Mass Spectrometry
DOI: 10.5702/massspectrometry.A0017

Vol. 2 (2013), A0017

Original Article

Tandem Mass Spectrometry of Nitric Oxide and Hydrogen Sulfide Releasing Aspirins: A Hint into Activity Behavior

Maria Elisa Crestoni,[1] Barbara Chiavarino,[1] Stefano Guglielmo,[2] Valentina Lilla,[1] and Simonetta Fornarini*,[1]

[1] *Dipartimento di Chimica e Tecnologie del Farmaco, Università degli Studi di Roma "La Sapienza," Roma, Italy*
[2] *Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Torino, Italy*

Aspirin (acetylsalicylic acid, ASA) is the most popular non-steroidal anti-inflammatory drug. However, due to its action on cyclooxygenase and its acid nature, aspirin is associated with adverse gastrointestinal effects. In an effort to minimize these side effects, NO-donor and H_2S-donor ASA co-drugs have been designed and tested. Their mass spectrometric behavior is now analyzed and reported. Positive ions were obtained by electrospray ionization involving protonation or alkali metal attachment. Their dissociation processes have been studied by collision induced dissociation in a triple quadrupole instrument. High mass accuracy measurements have been recorded on a Fourier transform ion cyclotron resonance mass spectrometer. The protonated molecules dissociate by an exclusive or largely prevailing path leading to acetyloxy-substituted benzoyl cation, namely an ASA unit. The process is reminiscent of the enzymatic hydrolysis, releasing intact ASA to a large extent. Only at higher collision energy does the formal ketene loss disrupt the ASA moiety. The gas phase chemistry of protonated ASA-releasing drugs develops along elementary dissociation steps analogous to the reactive processes in complex biological environments. This notion may provide a tool for preliminary testing of new compounds.

Keywords: electrospray ionization mass spectrometry, FT-ICR mass spectrometry, non-steroidal anti-inflammatory drugs, acetylsalicylic acid derivatives

(Received February 7, 2013; Accepted March 10, 2013)

INTRODUCTION

Non-steroidal anti-inflammatory drugs are among the most widespread drugs. In this class, aspirin (acetylsalicylic acid, ASA) has been the most popular one. However, the use of non-steroidal anti-inflammatory drugs is limited by their toxicity, due to their action on cyclooxygenase. In particular, ASA may cause ulcers and bleeding in the upper gastrointestinal tract and these adverse effects are the major limitation to its use. The important role of nitric oxide (NO)[1–5] and hydrogen sulfide (H_2S),[6–9] small gaseous inorganics, as regulatory molecules involved in various physiological functions and pathological processes in mammalian tissues is amply recognized. In particular, there is emerging evidence that these endogenous gaseous substance can modulate inflammatory processes and exert beneficial effects on the cardiovascular system as well as gastroprotection. On this basis, ester derivatives of ASA have been synthesized bearing nitric oxide or hydrogen sulfide releasing functionalities.[10] The new compounds, with promising performances regarding gastrointestinal safety and anti-inflammatory efficacy, have been evaluated as new ASA co-drugs. The structure of the products, named **1**–**6**, is illustrated in Fig. 1.

In order to perform as ASA, as well as NO or H_2S releasing drugs, the compounds should be resistant to deacetylation relative to hydrolytic cleavage of the benzoic ester function. Hydrolysis has been studied both in buffered aqueous solution and in human serum. In all cases salicylic acid and hydroxyl derivatives were observed as final metabolites.[10] All compounds were found to behave as efficient ASA producers, with a slightly lower performance of the aliphatic carbonates **1** and **2** relative to the aromatic ones **3** and **4**, performing about similarly to the sulfur-containing products **5** and **6**.[10] As part of the product characterization procedures, mass spectra were collected in the chemical ionization mode, yielding the corresponding $[M+H]^+$ posi-

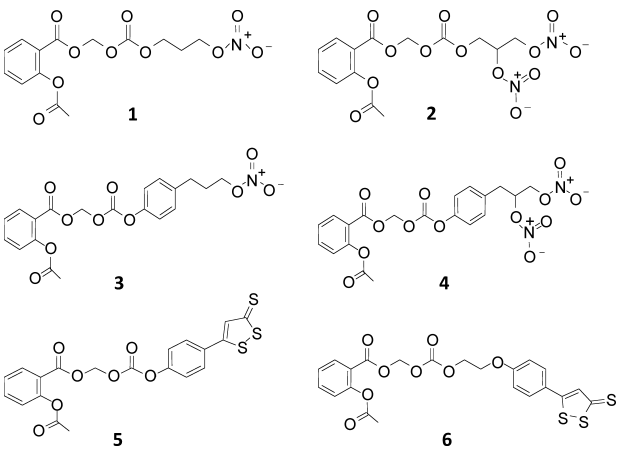


Fig. 1. Structural formulas of the sampled compounds.

* Correspondence to: Simonetta Fornarini, *Dipartimento di Chimica e Tecnologie del Farmaco, Università degli Studi di Roma "La Sapienza," P. le A. Moro 5, I–00185, Roma, Italy*, e-mail: simonetta.fornarini@uniroma1.it

Please cite this article as: S. Fornarini, *et al.*, Tandem Mass Spectrometry of Nitric Oxide and Hydrogen Sulfide Releasing Aspirins: A Hint into Activity Behavior, *Mass Spectrometry*, 2: A0017 (2013); DOI: 10.5702/massspectrometry.A0017

Table 1. ESI mass spectra of compounds **1**–**6** [*m*/*z* (relative abundance, %)].

	1	**2**	**3**	**4**	**5**	**6**
$[M+H]^+$	358 (100)	419 (100)	434 (76)	495 (46)	463 (100)	507 (100)
$[M+18]^+$	375 (58)	436 (63)	—	512 (31)	—	525 (7)
$[M+Na]^+$	380 (43)	441 (79)	456 (82)	517 (19)	485 (43)	529 (43)
$[M+K]^+$	396 (18)	457 (23)	472 (11)	533 (10)	501 (19)	545 (36)
$[C_9H_7O_3]^+$	163 (26)	163 (27)	—	163 (38)	—	—
$[C_7H_5O_2]^+$	121 (36)	121 (68)	121 (100)	121 (100)	—	—

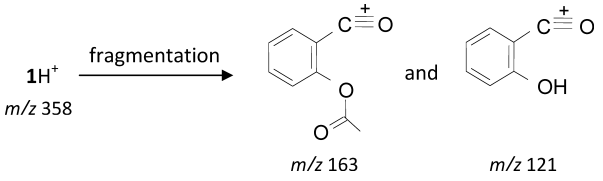
tive ions.[10] However, a comprehensive mass spectrometric analysis was not performed. Furthermore, beyond providing powerful analytical tools, mass spectrometry is well suited to reveal the reactivity behavior of naked ions in an unperturbed environment.[11–15] Aiming to investigate whether the gas phase chemistry of cationized **1**–**6** could reveal any signature of the chemical modifications responsible for their bimodal activity as ASA and NO/H_2S releasing drugs, a tandem mass spectrometric study has been undertaken and is presently reported. Interestingly, examples have been illustrated whereby mass spectrometry has aided in assessing the stability of new molecules of pharmacological potential, in predicting their behavior in biological environments, in yielding structure–reactivity relationships of active compounds.[16–19]

EXPERIMENTAL

Compounds **1**–**6** were available from a previous work where their synthesis and characterization are described.[10] Solutions (0.5–0.7 mM) were prepared in methanol–water–acetic acid (50 : 50 : 0.1, v/v) using HPLC grade solvents. Mass spectrometric experiments were carried out using a 2,000 Q-TRAP instrument (Applied Biosystems SCIEX, Concord, ON, Canada), a commercial hybrid triple quadrupole linear ion trap mass spectrometer ($Q1q2Q_{LIT}$), equipped with an electrospray ionization (ESI) source and a syringe pump. In this work the instrument was used as a classic triple quadrupole mass spectrometer. The MS conditions were set as follows: sheath gas at 34 psi, nebulizer gas at 15 psi, ion spray voltage at 5.5 kV, capillary temperature at 250°C, and declustering potential (the potential difference between ground, the skimmer, and the orifice plate) at +10 V. The sample solutions were introduced into the ESI source with the syringe pump at a flow rate of 5 μL/min. Mass spectra were scanned over the range 30–500 in positive ion mode.

In the collision induced dissociation (CID) experiments the ion of interest was mass selected using Q1 and dissociation was activated in the quadrupole collision cell q2 at nominal collision energies (E_{lab}) of 5–30 eV. The nominal pressure of the N_2 collision gas was typically set at 2.4×10^{-5} mbar. The intact precursor ion and its product ions were accumulated in Q_{LIT} and eventually detected. The standard deviation of the relative ion abundances is about ±10%.

High resolution measurements were performed on a Bruker BioApex Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Apollo I ESI source, a 4.7 T superconducting magnet, and a cylindrical infinity cell (Bruker Daltonics, Billerica, MA, USA). In the FT-ICR experiments the samples were continuously infused through a 50 mm i.d. fused-silica capillary to the ESI source at a rate of 4 μL/min and ions were accumulated in a rf-only hexapole ion guide for 1.0 s. Typical operating conditions for the ESI source were as follows: spray voltage 4.0 kV, capillary to skimmer potential difference 15 V, desolvation N_2 countercurrent gas at 380 K. The ions delivered into the FT-ICR cell at room temperature (300 K) were chirp excited and detected in direct mode (512 kword time-domain data). One hundred time-domain data sets were coadded, Hanning apodized, zero-filled once, and subjected to Fourier transform followed by magnitude calculation. The FT-ICR mass spectra were internally frequency-to-*m*/*z* calibrated with respect to ions of known elemental composition. All mass measurements are based on the "monoisotopic" ion (*i.e.*, the species in which all carbons are ^{12}C, all oxygens are ^{16}O, all nitrogens are ^{14}N, and so on). The average mass resolving power, $m/\Delta m50\%$, was 60,000 (where $\Delta m50\%$, is the mass spectral peak width at half-maximum peak height). FT-ICR mass spectral data were analyzed by use of the Xmass Analysis software package.



Scheme 1.

RESULTS AND DISCUSSION

Full scan ESI mass spectra of **1**–**6** have been recorded. The relative ion abundances appearing in the mass spectra of **1**–**6** are summarized in Table 1. The dominant ion in the spectrum of **1** is the protonated molecule ([**1**+H^+]) at *m*/*z* 358. At higher *m*/*z* values the alkali metal cationized species are observed (*m*/*z* 380, $[M+Na]^+$; *m*/*z* 396, $[M+K]^+$), while an ion at *m*/*z* 375, formally corresponding to ammonium ion attachment to M, has no straightforward attribution. Fragment ions at *m*/*z* 163 and *m*/*z* 121 can be assigned to $[C_9H_7O_3]^+$ and $[C_7H_5O_2]^+$, respectively. They conceivably arise from 'in source' collisional phenomena fragmenting the ASA portion of the molecule as depicted in Scheme 1. The appearance of these processes suggests that they are not hindered by high energy barriers, because ESI is known to impart little excess of internal energy to the desolvated ions.

The identity of the parent ion [**1**+$H]^+$, and of the alkali metal cationized species [**1**+$Na]^+$ and [**1**+$K]^+$, and the composition of the ions at *m*/*z* 163 and *m*/*z* 121 have been verified using a high resolution ESI FT-ICR mass analyzer. The relevant data listed in Table 2 confirm the assignments, showing an average error of ± 2 ppm between measured exact masses and theoretical values. The mass spectra of **2**–**6** are similar to the one of **1**. A notable difference, though, is the absence of any fragment ion in the case of compounds

Table 2. ESI-FT-ICR exact mass analysis of ions from compounds **1** and **5**.

Formula	Exact mass, Da (mass error, ppm)
$C_{14}H_{16}NO_{10}$ (**1**+H^+)	358.07793 (+1.4)
$C_{14}H_{15}NO_{10}Na$ (**1**+Na^+)	380.06052 (+3.0)
$C_{14}H_{15}NO_{10}K$ (**1**+K^+)	396.03261 (−1.8)
$C_9H_7O_3$	163.03918 (−2.1)
$C_7H_5O_2$	121.02916 (+1.7)
$C_{20}H_{15}O_7S_3$ (**5**+H^+)	462.99853 (+1.2)
$C_{20}H_{14}O_7S_3Na$ (**5**+Na^+)	484.98137 (+3.0)
$C_{20}H_{14}O_7S_3K$ (**5**+K^+)	500.96418 (+2.1)

5 and **6**. Accurate mass measurements have been carried out also on the ions formed from **5** (Table 2). The conditions favoring the formation of the *m/z* 163 and *m/z* 121 fragments have been studied by MS/MS experiments. Each potential precursor, $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, has been selected in Q1 and submitted to CID in q2. The protonated molecules $[M+H]^+$ of **1**–**4** undergo fragmentation forming ions at *m/z* 163 and *m/z* 121 as the only products. A certain difference is observed in the relative extent of fragmentation between compounds **1**–**2** and **3**–**4**. The latter ones appear more prone to undergo dissociation as judged from the relative abundance of the $[M+H]^+$ ion with respect to the products at *m/z* 163 and *m/z* 121. This behavior may arise from the presence of the aromatic ring in **3**–**4**, possibly conferring a lower energy path to the fragmentation reaction. In order to further elucidate the fragmentation process, CID experiments have been run at different collision energies. Figure 2a shows that already at a relatively low collision energy (E_{lab}=5 eV) a large fraction of the selected [**1**+H]$^+$ ions has fragmented. This result suggests a low critical energy for dissociation, consistent with the fragment ions already appearing as a result of 'in source' collisions. Upon increasing collision energy (Figs. 2b, c) the relative abundance of the product ion at *m/z* 121 increases at the expense of the ion at *m/z* 163. Overall it appears that the protonated molecule firstly releases the ASA unit at *m/z* 163 which may then undergo ketene loss when further energy is laid on the parent ion. This stepwise fragmentation mechanism has been confirmed submitting ions at *m/z* 163 coming from the ESI source to CID in q2. Fragment ions appeared at *m/z* 121. This mechanism is reminiscent of the acid-catalyzed hydrolysis responsible for the ASA releasing activity of the designed compounds. The driving force for the formation of the *m/z* 163 ion is likely the stability of the substituted benzoyl cation structure. Interestingly, the metal ion cationized molecules, $[M+Na]^+$ and $[M+K]^+$, do not release the benzoyl cations, probably due to the absence of mobile protons. However, the fragmentation paths may still lead to ASA-related ions. For example, the fragment at *m/z* 203 corresponds to sodiated ASA and may derive from the parent [**1**+Na]$^+$ ion by stepwise losses *via* intermediate ions at *m/z* 304 and *m/z* 260. The results of CID experiments conducted at the collision energy of 5 eV are summarized in Table 3. Under CID conditions the main fragmentations shown in Scheme 1 are now observed also for compounds **5** and **6**. However, they are clearly associated with a higher critical energy, appearing only upon collisional activation (Table 3). Furthermore, the general pattern of CID experiments on the ions obtained from **5** and **6** presents some different features with respect to the CID behavior of the corresponding species from **1**–**4**. The $[M+H]^+$ ions still yield ions at *m/z* 163 and *m/z* 121 but these fragments are accompanied by a product ion involving loss of 42 Da (at *m/z* 421 from **5** and at *m/z* 465 from **6**) besides further daughter ions. The 42 Da unit is likely ketene (CH_2CO) suggesting a tendency of these protonated molecules to undergo deacetylation at the ASA portion. At variance with other metal cationized species, [**6**+Na]$^+$ yields also the fragmentation products at *m/z* 163 and *m/z* 121.

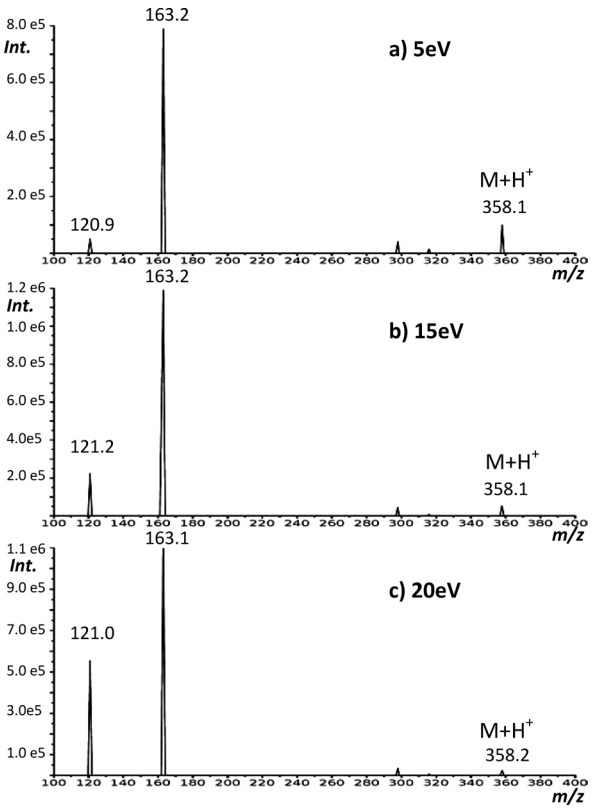


Fig. 2. Positive ion CID mass spectra obtained when [1+H]$^+$ ions are mass selected in Q1 and submitted to collision at variable energy (E_{lab}=5 eV in (a); 15 eV in (b) and 20 eV in (c)) in q2.

Table 3. CID mass spectra of $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions from compounds **1**–**6** [*m/z* (relative abundance, %)].[a]

	Parent ion	Product ions
[**1**+H]$^+$	358 (10)	163 (100); 121 (4)
[**1**+Na]$^+$	380 (58)	304 (30); 260 (15); 203(100)
[**2**+H]$^+$	419 (3)	163 (100); 121 (3)
[**2**+Na]$^+$	441 (40)	319 (100); 290 (8); 246 (7); 185 (12); 127 (4)
[**2**+K]$^+$	457 (80)	335 (100); 306 (9); 261 (5); 39 (8)
[**3**+H]$^+$	434 (4)	163 (100); 121 (4)
[**3**+Na]$^+$	456 (60)	412 (9); 380 (100)
[**3**+K]$^+$	472 (90)	428 (7); 396 (100)
[**4**+H]$^+$	495 (1)	163 (100); 121 (4)
[**4**+Na]$^+$	517 (30)	395 (80); 366 (100); 321 (9); 201 (10)
[**4**+K]$^+$	533 (57)	411 (100); 382 (90); 353 (5); 163 (5)
[**5**+H]$^+$	463 (100)	421 (25); 389 (30); 227 (95); 163 (37); 121 (8)
[**5**+Na]$^{+(b)}$	485 (100)	249 (70); 163 (30); 121 (23)
[**6**+H]$^+$	507 (100)	465 (60); 315 (40); 163 (30)
[**6**+Na]$^{+(b)}$	529 (100)	411 (23); 337 (15); 249 (46); 185 (38)
[**6**+K]$^{+(c)}$	545 (100)	513 (11); 265 (9); 121 (4)

[a] Collision energy (E_{lab}) is 5 eV for $[M+H]^+$ ions and 15 eV for $[M+Na]^+$ and $[M+K]^+$ ions, unless otherwise stated.
[b] Collision energy (E_{lab}) 20 eV for [**5**+Na]$^+$ and [**6**+Na]$^+$ ions.
[c] Collision energy (E_{lab}) 30 eV for [**6**+K]$^+$ ions.

CONCLUSION

Newly synthesized ASA co-drugs containing NO-donor or H_2S-donor functionalities have been analyzed in a mass spectrometric study of their ESI-formed ions. In a CID process the protonated ions from **1–6** are found to undergo fragmentation reactions releasing the ASA unit in the form of a highly stable benzoyl cation at *m/z* 163. Only when the energy imparted onto $[M+H]^+$ is further increased does the fragmentation proceed to the inactive deacetylated species at *m/z* 121. The ion chemistry of the protonated compounds in the gas phase thus appears to maintain some basic features that characterize the enzymatic degradation in human serum. These results suggest that under selected circumstances the gas phase ion chemistry of new molecules of potential biological activity may well be inspected for their reactivity behavior under mass spectrometric conditions. The ultimate, far reaching goal is towards obtaining a reliable measure for a preliminary screening of promising new compounds.

Acknowledgements

Financial support from the Università degli Studi di Roma 'La Sapienza' and from the Italian Ministry of Education, University and Research (MIUR) is gratefully acknowledged. The authors wish to thank Professor Alberto Gasco for helpful discussion and Marco Giampà for valuable experimental assistance.

REFERENCES

1) F. Murad. Discovery of some of the biological effects of nitric oxide and its role in cell signaling (Nobel lecture). *Angew. Chem. Int. Ed. Eng.* 38: 1857–1868, 1999.
2) L. J. Ignarro. Nitric oxide: A unique endogenous signaling molecule in vascular biology (Nobel lecture). *Angew. Chem. Int. Ed. Eng.* 38: 1882–1892, 1999.
3) R. F. Furchgott. Endothelium-derived relaxing factor: Discovery, early studies, and identifcation as nitric oxide (Nobel lecture). *Angew. Chem. Int. Ed. Eng.* 38: 1870–1880, 1999.
4) J. A. McCleverty. Chemistry of nitric oxide relevant to biology. *Chem. Rev.* 104: 403–418, 2004.
5) N. Lehnert, W. R. Scheidt. Preface for the inorganic chemistry forum: The coordination chemistry of nitric oxide and its significance for metabolism, signaling, and toxicity in biology. *Inorg. Chem.* 49: 6223–6225, 2010.
6) M. Whiteman, S. Le Trionnaire, M. Chopra, B. Fox, J. Whatmore. Emerging role of hydrogen sulfide in health and disease: Critical appraisal of biomarkers and pharmacological tools. *Clin. Sci.* 121: 459–488, 2011.
7) J. L. Wallace, G. Caliendo, V. Santagada, G. Cirino. Markedly reduced toxicity of a hydrogen sulphide-releasing derivative of naproxen (ATB-346). *Br. J. Pharmacol.* 159: 1236–1246, 2010.
8) J. L. Wallace. Physiological and pathophysiological roles of hydrogen sulfide in the gastrointestinal tract. *Antioxid. Redox Signal.* 12: 1125–1133, 2010.
9) G. Caliendo, G. Cirino, V. Santagada, J. L. Wallace. Synthesis and biological effects of hydrogen sulfide (H_2S): Development of H_2S-releasing drugs as pharmaceuticals. *J. Med. Chem.* 53: 6275–6286, 2010.
10) L. Lazzarato, K. Chegaev, E. Marini, B. Rolando, E. Borretto, S. Guglielmo, S. Joseph, A. Di Stilo, R. Fruttero, A. Gasco. New nitric oxide or hydrogen sulfide releasing aspirins. *J. Med. Chem.* 54: 5478–5484, 2011.
11) D. Schröder. Applications of electrospray ionization mass spectrometry in mechanistic studies and catalysis research. *Acc. Chem. Res.* 45: 1521–1532, 2012.
12) F. Coelho, M. N. Eberlin. The bridge connecting gas-phase and solution chemistries. *Angew. Chem. Int. Ed. Eng.* 50: 5261–5263, 2011.
13) R. H. Wu, T. B. McMahon. Structures, energetics, and dynamics of gas phase ions studied by FTICR and HPMS. *Mass Spectrom. Rev.* 28: 546–585, 2009.
14) B. Chiavarino, M. E. Crestoni, S. Fornarini, C. Rovira. Unravelling the intrinsic features of NO binding to iron(II)- and iron(III)-hemes. *Inorg. Chem.* 47: 7792–7801, 2008.
15) F. Angelelli, B. Chiavarino, M. E. Crestoni, S. Fornarini. Binding of gaseous Fe(III)-heme cation to model biological molecules: Direct association and ligand transfer reactions. *J. Am. Soc. Mass Spectrom.* 16: 589–598, 2005.
16) R. Seraglia, P. Traldi. Mass spectrometry as test bench for medicinal chemistry studies. *Anal. Bioanal. Chem.* 399: 2695–2710, 2011.
17) F. Tisato, F. Refosco, M. Porchia, M. Tegoni, V. Gandin, C. Marzano, M. Pellei, G. Papini, L. Lucato, R. Seraglia, P. Traldi. The relationship between the electrospray ionization behaviour and biological activity of some phosphino Cu(I) complexes. *Rapid Commun. Mass Spectrom.* 24: 1610–1616, 2010.
18) G. Tarzia, G. Diamantini, A. Tontini, D. Favretto, P. Traldi. Correlation of the mutagenic properties of aryl- and heteroaryltriazenes with their electron ionization induced fragmentation. *Rapid Commun. Mass Spectrom.* 10: 1156–1158, 1996.
19) E. Basso, A. Duranti, M. Mor, D. Piomelli, A. Tontini, G. Tarzia, P. Traldi. Tandem mass spectrometric data-FAAH inhibitory activity relationships of some carbamic acid *O*-aryl esters. *J. Mass Spectrom.* 39: 1450–1455, 2004.

