

Neurochemical analysis of amino acids, polyamines and carboxylic acids: GC–MS quantitation of tBDMS derivatives using ammonia positive chemical ionization

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Abstract

The GC–MS quantitation of a large number of neurochemicals utilizing a single derivatization step is not common but is provided by the reagent *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoro-acetamide (MTBSTFA). Previous workers have utilized this derivative for GC–MS analyses of amino acids, carboxylic acids and urea with electron impact (EI) and with positive chemical ionization (PCI; methane as reagent gas). However, these conditions yield significant fragmentation, decreasing sensitivity and in some cases reducing specificity for quantitation with selected ion monitoring (SIM). Additionally, the majority of studies have used a single internal standard to quantitate many compounds. In this study we demonstrate that using isotopic dilution combined with ammonia as the reagent gas for PCI analyses, results in high precision and sensitivity in analyzing complex neurochemical mixes. We also demonstrate for the first time the utility of this derivative for the analysis of brain polyamines and the dipeptide cysteinyl glycine. In the case of ammonia as the reagent gas, all amino acids, polyamines and urea yielded strong $[MH]^+$ ions with little or no fragmentation. In the case of carboxylic acids, $[M + 18]^+$ ions predominated but $[MH]^+$ ions were also noted. This approach was used to analyze superfusates from hippocampal brain slices and brain tissue extracts from brain lesion studies. The advantages of this methodology include: (i) simple sample preparation; (ii) a single derivatization step; (iii) direct GC–MS analysis of the reaction mix; (iv) high precision as a result of isotopic dilution analyses; (v) high sensitivity and specificity as a result of strong $[MH]^+$ ions with ammonia reagent gas; (vi) no hydrolysis of glutamine to glutamate or asparagine to aspartate; and (vii) applicability to a wide range of neurochemicals.

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1. Introduction

tert-Butyldimethylsilyl (tBDMS) derivatives have been utilized to monitor amino acids [1–5], sulfur amino acids [6,7], urea, carboxylic acids including lactate and pyruvate, α -keto acids, ketone bodies and free fatty acids [3,8]. This simple and robust derivatization procedure has proven extremely useful in the selected ion monitoring (SIM) quantitation of all of these compounds as well as in studies of amino acid synthesis and dynamics [9–11]. With regard to neurochemical analyses, tBDMS derivatives have been used to study regional GABA

turnover in the brain via labeling with stable isotope precursors [12], GABA synthesis in synaptosomes [13,14] and glutamate synthesis [15,16] and glutamate release in brain dialysis studies [15]. However, analyses using electron ionization (EI) or PCI with methane have resulted in significant fragmentation and weak molecular ions which are the most characteristic markers for each of the analytes listed in Table 1. In this study, we demonstrate for the first time that MTBSTFA is also useful for the analysis of polyamines in complex neurochemical samples. Additionally, we have demonstrated that ammonia as the reagent gas for PCI results predominantly in the production of strong $[MH]^+$ ions that are extremely useful for SIM of complex biological matrices that are only partially resolved. The sensitivity provided by SIM of predominant molecular ions also allows the analysis of compounds present in low concentrations along

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Table 1
Retention times and ions utilized for SIM of tBDMS amino acids, polyamines, carboxylic acids and urea with ammonia PCI

Compound	MW	Number of tBDMS groups (1 1 4)	Derivative MW	RT	[MH] ⁺	IS [MH] ⁺
Pyruvate	88	2	316	4.8	334 ^a /317	336 ^a /319
Lactate	90	2	318	4.9	319	322
Glycine	75	2	303	5.1	304	307
Urea	60	2	288	5.5	289	292
Leucine	131	2	359	5.6	360	–
GABA	103	2	331	5.8	332	338
Putrescine	88	2	316	5.85	317	321
Proline	115	2	343	5.9	344	351
Aspartic acid	133	3	475	5.9	476	479
Pyroglutamate	129	2	357	6.4	358/375 ^a	–
Serine	105	3	447	6.7	448	451
Methionine	149	2	377	6.8	378	381
Spermidine	145	2	373	7.2	374	–
Cysteine	121	3	463	7.4	464	468
Glutamic acid	147	3	489	7.5	490	495
Ornithine	132	3	474	7.6	475	481
Glutamine	146	3	488	8.0	489	494
Cysteine sulfinate	153	3	495	8.6	496	–
Cysteinyl glycine	178	3	520	10.1	521	–
Cystathionine	222	4	678	11.0	679	683
Cystine	240	4	696	12.8	697	699

^a [M + 18]⁺; MW, molecular weight; RT, retention time (min); IS, stable isotope internal standard.

with compounds in high concentration in a single GC–MS run. The only other method that has also yielded strong molecular ions of these derivatives has utilized atmospheric photochemical ionization [17]. The current study demonstrates the utility of ammonia PCI, a simpler method than atmospheric photochemical ionization, via presentation of data from different complex neurochemical samples.

2. Experimental

2.1. Reagents

Amino acids, polyamines and carboxylic acids were all purchased from Sigma (St. Louis, MO). Acetonitrile, extra dry, was purchased from Acros Organics (Fisher Scientific), *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane from Sigma.

2.2. Stable isotope internal standards

L-[²H₅]glutamic acid, [²H₆]GABA, L-[²H₅]glutamine, DL-[²H₄]cystine, [²H₅]glycine, DL-[²H₃]serine, [²H₆]ornithine, [²H₄]putrescine, and [²H₄]cystathionine were purchased from CDN Isotopes (Pointe Claire, Quebec). L-[¹³C₃¹⁵N₁]cysteine, [¹³C₂¹⁵N₁]urea, [²H₇]proline and [¹³C₂]pyruvate were purchased from Cambridge Isotopes Labs (Woburn, MA). L-[²H₃]aspartate and L-[²H₃]methionine were purchased from Sigma (St. Louis, MO).

All unlabelled compounds and associated stable isotopic variants were stored frozen as 5 mM solutions in 1N HCl. These stock solutions were used to make the appropriate dilutions for analysis of biological samples on the same day of sample

preparation. Standards for the standard curve (five points) for all analytes underwent identical processing as the biological samples.

2.3. Samples

Male Sprague–Dawley rats (200 g) were administered trimethyltin (8 mg, sc) 14 days prior to decapitation and isolation of the lesioned hippocampi. A single hippocampus from each rat was sonicated in 1N HCl containing stable isotope internal standards and processed as described in Section 2.4.

Untreated rats were decapitated and the hippocampus isolated in Hanks Balanced Salt Solution containing 20 mM HEPES (HBSS-H, 4 °C). The hippocampus was next sliced into 300 μM slices. A single slice was incubated in 2 ml of HBSS-H in 12-well culture plates at 37 °C for 30 min. The media was discarded and the slice incubated for two further 10 min periods, each with fresh media. Next the slices were incubated for 5 min after which the media was isolated for amino acid analyses (pre sample), followed by a 5 min incubation in which 50 mM KCl was added to evoke neurotransmitter release from the slices (stimulation sample). Next a final 5 min incubation in HBSS-H was collected (post sample). The amino acids released into the media were isolated by cation exchange chromatography as described in Section 2.4.

2.4. Sample preparation

Tissues were sonicated in 1.5 ml screwtop microfuge tubes (Sarstedt, Newton, SC) containing 750 μl of 1N HCl and appropriate stable isotope internal standards. Extraction efficiency

from brain tissues was 90–95% for all analytes examined. The samples were centrifuged for 40 min at $25,000 \times g$ at 4°C . The supernatants were dried overnight in a Savant concentrator. The dried samples were reacted as described in Section 2.5.

Tissue culture media, tissue superfusates, CSF or plasma samples are transferred to 18 mm \times 100 mm glass tubes containing appropriate stable isotope internal standards and mixed with 500 μl of cation-exchange resin, Dowex AG 50W-X8 (200–400 mesh, hydrogen form, Biorad, Hercules, CA). The tubes were shaken for 5 min, the resin allowed to settle and the supernatant aspirated. The resin was next washed three times via brief vortexing with 4 ml of water. The washes were aspirated each time after the resin settled. The acids and polyamines were then eluted with 1 ml of 8N NH_4OH and 900 μl added to 1.5 ml screwtop microfuge tubes which were dried overnight in a Savant concentrator. The dried samples were directly reacted as described in Section 2.5.

In the case of lactic acid and pyruvic acid, these compounds were isolated from fluids via absorption to anion-exchange resin, AG1-X8 (100–200 mesh, acetate form, Biorad), washing with water and elution of analytes with 0.1N HCl.

2.5. Derivatization

To the Savant-dried samples, were added 50 μl of acetonitrile and 50 μl of MTBSTFA containing 1% *t*-butyldimethylchlorosilane (TBDMS-Cl) as a catalyst. The tubes were capped and heated at 80°C for 1 h in a dry-block. After cooling, the samples were centrifuged at $25,000 \times g$ for 5 min in a microfuge to precipitate any salts or particulate material. The clear reaction supernatants were transferred to 0.1 ml autosampler vials for GC-MS analyses. This derivatization procedure results in the addition of a TBDMS group (+114) to each $-\text{COOH}$, $-\text{NH}_2$, $-\text{SH}$, $-\text{SO}_2\text{H}$ and $-\text{OH}$ functional group. In the case of pyruvic acid, derivatization of the $-\text{C}=\text{O}$ group also occurred.

2.6. GC-MS analysis

An Agilent bench-top GC-MSD (HP6890/MSD5973) system was autotuned (41, 267 and 599) under PCI conditions with methane as the reagent gas. Next the reagent gas was switched to ammonia and gas flow optimized via monitoring m/z 52. The GC-MS conditions included: source (150°C), quadrupole (150°C), interface (320°C), injector (250°C), emission (150 μA) and eV (150). The injection port liner was packed with 2% SP-2250 on 100/200 Supelcoport (Sigma), serving as a precolumn. Aliquots of 1 μl were injected splitless. The HP-5ms capillary column (25 m, 0.25 mm i.d. and 0.25 μm thickness; 5%-phenyl-methylpolysiloxane bonded and crosslinked) was held at 100°C for 1 min followed by 30°C gradient to 300°C , with carrier gas (He) flow of 1.2 ml/min. For most analyses this resulted in a run time of 10 and 3 min between injections. In cases of samples with interfering peaks, the temperature gradient was reduced to $20^\circ\text{C}/\text{min}$.

2.7. Standard curves and assay variability

Pilot experiments were used to define the level of stable isotope internal standard that needed to be spiked for each compound in different biological samples. Once established, this was used for repeated experiments with a five point standard curve evaluated with each experiment (unlabelled:internal standard = 0.25:1, 0.5:1, 1:1, 2:1, 4:1). Calibration curves plotting the peak area between the endogenous compound and its associated labeled internal standard were all linear ($r = 0.999$) and reproducible.

Reproducibility was evaluated both with regard to intra-day relative standard deviation (R.S.D.) and inter-day R.S.D. These were ≤ 2 and $\leq 8\%$, respectively. Biological samples were found to be stable for at least 24 h at room temperature, allowing for overnight autosampler loading. Reaction mixes, stored at -70°C , were stable for several weeks.

3. Results

3.1. Spectra: methane versus ammonia reagent gas

PCI with methane as reagent gas yielded very similar fragmentation for all analytes, namely key fragment ions of $[\text{MH}]^+$, $[\text{M} - 15]^+$ from the loss of a methyl from the TBDMS group, $[\text{M} - 57]^+$ from the loss of a *t*-butyl from the TBDMS group, and $[\text{M} + 29]^+$ from a methane adduct (see Fig. 1). In contrast, PCI with ammonia as the reagent gas yielded spectra dominated by the $[\text{MH}]^+$ ion (see Fig. 1). In the cases of pyroglutamate, pyruvate and lactate, $[\text{MH}]^+$ and $[\text{M} + 18]^+$ ions dominated the spectra.

3.2. SIM parameters for PCI analysis of compounds with ammonia reagent gas

Table 1 lists the compounds derivatized in a single reaction step. For all analytes we utilized the $[\text{MH}]^+$ ion for SIM, except for pyruvate and lactate where the $[\text{M} + 18]^+$ ions were utilized. The retention times are presented based on a steep temperature gradient. In the case of interfering peaks in complex samples, the gradient can be modified to provide improved chromatography. Representative SIM traces of hippocampal amino acids isolated from tissue superfusates by ion exchange chromatography and from simple acid extracts of hippocampal brain slices are presented in Fig. 3.

3.3. Neurochemical analysis of rat hippocampal lesions

Analysis of control and trimethyltin (TMT) lesioned hippocampus in rats demonstrated no changes in steady state levels of the amino acids GABA, glutamate, glycine and serine but decreases in cysteine levels, an index of the oxidative stress invoked by TMT (Table 2). Urea levels were unchanged as were ornithine levels. However, putrescine levels were increased, an index of increased polyamine oxidase activity in this lesion model.

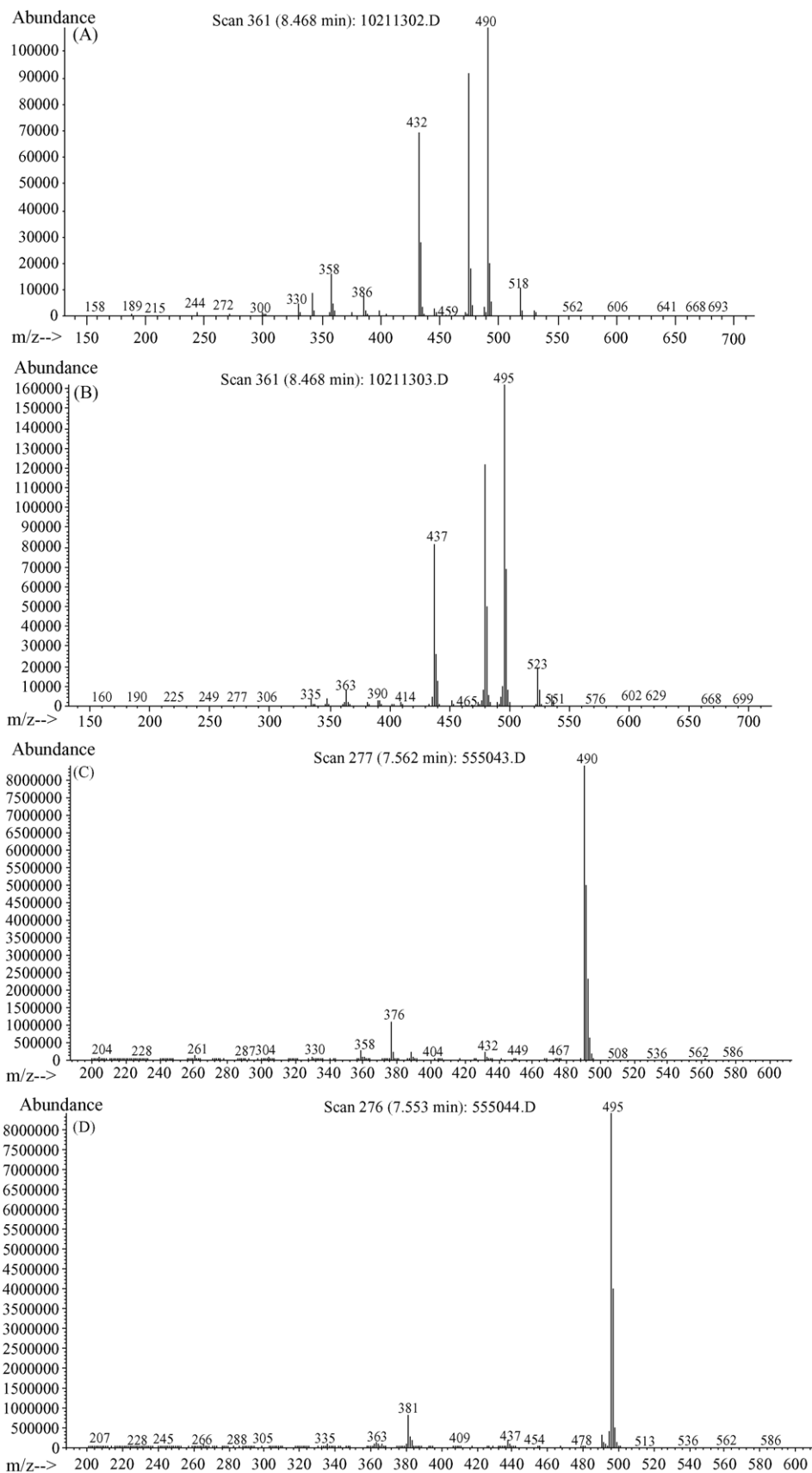


Fig. 1. PCI spectra of glutamate (A) and [$^2\text{H}_5$]glutamate (B) with methane as the reagent gas. Note the strong $[\text{MH}]^+$, $[\text{M} - 15]^+$, and $[\text{M} - 57]^+$ ions along with an $[\text{M} + 29]^+$ ion from the methane reagent gas. In contrast, the ammonia PCI spectra for glutamate (C) and [$^2\text{H}_5$]glutamate (D) are dominated by the $[\text{MH}]^+$ ion. A characteristic fragment resulting from the loss of 1 tBDMS group $[\text{MH} - 114]^+$ was also noted in ammonia PCI ($d_0 = 376$; $d_5 = 381$).

Table 2
Amino acid, polyamine and urea levels of rat hippocampus 14 days after lesions with the toxin trimethyltin (8 mg/kg, sc)

Analyte	Units	Control	TMT
Cysteine	nmol/mg protein	10.0 ± 0.63	7.1 ± 0.85*
GABA	nmol/mg protein	65.4 ± 3.9	66.2 ± 2.3
Glycine	nmol/mg protein	19.5 ± 1.1	14.5 ± 0.6
Putrescine	pmol/mg protein	174 ± 15	286 ± 30*
Serine	nmol/mg protein	21.8 ± 0.8	19.2 ± 0.4
Glutamate	nmol/mg protein	149 ± 4.4	143 ± 4.6
Ornithine	nmol/mg protein	1.0 ± 0.07	1.0 ± 0.09
Urea	nmol/mg protein	56.4 ± 3.5	56.1 ± 1.9

Mean ± S.E.M.

* $p < 0.05$ ($N = 7$ per group). These data agree well with previously published results, except for urea, in which case, regional brain levels are not available.

3.4. Analysis of neurotransmitter release from rat hippocampal slices

Analysis of the media from hippocampal incubations demonstrated KCl-evoked neurotransmitter release of glutamate and GABA but not serine, which is not a classical neurotransmitter released from nerve endings (Fig. 2).

4. Discussion

GC–MS analytical methods that provide versatility with regard to the range of neurochemicals that can be analyzed, along with high precision provided by isotopic dilution analyses, are valuable tools for the neurochemist and neuropharmacologist. However, multiple derivatization procedures are often needed for the analysis of complex neurochemical samples [18]. In the case of tBDMS derivatives, a wide versatility has already been demonstrated in neurochemical analyses [1–16]. In this study we have utilized TBDMS compound derivatization and demonstrated the utility of ammonia PCI and isotopic dilution analyses both for the sensitive and precise quantitation of a variety of neurochemicals. With the majority of neurochemicals analyzed with ammonia PCI, almost the entire ion current was made up by the $[MH]^+$ ions, providing both specificity and sensitivity for SIM (Fig. 3).

In the first example of a biological sample, we found as described by previous workers [19], that the majority of amino acids are not altered in the hippocampus of TMT-lesioned rats. However, our data are the first to demonstrate increased polyamine metabolism, as reflected by increases in putrescine levels and increases in oxidative stress as indicated by decreases in hippocampal cysteine levels. The analysis of this tissue extract in a single GC–MS run demonstrated the flexibility and accuracy of this methodology for neurochemical analyses. These observations were further validated in a second study examining KCl-evoked neurotransmitter release from rat hippocampal brain slices. A simple ion exchange clean-up procedure followed by a one step reaction and direct injection of the reaction mix made this a very simple assay to operate in the acquisition of precise quantitative data from a complex tissue superfusate.

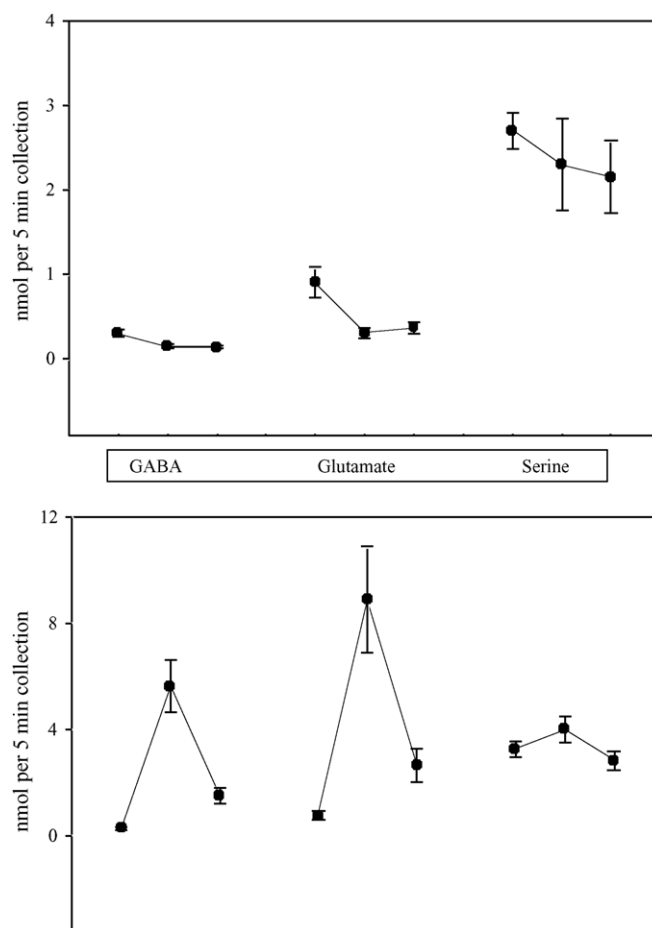


Fig. 2. Release of GABA, glutamate and serine from single rat hippocampal slices. The data points are from four separate samples and are 5 min collection periods after a 50 min equilibrium period with three washes. In the upper graph, no stimulus was given, while in the lower graph, 50 mM KCl was applied during the second 5 min collection period. Mean ± S.E.M.

In summary, the advantages of tBDMS derivatives for GC–MS analyses that have been established by previous workers and by the current studies include:

- Ability to quantitate a wide range of neurochemicals, including amino acids, sulfur amino acids, dipeptides, carboxylic acids, urea, and polyamines; functional groups derivatized include $-\text{COOH}$, $-\text{NH}_2$, $-\text{SH}$, $-\text{SO}_2\text{H}$, $-\text{OH}$ and $-\text{C}=\text{O}$ in the case of pyruvate.
- Sample preparation is simple, not requiring extensive purification procedures.
- The reaction is a simple one step procedure.
- The reaction mix can be injected directly for GC–MS; however, in the case of trace analyses, the reaction mix can also be subjected to aqueous–organic washing protocols with hexane to extract the reaction products and water to hydrolyze the unreacted derivatizing reagents.
- There is no hydrolysis of glutamine or pyroglutamate to glutamate or of asparagine to aspartate, in contrast to harsh alkylation protocols [20].

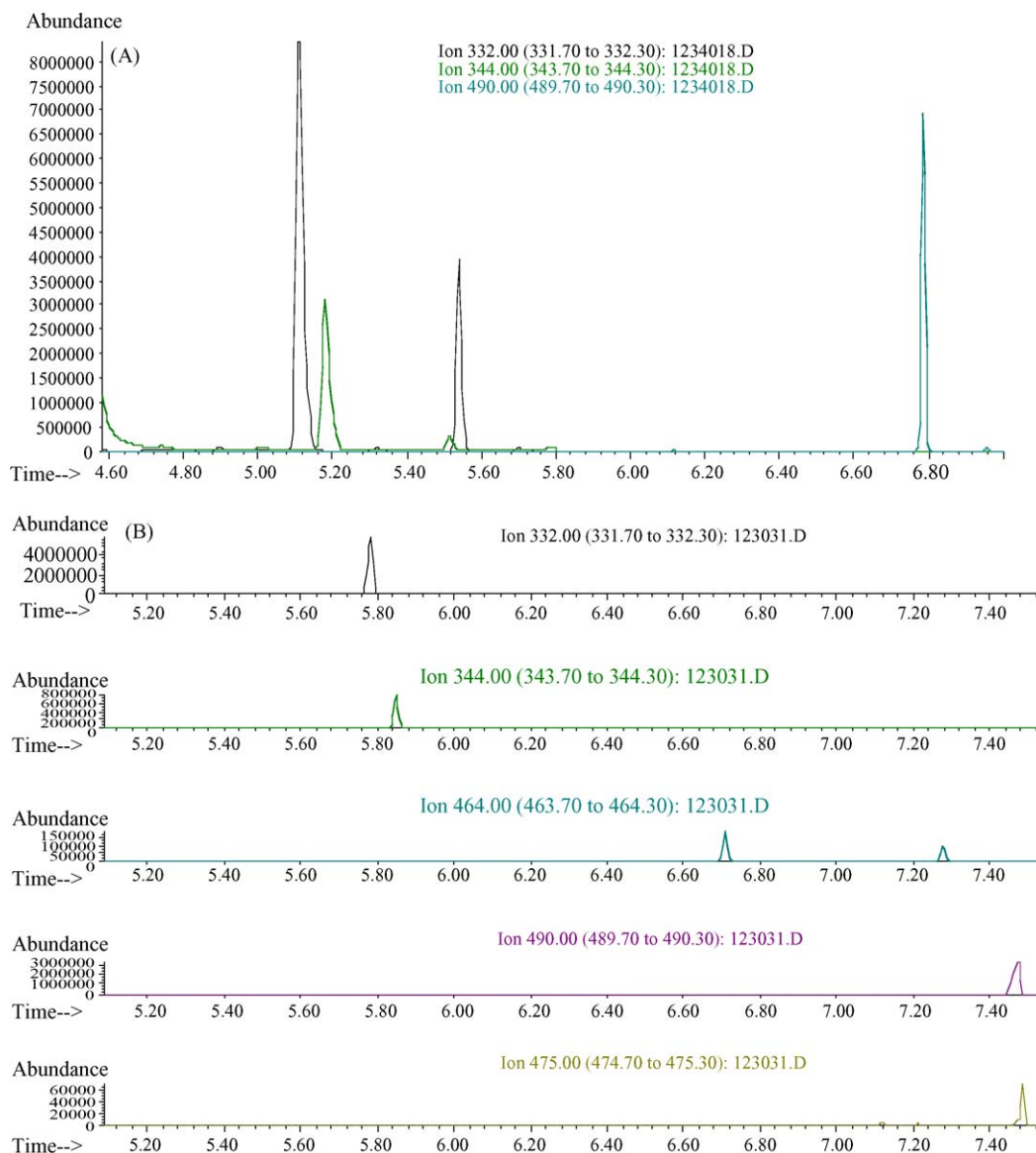


Fig. 3. Representative SIM scans from biological samples. In (A), KCl-evoked release of GABA (332, first peak), proline (344, second peak) and glutamate (490, fourth peak) from a single hippocampal slice were monitored; the third peak is an unknown. In this example, the column temperature was held at 120 °C for 1 min followed by a 30 °C/min temperature gradient. In (B), the SIM scans of an acid extract of a single hippocampal slice are presented. The upper trace was GABA (332), next was proline (344), cysteine (464), glutamate (490) and the lowest trace was ornithine (475). In this case the column was held at 100 °C for 1 min followed by a gradient of 30 °C/min.

- Ammonia PCI yields the greatest ion current for the $[MH]^+$ ion, resulting in both enhanced sensitivity and in specificity for SIM.
- Isotopic dilution analyses yield the most accurate quantitation possible.

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References

- [1] T.P. Mawhinney, R.S. Robinett, A. Atalay, M.A. Madson, J. Chromatogr. 358 (1986) 231.
- [2] A.G. Calder, K.E. Garden, S.E. Anderson, G.E. Lobley, Rapid Comm. Mass Spectrom. 13 (1999) 2080.
- [3] W.F. Schwenk, P.J. Berg, B. Beaufriere, J.M. Miles, M.H. Haymond, Anal. Biochem. 141 (1984) 101.
- [4] H.J. Chaves Das Neves, A.M. Vasconcelos, J. Chromatogr. 392 (1987) 249.
- [5] S.L. MacKenzie, D. Tenaschuk, G. Fortier, J. Chromatogr. 387 (1987) 241.
- [6] S.P. Stabler, P.D. Marcell, E.R. Podell, R.H. Allen, Anal. Biochem. 162 (1987) 185.
- [7] C.R. Santhosh-Kumar, J.C. Deutsch, J.C. Kolhouse, K.L. Hassell, J.F. Kolhouse, Anal. Biochem. 220 (1994) 249.
- [8] T.P. Mawhinney, R.S.R. Robinett, A. Atalay, M.A. Madson, J. Chromatogr. 361 (1986) 117.
- [9] B.W. Patterson, F. Carraro, R.R. Wolfe, Biol. Mass Spectrom. 22 (1993) 518.
- [10] B.D. Williams, R.R. Wolfe, Biol. Mass Spectrom. 23 (1994) 682.

- [11] G.N. Thompson, P.J. Pacy, H. Merritt, G.C. Ford, M.A. Read, K.N. Cheng, D. Halliday, *Am. J. Physiol.* 256 (1989) E631.
- [12] G. Carboni, P. Tueting, L. Tremolizzo, I. Sugaya, J. Davis, E. Costa, A. Guidotti, *Neuropharmacology* 46 (2004) 1070.
- [13] J. Palaty, R. Burton, F.S. Abbott, *J. Chromatogr. B* 662 (1994) 85.
- [14] H.S. Waagepetersen, U. Sonnewald, O.M. Larsson, A. Schousboe, *J. Neurosci. Res.* 57 (1999) 342.
- [15] R.W. Kondrat, K. Kanamori, B.D. Ross, *J. Neurosci. Methods* 120 (2002) 179.
- [16] M. Yudkoff, Y. Daikhin, I. Nissim, A. Lazarow, I. Nissim, *J. Neurosci. Res.* 66 (2001) 272.
- [17] I.A. Revelsky, Y.S. Yashin, T.G. Sobolevsky, A.I. Revelsky, B. Miller, V. Oriedo, *Eur. J. Mass Spectrom.* 9 (2003) 497.
- [18] P.L. Wood, H.S. Kim, D.L. Cheney, C. Cosi, M. Marien, T.S. Rao, L.L. Martin, *Neuropharmacology* 27 (1988) 669.
- [19] W.E. Wilson, P.M. Hudson, T. Kanamatsu, T.J. Walsh, H.A. Tilson, J.S. Hong, R.R. Marenpot, M. Thompson, *Neurotoxicology* 7 (1986) 63.
- [20] P.L. Wood, D.L. Cheney, *Neuromethods* 3 (1985) 51.