorism activities [22]. Therefore, methodological developments of exact methods for MS data-processing of measurands is highly justifiable, due to broad practical interdisciplinary applications of the *mass spectrometry* to different fields of fundamental science, industry, and control authorities, respectively.

# Experimental

The contribution has used experimental MS database on MS measurements available in [23,24].

[https://doi.org/10.5281/zenodo.6543678] [https://doi.org/10.5281/zenodo.6545447]

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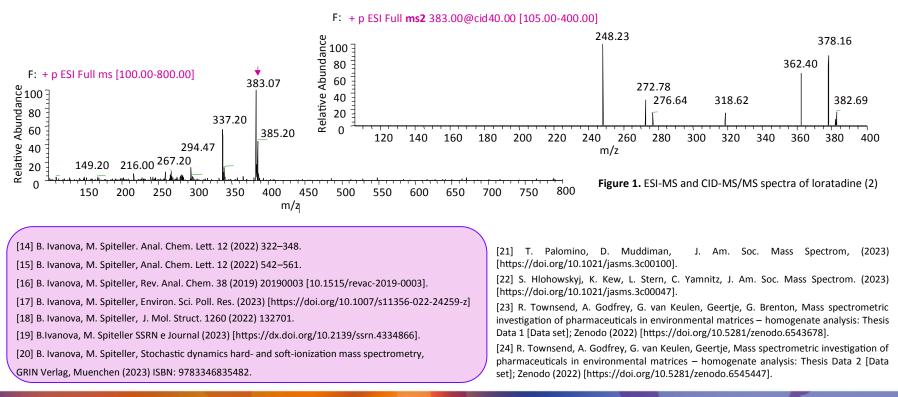
# Results and discussion

## 1. Mass spectrometric data

ESI-MS spectra of analytes (1) and (2) are depicted in **Figures 1** and **2. Figures 3** and **4** depict their fragmentation schemes. Fragmentation reactions of (1) depending on conditions of MS measurements are discussed [25–32]. The GC-SIM-MS analysis of (1) is based on fragmentation reaction: (m/z 318; [M+Na]$^+$) 214 (100); 242 (70); 277 (50) [25]. Data on relative abundance of fragmentation ions [%] are show in parentheses.



**Figure 1.** ESI-MS and CID-MS/MS spectra of loratadine (2)

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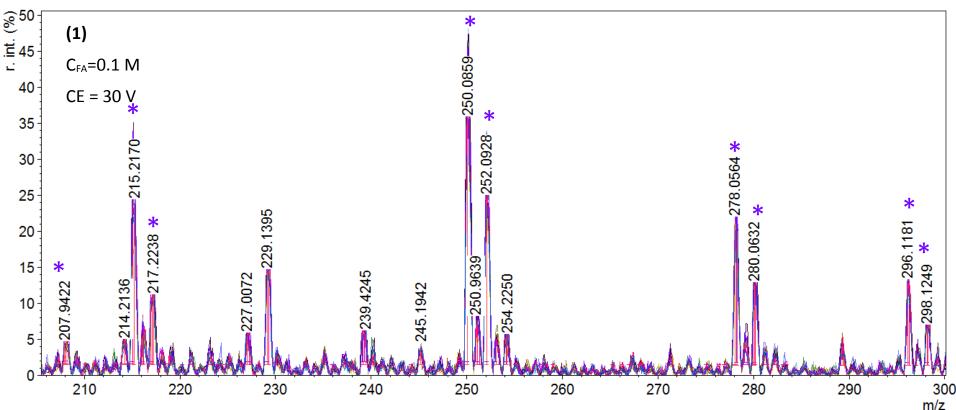
**Figure 2.** ESI-MS spectra of diclofenac (1) *per* short span of scan time and at presence of formic acid ($C_{FA}$ = 0.1 M) and collision energy CE = 30 V.

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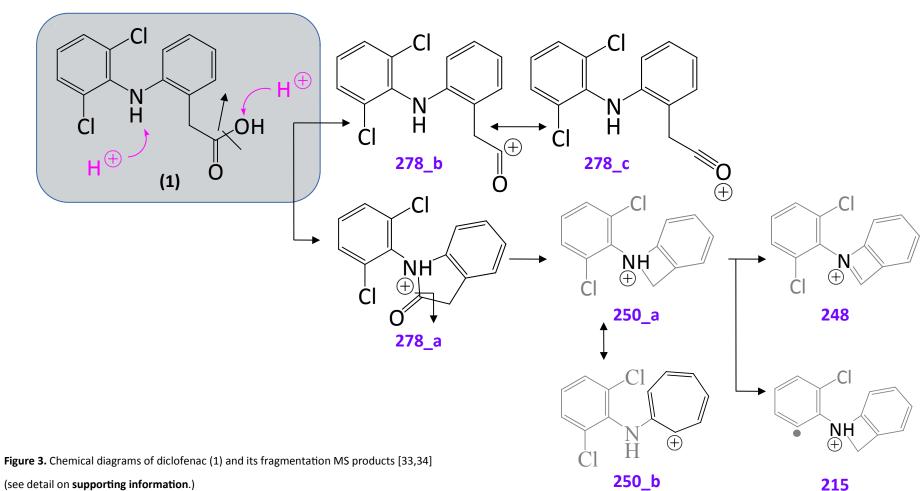
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**Figure 3.** Chemical diagrams of diclofenac (1) and its fragmentation MS products [33,34]

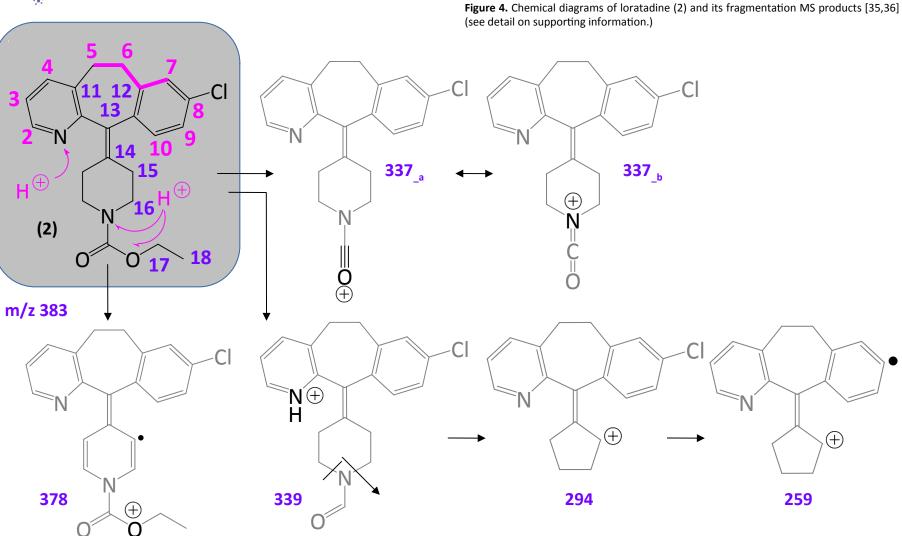(see detail on **supporting information**.)

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**Figure 4.** Chemical diagrams of loratadine (2) and its fragmentation MS products [35,36] (see detail on supporting information.)

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For the purpose of this study, as the title highlights, there are examined quantitatively isotope shapes of molecular and fragment ions of analytes (1) and (2). In this context, the so-called *monoisotopic* MS peak (MP), denotes observable peak of charged molecular ion [M]$^+$ or protonated cation [M+H]$^+$ of analyte consisting of only main isotopes of atoms such as, for example, $^{12}$C-, $^1$H-, $^{14}$N-, $^{35}$Cl-, $^{16}$O-, *et cetera* [37–40]. There are observed isotopic clusters of peaks next to MP of the analyte in a difference in m/z values $\Delta$(m/z)=1. The first of them, [MP+1] contains main isotopes and only one isotope of $^{13}$C-atom. The isotope cluster [MP+2], contains two $^{13}$C-atoms or one $^{13}$C- and one $^{15}$N-atom, for example (**Figure 5**.) Those, observable multiple-peak systems of isotope clusters can be very complex: thus, remaining hidden fine structures of each of the cluster MS peaks. Despite, employment in ultra-high MS resolving power allows us to resolve fine structure of discussed peaks of isotope shape of cationic species of analyte. Generally, in cases of organics the cluster peaks [MP+n] (n = 1, 2, 3…) refer to $^{13}$C-peaks (**Figure 6**.)



[(2)+H]$^+$

m/z 383

[C$_{22}$H$_{24}$ClN$_2$O$_2$]$^+$

Exact Mass: 383.15208

Theor. m/z:

383.15263 (100.0%): $^{35}$Cl,$^{12}$C

385.14968 (32.0%): $^{37}$Cl,$^{12}$C

384.15599 (24.5%): $^{35}$Cl,$^{13}$C

386.15304 (7.8%):  $^{37}$Cl,$^{13}$C

385.15934 (2.9%): $^{35}$Cl,$^{13}$C$_2$,

**Figure 5.** Experimental ESI-MS spectrum of loratadine (2), showing isotope shape at m/z 383 of [M+H]$^+$ cation; theoretical exact mass, m/z values, and intensity ratios (%) of MS peaks of the isotope shape; chemical diagram of protonated cationic form of (2).

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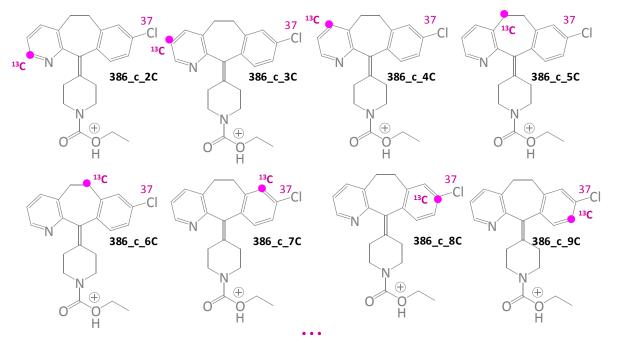[40] J. Claesen, A.  Rockwood, M. Gorshkov, D. Valkenborg,  Mass Spec. Rev. (2023) 1–21.

**Figure 6.** ^{13}C-isotope specific positions of loratadine (2) examining the MS peak m/z 386 of [M+H]+ cation of the analyte (see **Figure 2;**)

*Molecular isotopologies* provide *relative isotopic abundance* of analytes. It increases in reliability of data on analyte identification and annotation, which is of particular importance for purposes of routine implementation of omics-protocols into *clinical precision medicine*, because of high sandard chemometric method performances of omics-analyses are compulsory. However, *fluctuations* in elemental composition are caused by isotope fractionation determined by (bio)chemical and geochemical processes [40]. They induce differences in isotope ratio among isotopes of the same atom: thus causing for errors in *proteomics* [41] or *isotomics* [40]. Highly precise MS measurements of molecular isotopologies contribute crucially not only to the field of medicine and clinical diagnostics, but also to ecology, geology or history, in addition to *forensic anthropology,* where so-called *isoscapes* from human body tissues such as teeth, nails, bone and hair, allow to predict region-of-origin of human remains [42]. Due to, these reasons, fractional labelling of analytes must be obtained accurately. Accurate measurands of *fractional abundance* of molecular isotopologies of analyte refers to so-called *spectral accuracy* [43].

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| CE = 10 V | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| $C_{FA}$=0.2M | | | | | | | |
| Scan | m/z | I [arb.units] | m/z | I [arb.units] | m/z | I [arb.units] | m/z | I [arb.units] |
| 01 | 296.11807 | $2.13 \cdot 10^6$ | 297.24692 | 291324.328 | 298.12494 | $1.29 \cdot 10^6$ | 301.38605 | 107508.059 |
| 02 | 296.11807 | $2.27 \cdot 10^6$ | 297.12149 | 375839.217 | 298.12494 | $1.35 \cdot 10^6$ | 301.26062 | 122320.882 |
| 03 | 296.11807 | $2.09 \cdot 10^6$ | 297.24692 | 331929.321 | 298.12494 | $1.27 \cdot 10^6$ | 301.13522 | 98668.778 |
| 04 | 296.11807 | $2.16 \cdot 10^6$ | 297.12149 | 277727.366 | 298.12494 | $1.43 \cdot 10^6$ | 301.26062 | 98168.7215 |
| 05 | 296.11807 | $2.09 \cdot 10^6$ | 297.12149 | 374971.519 | 298.12494 | $1.44 \cdot 10^6$ | 301.38605 | 99537.7983 |
| 06 | 296.11807 | $2.22 \cdot 10^6$ | 297.12149 | 305343.558 | 297.99948 | $1.28 \cdot 10^6$ | 301.26062 | 82198.3022 |
| 07 | 295.99265 | $1.90 \cdot 10^6$ | 297.12149 | 258503.749 | 298.12494 | $1.19 \cdot 10^6$ | 301.26062 | 119635.458 |
| 08 | 296.11807 | $1.97 \cdot 10^6$ | 297.12149 | 349880.275 | 298.12494 | $1.19 \cdot 10^6$ | 301.26062 | 121683.97 |
| 09 | 296.24347 | $2.04 \cdot 10^6$ | 297.12149 | 293467.267 | 298.25037 | $1.15 \cdot 10^6$ | 301.13522 | 82016.6479 |
| 10 | 296.11807 | $2.07 \cdot 10^6$ | 297.12149 | 386088.469 | 298.12494 | $1.38 \cdot 10^6$ | 301.26062 | 128442.379 |
| 11 | 296.11807 | $2.04 \cdot 10^6$ | 296.99609 | 283941.018 | 298.12494 | $1.45 \cdot 10^6$ | 301.13522 | 105097.172 |
| 12 | 296.11807 | $1.96 \cdot 10^6$ | 297.12149 | 332191.988 | 298.12494 | $1.36 \cdot 10^6$ | 301.26062 | 99901.9127 |
| Mean | 296.11807 | | 297.13195 | | 298.12494 | | 301.25018 | |
| sd(yEr±) | 0.05348 | | 0.06458 | | 0.05349 | | 0.08384 | |
| se(yEr±) | 0.01544 | | 0.01864 | | 0.01544 | | 0.0242 | |
| <I> | | $2.07745 \cdot 10^6$ | | 321767.33964 | | $1.31493 \cdot 10^6$ | | 105431.67333 |
| <I>$^2$ | | $4.3157985025 \cdot 10^{12}$ | | $1.03534220859 \cdot 10^{11}$ | | $1.7290409049 \cdot 10^{12}$ | | $1.111583774 \cdot 10^{10}$ |
| <I$^2$> | | $4.32613 \cdot 10^{12}$ | | $1.05223 \cdot 10^{11}$ | | $1.73862 \cdot 10^{12}$ | | $1.13272 \cdot 10^{10}$ |
| <I$^2$>−<I>$^2$ | | $1.03314975 \cdot 10^{10}$ | | $1.688779140997 \cdot 10^9$ | | $9.5790951 \cdot 10^9$ | | $2.1136225884 \cdot 10^8$ |
| D"$_{SD}$ | | $2.7262755603 \cdot 10^{-7}$ | | $4.456350397263 \cdot 10^{-8}$ | | $2.5277316149 \cdot 10^{-7}$ | | $5.5774272862 \cdot 10^{-9}$ |

| CE = 20 V | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| $C_{FA}$=0.2M | | | | | | | |
| Scan | m/z | I [arb.units] | m/z | I [arb.units] | m/z | I [arb.units] | m/z | I [arb.units] |
| 01 | 295.99265 | $2.59 \cdot 10^6$ | 297.12149 | $4.85 \cdot 10^5$ | 298.12494 | $1.89 \cdot 10^6$ | 301.13522 | $1.93 \cdot 10^5$ |
| 02 | 296.11807 | $2.86 \cdot 10^6$ | 296.99609 | 349833.532 | 298.12494 | $1.73 \cdot 10^6$ | 301.26062 | $1.66 \cdot 10^5$ |
| 03 | 296.11807 | $2.92 \cdot 10^6$ | 297.12149 | $3.63 \cdot 10^5$ | 298.12494 | $1.85 \cdot 10^6$ | 301.26062 | $1.82 \cdot 10^5$ |
| 04 | 296.11807 | $2.99 \cdot 10^6$ | 296.99609 | 360901.115 | 298.12494 | $1.78 \cdot 10^6$ | 301.13522 | $2.26 \cdot 10^5$ |
| 05 | 296.11807 | $2.86 \cdot 10^6$ | 297.12149 | 378684.441 | 298.12494 | $1.79 \cdot 10^6$ | 301.13522 | 167262.776 |
| 06 | 296.11807 | $2.96 \cdot 10^6$ | 297.12149 | 450949.68 | 298.12494 | $1.70 \cdot 10^6$ | 301.26062 | 221801.268 |
| 07 | 296.11807 | $2.33 \cdot 10^6$ | 297.12149 | 342321.674 | 298.12494 | $1.81 \cdot 10^6$ | 301.13522 | 131386.564 |
| 08 | 296.11807 | $2.95 \cdot 10^6$ | 297.12149 | 472503.448 | 298.12494 | $2.13 \cdot 10^6$ | 301.13522 | 182346.207 |
| 09 | 296.11807 | $3.00 \cdot 10^6$ | 296.99609 | 504985.083 | 298.12494 | $1.88 \cdot 10^6$ | 301.13522 | 107686.17 |
| 10 | 296.11807 | $2.91 \cdot 10^6$ | 297.12149 | 492028.32 | 298.12494 | $1.90 \cdot 10^6$ | 301.13522 | 230684.695 |
| 11 | 296.11807 | $2.73 \cdot 10^6$ | 297.12149 | 409902.594 | 298.12494 | $2.10 \cdot 10^6$ | 301.13522 | 153614.439 |
| Mean | 296.10667 | | 297.08729 | | 298.12494 | | 301.16942 | |
| sd(yEr±) | 0.03782 | | 0.05857 | | 0 | | 0.05857 | |
| se(yEr±) | 0.0114 | | 0.01766 | | 0 | | 0.01766 | |
| <I> | | $2.82853 \cdot 10^6$ | | 419090.61195 | | $1.86618 \cdot 10^6$ | | 178354.04928 |
| <I>$^2$ | | $8.00058196 \cdot 10^{12}$ | | $1.75636941025 \cdot 10^{11}$ | | $3.4826277924 \cdot 10^{12}$ | | $3.18101669 \cdot 10^{10}$ |
| <I$^2$> | | $8.03832 \cdot 10^{12}$ | | $1.79257 \cdot 10^{11}$ | | $3.49978 \cdot 10^{12}$ | | $3.31964 \cdot 10^{10}$ |
| <I$^2$>−<I>$^2$ | | $3.77380391 \cdot 10^{10}$ | | $3.620058975375 \cdot 10^9$ | | $1.71522076 \cdot 10^{10}$ | | $1.386233105 \cdot 10^9$ |
| D"$_{SD}$ | | $9.95831376 \cdot 10^{-7}$ | | $9.55261162422 \cdot 10^{-8}$ | | $4.52612454 \cdot 10^{-7}$ | | $3.657991918 \cdot 10^{-8}$ |

Equation (4) it is derived from equation (1) [15]. It connects between $<I^{TOT}_{Theor}>$ values of analyte fragmentation ions measured toward experimental parameters such as collision energyand $D''_{SD}$ data on equation (2). The statistical factors $A_D^q$ and $A_I^q$ are *functional amplitudes* of SineSqr function. It is fitted of relations $D''_{SD}{}^q=f(CE)$ and $<I>^q=f(CE)$ of $q^{th}$ MS analyte fragmentation ion. Looking at new empirical proof of equation (4), let us detail on measurands of analytes (1) and (2) (**Table 1**.) Relations $<I>^q=f(CE)$ and $<I>^q=f(C_{FA})$ of ions of (1) and (2) reveal nonlinear relationship showing $|r|$ =0.9769–0.49583 (1). Loratadine (2) exhibits $<I>^q=f(CE)$ function, showing $|r|=0.99962$–0.95594 (**Figures 7** and **8**). Generally, there is inapplicability of linear model to fit quantitatively $<I>^q=f(CE)$ and $<I>^q=f(C_{FA})$ relations.

**Table 1**

Mass spectrometric measurands such as m/z and intensity values [arb.units] *per* scan number of diclofenac (1) with respect to concentration of formic acid $C_{FA}$ = 0.2 M, and collision energy CE=10 and 20 V; descriptive statistics; data on equation (2)
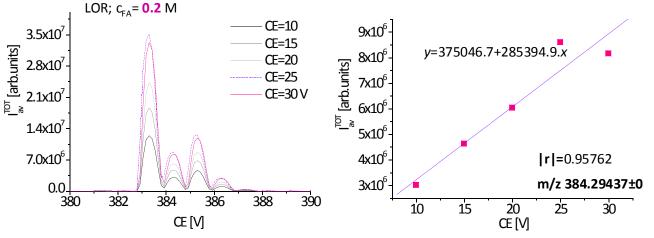
**Figure 7**. Relationships between average total intensity [arb.units] with respect to collision energy (CE=10–30V) of ESI(+)-MS spectrometric variables at concentration of HCOOH ($C_{FA}$=0.2 M) and within spectrometric range m/z=370–410; analyte (LOR) concentration 10 pmol; chemometrics.
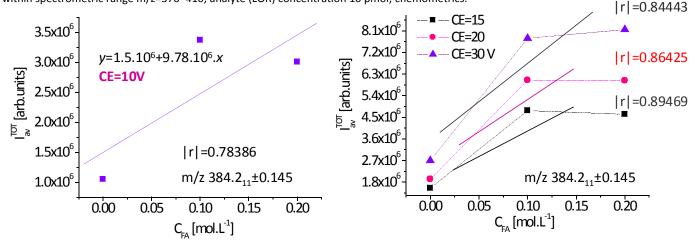


**Figure 8.** Relationships between average total intensity [arb.units] of ions at m/z 383 and 384 of the isotope shape of analyte loratadine with respect to collision energy (CE=10–30V) of ESI(+)-MS spectrometric variables at concentrations of HCOOH ($C_{FA}$=0–0.2 mol.L$^{-1}$) and within spectrometric range m/z=370–410; analyte concentration 10 pmol; descriptive statistics; chemometrics.
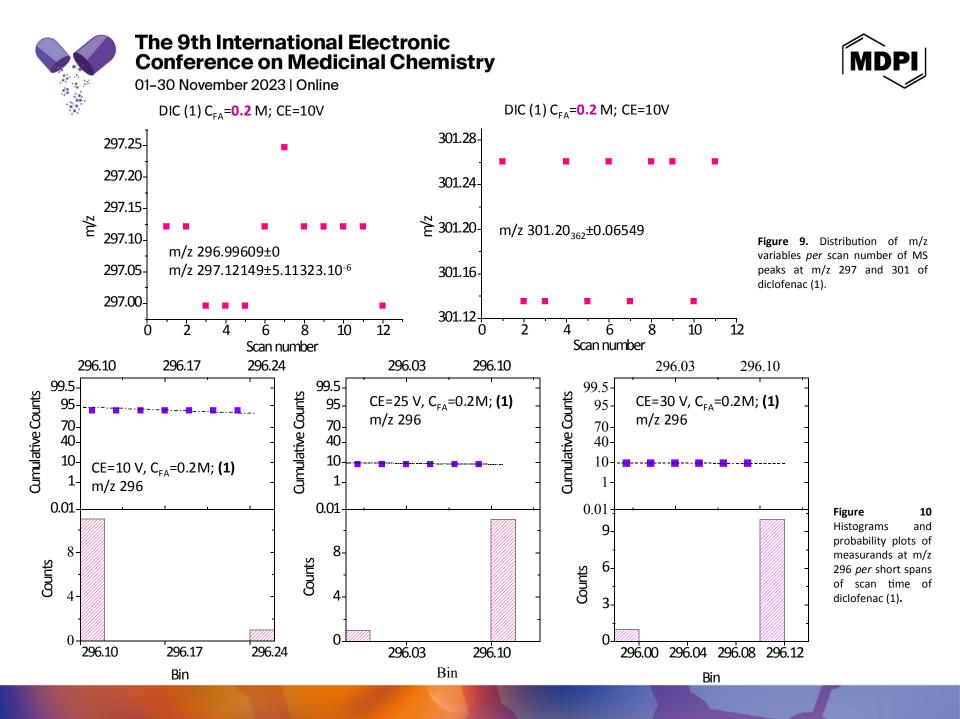
**Table 2**
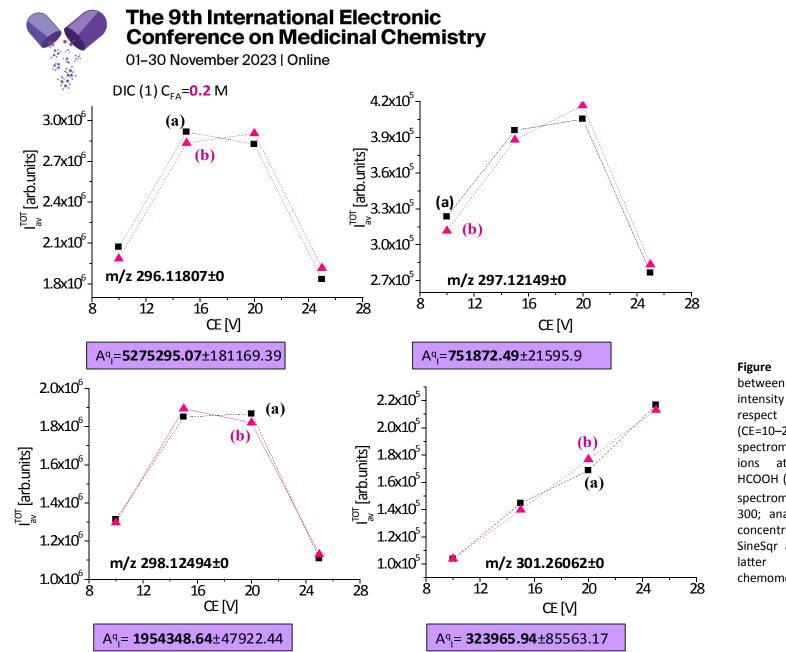
ANOVA data on m/z 296 and 301 of diclofenac (1) with respect to concentration of formic acid $C_{FA}$ = 0.2 M, and collision energy CE=10–30 V

| Dataset | N | Mean | sd(yEr±) | se(yEr±) | |
|---|---|---|---|---|---|
| Data61_A | 6 | 301.26062 | 0 | 0 | |
| Data61_B | 6 | 301.19794 | 0.15362 | 0.06271 | |
| H0: The means of all selected datasets are equal | | | | | |
| H1: The means of one or more selected datasets are different | | | | | |
| Source | DoF | Sum of Squares | Mean Square | F Value | P Value |
| Model | 1 | 0.0117875150 | 0.0117875150 | 0.99903 | 0.34112 |
| Error | 10 | 0.117989955 | 0.0117989955 | | |
| At the 0.01 level, the population means are not significantly different. | | | | | |
| Means Comparison using Bonferroni Test | | | | | |
| Dataset | Mean | Difference between | Simultaneous Confidence Intervals | | Significant at 0.01 Level |
| Data64_20 | 301.26062 | means | Upper Limit | Lower Limit | |
| Data64_25 | 301.19794 | 0.06268 | -0.13607 | 0.26144 | No |
| Dataset | N | Mean | sd(yEr±) | se(yEr±) | |
| | 4 | 296.99609 | 0 | 0 | |
| | 7 | 297.12149 | $5.11323.10^{-6}$ | $1.93262.10^{-6}$ | |
| H0: The means of all selected datasets are equal | | | | | |
| H1: The means of one or more selected datasets are different | | | | | |
| Source | DoF | Sum of Squares | Mean Square | F Value | P Value |
| Model | 1 | 0.0400236805 | 0.0400236805 | 2.29624E9 | 0 |
| Error | 9 | $1.56870629.10^{-10}$ | $1.74300699.10^{-11}$ | | |
| At the 0.01 level, the population means are not significantly different. | | | | | |
| Means Comparison using Bonferroni Test | | | | | |
| Dataset | Mean | Difference between | Simultaneous Confidence Intervals | | Significant at 0.01 Level |
| Data64_20 | 296.99609 | means | Upper Limit | Lower Limit | |
| Data64_25 | 297.12149 | -0.12539 | -0.1254 | -0.12539 | Yes |

Functional relationships according to equation (4) are determined within the same peaks of isotope shape of analyte cations. Theoretical $<I>_{theor}$ data on ions with respect to CEs, and $A_D^q$, $A_I^q$ parameters as listed in **Figures 11** and **12** are correlated with experimental $<I>_{exp}$ data on (1) (**Figure 13**) There is |r|=0.96701. The deviation of the coefficient from exact value |r|=1 is, due to error contribution to $I^{TOT}_{av;exp}$ data on intensity of MS ions of different m/z measurants *per* short span of scan time, which are statistically significantly different (**Figure 9**.) MS peaks at m/z 296.24347 and 295.99265 are different from 296.11807 one, which, however, is mainly observed within the studied spans of scan time of MS spectra of (1) at $C_{FA}$=0.2M and CE=10 V.

ANOVA tests are applied to normally distributed variables (**Figure 9**.) Looking at histograms and probability plots (**Figure 10**,) when the plot lies from a line, then random quantities are normally distributed. Conversely, when there is a deviation from linear plot, then variables can be outliner. The histogram and probability plots of m/z-data show a lack of point out of the line. The variables are normally distributed. However, W-statistics show small W-values, thus assuming a not normal distribution. This result could be interpreted as follows. Shapiro-Wilk the test is not robust. it is used to histogram and probability plots of measurands, assuming normal distribution of random variables. Moreover, detail on measurands at m/z 297, 298 and 301 shows a discrete sets of variables, belonging to molecular *isotopologies. ANOVA data* on m/z 301 reveals that variables are not statistically significantly different. Therefore, there is a single peak at m/z $301.20_{362}$±0.06549. Conversely, results from variables at m/z 297 show two sets of variables, which are statistically significantly different (**Table 2**.) There are two sets of values at m/z 296.99609±0 and 297.12149±$5.11323.10^{-6}$. The data on latter table clearly underline that without employment in ANOVA test on distinguishing statistically variables, there is unable to assign correctly isotope MS peaks of an isotope shape.

DIC (1) $C_{FA}$=**0.2** M; CE=10V

DIC (1) $C_{FA}$=**0.2** M; CE=10V

m/z 296.99609±0
m/z 297.12149±5.11323.10$^{-6}$

m/z 301.20$_{362}$±0.06549

**Figure 9.** Distribution of m/z variables *per* scan number of MS peaks at m/z 297 and 301 of diclofenac (1).

CE=10 V, $C_{FA}$=0.2M; **(1)**
m/z 296

CE=25 V, $C_{FA}$=0.2M; **(1)**
m/z 296

CE=30 V, $C_{FA}$=0.2M; **(1)**
m/z 296

**Figure 10** Histograms and probability plots of measurands at m/z 296 *per* short spans of scan time of diclofenac (1).

DIC (1) $C_{FA}$=**0.2** M



m/z 296.11807±0

$A^q_I$=**5275295.07**±181169.39

m/z 297.12149±0

$A^q_I$=**751872.49**±21595.9

m/z 298.12494±0

$A^q_I$= **1954348.64**±47922.44

m/z 301.26062±0

$A^q_I$= **323965.94**±85563.17

**Figure 11.** Relationships between average total intensity [arb.units] with respect to collision energy (CE=10–25V) of ESI(+)-MS spectrometric variables of MS ions at concentration of HCOOH ($C_{FA}$=0.2 M) and within spectrometric range m/z=200–300; analyte diclofenac (DIC) concentration 15 pmol (a); SineSqr approximation of the latter relationship (b); chemometrics.
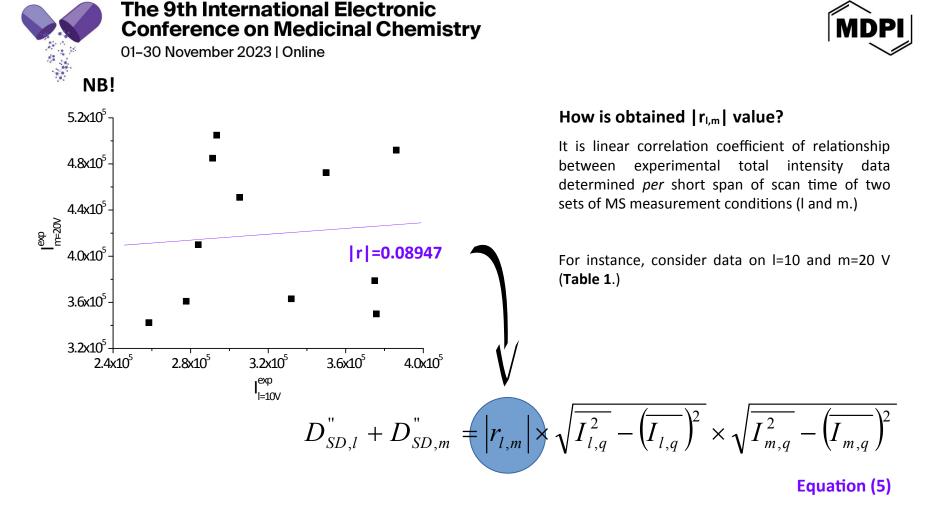
DIC (1) $C_{FA}$=**0.2** M

m/z 296.11807

$A^{q}_{D}$=**1.1262.10$^{-6}$**±3.213.10$^{-8}$

m/z 297.12149

$A^{q}_{D}$= **9.8877.10$^{-8}$**± 7.10$^{-9}$

m/z 298.12494

$A^{q}_{D}$= **4.4832.10$^{-7}$**± 5.47.10$^{-8}$

m/z 301.26062

$A^{q}_{D}$= **1.7121.10$^{-7}$**± 1.88.10$^{-8}$

**Figure 12**. Relationships between $D^{''}_{SD}$ [cm$^2$.s$^{-1}$] parameters of equation (2) with respect to collision energy (CE=10–25V) of ESI(+)-MS spectrometric variables of MS ions at concentration of HCOOH ($C_{FA}$=0.2 M) and within spectrometric range m/z=200–300; analyte diclofenac (DIC) concentration 15 pmol (a); SineSqr approximation of the latter relationship (b); chemometrics.

**NB!**



$|r|=0.08947$

## How is obtained $|r_{l,m}|$ value?

It is linear correlation coefficient of relationship between experimental total intensity data determined *per* short span of scan time of two sets of MS measurement conditions (l and m.)

For instance, consider data on l=10 and m=20 V (**Table 1**.)

$$D_{SD,l}^{"} + D_{SD,m}^{"} = \left| r_{l,m} \right| \times \sqrt{\overline{I_{l,q}^2} - \left(\overline{I_{l,q}}\right)^2} \times \sqrt{\overline{I_{m,q}^2} - \left(\overline{I_{m,q}}\right)^2}$$

**Equation (5)**

**NB!**

The contribution uses term *fragmentation ion* instead of *fragment ion;* thus, following IUPAC standards: K. Murray, R. Boyd, M. Eberlin, G. Langley, L. Li, Y. Naito, Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013). Pure Applied Chemistry. 85 (2013) 1515–1609.

**Figure 13**. Relationships between theoretical and experimental average total intensity [arb.units] data on analyte (1) with respect to collision energy (CE=10–25V) of ESI(+)-MS spectrometric variables of MS ions at concentration of HCOOH ($C_{FA}$=0.2 M) according to **equation (4)** (A); correlation between parameters according to **equation (5)** of the same analyte with respect to collision energy l=10 and m=20 V; the $|r_{l,m}|$ parameters are 0.45902 (m/z 296), 0.08947 (m/z 297), and 0.12502 (m/z 301) (B); chemometrics.

## 2. Theoretical quantum chemical data

For our purposes important computational quantum chemical method, amongst others, is that of determining molecular vibrations by means of **harmonic approximation of Born-Oppenheimer potential energy surface** [41–44]. It exhibits great success in description of molecular properties such as specific heat capacity, thermal expansion, entropy, electronic structure of stationary points, trajectories, reaction dynamics, thermochemistry of GS and TS (**Figure 14**,) molecular kinetics, spectrocopic quantities, *et cetera*, due to the fact that method employs *statistical thermodynamics.* It bridges between microscopic atomic level description of molecular dynamics or quantum-mechanical vibrational both GS and TS of molecules and macroscopic (thermal) properties. The study uses DFT-BOMD computations (**Figure 15**). DFT (**Figure 16**) and MP2 methods with modest basis set are generaly recommendable as providing accurate theoretical parameters balancing between accuracy and computational costs [45–47]. As work [47] comprehensively illustrates B3LYP-D3 method, for instance, computing relative and reaction energy values of solvate coordination compounds of transition metal ions, provides accurate energetics comparing with experiment.
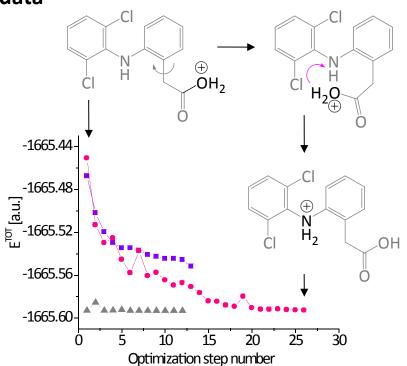


**Figure 14**. DFT (M062X) optimization of protonated [M+H]+ cation of analyte (1) showing intramolecular proton transfer: Total energy ($E^{TOT}$) [a.u.] with respect to optimization step number.

[41] A. Erba, J. Maul, M. Ferrabone, P. Carbonnière, M. Rérat, R. Dovesi,  J. Chem. Theory Comput. 15 (2019) 3755–3765.

[42]  A. Maradudin, E. Montroll, G. Weiss, Theory of lattice dynamics in the harmonic approximation; Academic Press: New York and London (1963) Suppl. 3: Solid state physics, pp. 1–319.

[43]. A. Turney, B. Zhang, D. Smith, D. Altarawy, H. Schaefer, III, J. Chem. Theory Comput. 15 (2019) 4386–4398.

[44] R. Conte, C. Aieta, G. Botti, M. Cazzaniga, M. Gandolfi, C. Lanzi, G. Mandelli, D. Moscato, M. Ceotto, Theor. Chem. Accts 143 (2023) 53.

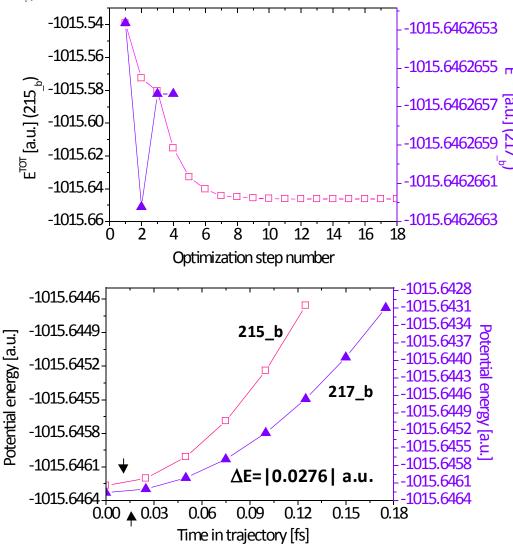[45] T. Gyori, G. Czako, J. Chem. Theory Comput. 16 (2020) 51–66.

[46] D. Villablanca, S. Gazzari, B. Herrera, Theor. Chem. Accts 142 (2023) 32.

[47] Y. Kumar, N. Kumar, G. Sastry,  Theor. Chem. Accts 142 (2023) 35.
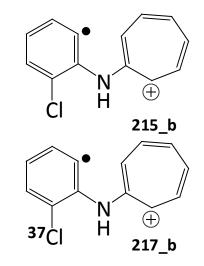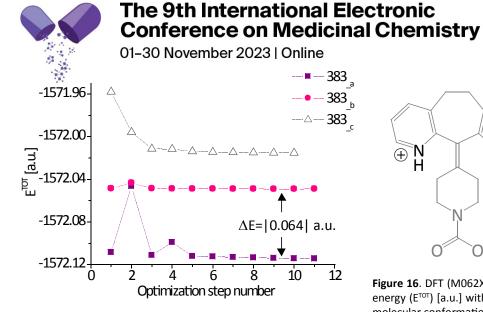
**Figure 15**. DFT (M062X) optimization of fragmentation cation of analyte (1) at m/z 215 and 217: Total energy ($E^{TOT}$) [a.u.] with respect to optimization step number; BO-molecular dynamics data: Potential energy [a.u.] *versus* time in trajectory [fs]; difference in energy data ($\Delta$E.)

The frequency analysis of species in their ground and transition states is used to compute $D_{QC}$ data on equation (3) (**Table 3**.)
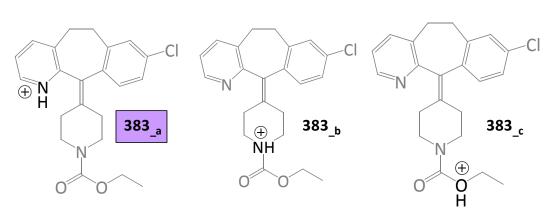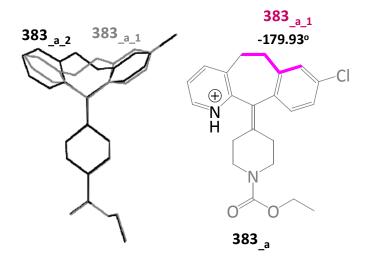
**Figure 16**. DFT (M062X) optimization of [M+H]$^+$ cation of analyte (2) depending on position of proton: Total energy ($E^{TOT}$) [a.u.] with respect to optimization step number; chemical diagrams of species; most stable 3D molecular conformation depending on the $C^5$-$C^6$-C-$C^7$ dihedral angle value (**Figure 5.**)

Most stable 3D molecular geometry is ion **383$_{a\_1}$**, showing protonation at N$^1$-position of the molecule of analyte (2) and having $C^5$-$C^6$-C-$C^7$ dihedral angle value ∠ -179.9$_3$°

## 3. Correlative analysis between experiment and theory

Following general calculation tasks and *chemometrics* of $D''_{SD}$ parameters of equation (2) of pharmaceutics we must begin, with the fact that there are a set of MS measurands of diclofenac (1). The ANOVA tests of variables show statistically significantly different m/z pairs of values at m/z 208.067161±0/208.19302±0; 215.09839±0/215.217±0; 250.08592±0/250.21136±0; 278.18182±1.52277.$10^{-6}$/278.0564±0; 296.11807±7.94.$10^{-7}$/296.2434±0; and 298.12494±3.273.$10^{-7}$/298.25037±0, respectively. The ratio of abundance of m/z data on by pairs varies within 8:3–10:2. The results, agree well with reported studies, so far, examining tautomers of analyte fragmentation ions [13,14]; thus, showing the same pairs of discrete m/z variables, which have been assigned quantitatively to tautomers, again, correlating between $D''_{SD}$ and $D_{QC}$ parameters of equations (2) and (3) (**Table 3**). There have been obtained excellent-to-exact |r|-parameters. Conversely, ANOVA tests of variables of m/z 280.06323 and 280.18869 of (1) indicate that they are not statistically significantly different (**supporting information**.) Therefore, there is a value m/z 280.0946±0.05674, belonging to only one 3D molecular and electronic structure of fragmentation ion. Owing to the fact that in the case of diclofenac, the ratio of those different electronic structures or molecular conformations of both of these is chiefly at about 80–62.5 % correlative analysis presented, herein, does not account for presence of small amount of second form of fragmentation ions. Therefore, **Figure 17** has to be understood in the following manner. Due to, aforementioned reasons, there should be a deviation of absolute |r| =1 value, which has been obtained, examining tautomers of metronidazole, where quantification of measurands has been carried out *per* tautomeric form [14]. Despite, this fact, results from **Figure 17** show |r|=0.99862, examining MS ions of diclofenac (1).

| Diclofenac (1) | | | | | |
|---|---|---|---|---|---|
| m/z | Form | $D_{QC}$ | m/z | Form | $D_{QC}$ |
| 296 | 296[(1)] b | 140.7023 | 277 | 277[(1)] | 419.6155$_{78}$ |
| 278 | 278[(1)] a | 91.1693 | 250 | 250[(1)] a | 105.6881$_8$ |
| | 278[(1)] b | 55.5705$_5$ | | 250[(1)] b | 202.9523$_{58}$ |
| | 278[(1)] c | 53.5619$_{01}$ | 248 | 248[(1)] | 79.6607$_9$ |
| 215 | 215[(1)] a | 37.4958$_{28}$ | 243 | 243[(1)] | 146.6788$_3$ |
| | 215[(1)] b | 75.8947$_9$ | 208 | 208[(1)] | 105.1445$_5$ |
| 217 | 217 b | 38.42796 | 178 | 178[(1)] | 109.2804$_1$ |
| Loratadine (2) | | | | | |
| m/z | Form | $D_{QC}$ | m/z | Form | $D_{QC}$ |
| 383 | 383[(2)] c 1a | 66.4553$_5$ | 384 | 384[(2)] a 1a C4 | 83.84224 |
| | 383[(2)] a 1a | 89.4761$_5$ | 248 | 248[(2)] a | 51.5607 |
| 384 | 384[(2)] a 1a C2 | 89.9996$_1$ | | 248[(2)] b | 38.2864 |
| | 384[(2)] a 1a C3 | 83.32625 | 337 | 337[(2)] a | 119.6811$_3$ |
| 385 | 385[(2)] c 1a | 124.2983$_5$ | | 337[(2)] b | 150.8789 |
| 259 | 259[(2)] a | 39.3346$_8$ | | | |
| | 259[(2)] b | 2428.6197 | | | |

**Table 3**

$D_{QC}$ data on equation (3) of fragmentation ions of analytes (1) and (2)

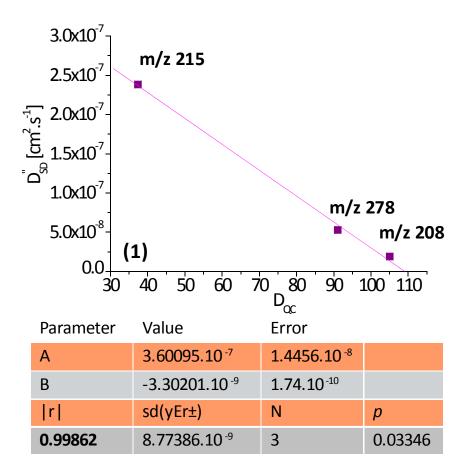| Parameter | Value | Error | |
|-----------|-------|-------|---|
| A | $3.60095 \cdot 10^{-7}$ | $1.4456 \cdot 10^{-8}$ | |
| B | $-3.30201 \cdot 10^{-9}$ | $1.74 \cdot 10^{-10}$ | |
| $|r|$ | sd(yEr±) | N | $p$ |
| **0.99862** | $8.77386 \cdot 10^{-9}$ | 3 | 0.03346 |

**Figure 17**. Correlative analysis between $D''_{SD}$ and $D_{QC}$ data on equations (2) and (3) of MS fragmentation ions of analyte (1); chemometrics.

What is most important from results of the current study is the next correlative analysis of experimental mass spectrometric and theoretical data on **equations (2)** and **(3)**, examining *molecular isotopologies* of loratadine (2). Let us look at experimental variables depicted and summarized in **Figure 18** and **Table 4**. It should be obvious from shown MS spectrum that, due to complexity of isotope shape employment in classical quantitative approaches to process data on MS measurands such as calculation of MS peak area should decrease crucially reliability of analytical information as a result from increasing in standard deviation of variables (see sd(yEr) parameters of **Table 4**.) Conversely, our stochastic dynamic approach and equation (2) processing MS variables *per* short span of scan time provide realiable results, despite, complexity of the shown isotope MS pattern. Due to, this reason chemometric assessment of relationship $D''_{SD}=f(D_{QC})$ is characterized by excellent-to-exact method performances (**|r|=0.99925**) (**Figure 19**.) The deviation of exact |r|=1 parameter is a result from great dispersion of MS measurands (**Table 4**;) thus causing for quantifying variables having very close mean values, despite, the fact that are statistically significantly different.
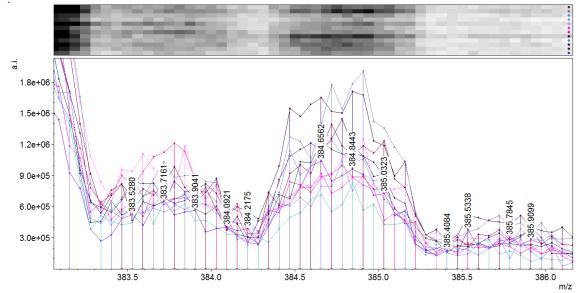
**Figure 18.** ESI-MS spectrum of loratadine (2) *per* span of scan time with respect to concentration of formic acid $C_{FA}$ = 0 M, and collision energy CE=50 V.

| Scan | m/z | I | m/z | I | m/z | I | m/z | I | m/z | I | m/z | I |
|------|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|
| 01 | 384.71893 | $1.39.10^6$ | 385.03235 | $1.23.10^6$ | 383.46533 | 821065.613 | 383.77869 | 984457.673 | 383.96674 | 789193.71 | 248.07336 | 34271.3789 |
| 02 | 384.71893 | $1.78.10^6$ | -- | -- | 383.52802 | 876073.327 | 383.71606 | $1.03.10^6$ | 383.90405 | 915113.401 | - | - |
| 03 | 384.78162 | $1.04.10^6$ | 385.03235 | 875817.698 | 383.52802 | 559912.231 | 383.65338 | 588072.353 | 383.84137 | 736488.536 | 248.01071 | 42588.0848 |
| 04 | 384.96967 | 621377 | 385.15771 | 450065 | 383.5907 | 669889 | 383.71606 | 659521 | 383.84137 | 628865 | 248.19873 | 11381 |
| 05 | 384.90698 | $1.26.10^6$ | 385.15771 | 795780.823 | 383.5907 | $1.11.10^6$ | - | - | 383.84137 | $1.19.10^6$ | 248.01071 | 24418.3919 |
| 06 | 384.8443 | 883137 | 385.09503 | 537793 | 383.52802 | 647233 | 383.65338 | 753025 | 383.84137 | 686529 | 248.01071 | 8369 |
| 07 | 384.78162 | 997404.644 | 385.03235 | 782748.685 | 383.46533 | 905116.429 | 383.77869 | $1.21.10^6$ | 384.02942 | 813532.521 | 248.07336 | 61.33003 |
| 08 | 384.8443 | $1.71.10^6$ | 385.15771 | $1.04.10^6$ | 383.46533 | 819017.613 | 383.77869 | 843017.673 | - | - | 248.13605 | 19350.391 |
| 09 | 384.71893 | $1.24.10^6$ | - | - | - | - | 383.71606 | 728449 | - | - | 248.01071 | 25146 |
| 10 | 384.78162 | $1.45.10^6$ | - | - | - | - | - | - | - | - | 248.07336 | 25292 |
| 11 | 384.8443 | $1.12.10^6$ | 385.15771 | 560513 | - | - | - | - | - | - | 248.26141 | 8987 |
| 12 | 384.8443 | 965313 | 385.03235 | 726337 | - | - | - | - | - | - | 248.07336 | 7440 |
| Mean | 384.81296 | - | 385.09503 | - | 383.52018 | - | 383.72388 | - | 383.8951 | - | 248.08477 | - |
| sd(yEr±) | 0.07793 | - | 0.06268 | - | 0.05231 | - | 0.05228 | - | 0.07616 | - | 0.08323 | - |
| se(yEr±) | 0.0225 | - | 0.02089 | - | 0.01849 | - | 0.01848 | - | 0.02879 | - | 0.0251 | - |
| <I> | - | $1.20.10^6$ | - | 777662.562 | - | 800984.985 | - | 850312.568 | - | 822125.552 | - | 18845.8706 |
| <I>$^2$ | - | $1.45.10^{12}$ | - | $6.05.10^{11}$ | - | $6.42.10^{11}$ | - | $7.23.10^{11}$ | - | $6.76.10^{11}$ | - | $3.55.10^8$ |
| <I$^2$> | - | $1.55.10^{12}$ | - | $6.60.10^{11}$ | - | $6.68.10^{11}$ | - | $7.62.10^{11}$ | - | $7.05.10^{11}$ | - | $5.06.10^8$ |
| <I$^2$>−<I>$^2$ | - | $1.05.10^{11}$ | - | $5.55.10^{10}$ | - | $2.64.10^{10}$ | - | $3.91.10^{10}$ | - | $2.92.10^{10}$ | - | $1.51.10^8$ |
| D"$_{SD}$ | - | $2.77.10^{-6}$ | - | $1.47.10^{-6}$ | - | $6.97.10^{-7}$ | - | $1.03.10^{-6}$ | - | $7.72.10^{-7}$ | - | $3.98.10^{-9}$ |

**Table 4**

Mass spectrometric measurands *per* span of scan time (m/z and intensity (I) [arb.units]) *per* scan number of loratadine (2) toward concentration of formic acid $C_{FA}$ = 0 M, and collision energy CE=50 V (see **Figure 18**,) used to calculate D"$_{SD}$ parameters of equation (2) according to chemometric criteria on normality and method performances; parameters of equation (2); chemometrics
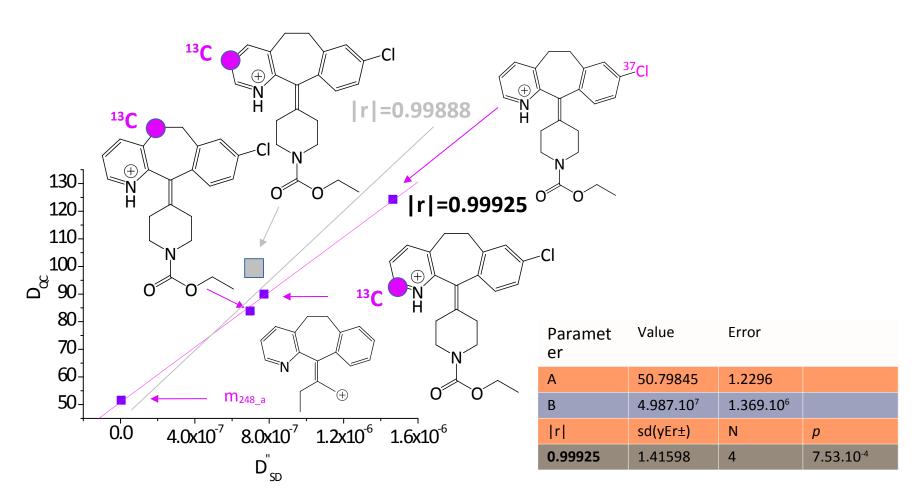
**Figure 19**. Correlative analysis between $D''_{SD}$ and $D_{QC}$ data on equations (2) and (3) of MS fragmentation ions of analyte (2); chemometrics (see **Tables 3** and **4**).

# Conclusions

To sum up, looking at e results presented, herein, a general conclusion is obvious: The innovative stochastic dynamic model **equations (2)**, **(4)** and **(5)** offer new perspectives for methodological developments of the *analytical mass spectrometry;* thus, underlying its both quantitative and 3D molecular structural analytical sub-fields. It emerges from application of formulas, so far, to problems of quantifying and determining 3D structurally pharmaceutics in complex environmental and food samples as well as biological fluids, that equations provide exact quantitative and structural analyses from perspective of *chemometrics* ($|r|=1$).

It seems highly relevant to examine systematically capability of the formulas, because of they make arguments in this study for universal application to the analytical practice, and determination about not only amount of a substance quantitatively and 3D molecular structurally, but also its molecular *isotopologies*. As the current contribution notes, application of equations (2), (4) and (5) to examine mass spectrometric measurands of diclofenac and loratadine has resulted to excellent-to-exact correlation coefficients between theory and experiment. Particularly, the relationship $D''_{SD}=f(D_{QC})$ of examining **molecular isotopologies** of loratadine yields to excellent correlation coefficient **$|r|=|$ 0.99925|**, examining statistically representative set of fragmentation peaks of analyte (2). This is the second molecular example, highlighting persuasively the great prospect of application of equation (2) to quantify and determine 3D structurally fragmentation species exhibiting subtle electronic effects, including determining species, according to their isotopic specific positions of isotopes of atoms in a molecule. Therefore, this contribution highlights the prospective impact of **equation (2)** on the field of *isotomics*, among other interdisciplinary areas of application employing broadly *omics-methods*. Despite, the fact that, currently, there are conceptual difficulty to determine concentrations of individual molecular isotopologies of analyte mass spectrometrically, the results from this study underline clearly that further research effort devoted to this issue within the framework of innovative model equations presented, herein, are justifiable, due to reliability of data evaluated by methods of *statistics* and *chemometrics*.

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