ELSEVIER

Journal of Chromatography B, 730 (1999) 81–93

JOURNAL OF CHROMATOGRAPHY B

# High-performance liquid chromatographic analysis of physiological amino acids in human brain tumors by pre-column derivatization with phenylisothiocyanate

Arturo Battaglia[a,*,1], Alessandro Bertoluzza[b], Fabio Calbucci[c], Vincenzo Eusebi[d], Patrizia Giorgianni[a], Renata Ricci[e], Raffaella Tosi[f], Vitaliano Tugnoli[b]

[a]*Istituto CNR dei Composti del Carbonio Contenenti Eteroatomi "I.Co.C.E.A.", Via Gobetti, 101-40129 Bologna, Italy*
[b]*Dip. di Biochimica "G. Moruzzi" Sez. di Chimica e Propedeutica Biochimica, Univ. Bologna, Via Belmeloro 8/2, 40126 Bologna, Italy*
[c]*Divisione di Neurochirurgia, Ospedale Bellaria, Via Altura 3, 40139 Bologna, Italy*
[d]*Sezione di Anatomia, Istologia e Citologia Patologica "M. Malpighi" Univ. Bologna, Via Altura 3, 40139 Bologna, Italy*
[e]*Servizio di Neuroradiologia, Ospedale Bellaria, Via Altura 3, 40139 Bologna, Italy*
[f]*Inst. Di Citomorfologia Normale e Patologica, CNR, Via di Barbiano 1/10, 40136 Bologna, Italy*

Received 16 November 1998; received in revised form 23 April 1999; accepted 23 April 1999

**Abstract**

A reversed-phase high-performance liquid chromatographic technique for the determination of free amino acids in five biopsies of human brain tumors (two meningiomas, one glioblastoma and two oligodendrogliomas) is described. The frozen tissues were homogenized, deproteinized with perchloric acid and neutralized with potassium hydroxide. Aliquots of the supernatant containing the physiological amino acids are used for pre-column derivatization with phenylisothiocyanate. The derivatized PTC-amino acids (phenylthiocarbamyl derivatives) are stable for a five day period if stored as a powder at −20°C in an inert atmosphere and they can be analyzed on a reversed-phase column (PicoTag) using a gradient of two eluents with absorption detection at a wavelength of 254 nm. Good resolution of several amino acids (>30) is achieved within ca. 60 min. For most amino acids this method is suitable for an accurate measurement over a wide range of physiological concentrations (50–400 pmol) starting from a very small amount of sample. 

*Keywords:* Derivatization, LC; Amino acids; Phenylisothiocyanate

## 1. Introduction

Non-invasive investigations of human brain tumors by in vivo localized proton magnetic resonance spectroscopy ($^1$H MRS) have revealed that tumor spectra exhibit metabolic alterations, induced by tumor growth, with respect to the spectra of healthy contra-lateral hemisphere [1–3]. The most common alterations are related to a number of metabolites, such as lactate, choline, inositol, *N*-acetylaspartate, phosphocreatine, creatinine, phos-

*Corresponding author.
*E-mail address:* battaglia@area.Bo.CNR.it (A. Battaglia)
[1]Dedicated to my beloved wife Maria Grazia and to my nephews Cristina and Lorenzo.

0378-4347/99/$ – see front matter 
PII: S0378-4347(99)00188-7

phorylcholine, glycerophophorylcholine and succinate. Such metabolic alterations may offer an in vivo non-invasive evaluation of malignancy grading. However, the interpretation of $^{1}H$ MRS spectra is difficult because of the low-frequency resolution in vivo. Potential clinical applications of MRS necessitates the development of higher-field MRS, but there are also been questions raised regarding the long term safety of magnetic field exposure including possible increased cancer rates. For this reason in vitro high-field $^{1}H$ nuclear magnetic resonance (NMR) studies of tumor biopsies have been undertaken [4–6]. These studies have shown additional signals from a variety of metabolites, such as the physiological amino acids. However, at present, a number of these metabolites are beyond the resolution of NMR spectroscopy. Furthermore, quantitative analysis is difficult and can be complicated by the presence of composite resonances of the NMR visible protons. Recognizing these NMR potential problems and the importance of obtaining a detailed profile of the physiological amino acids for metabolism studies of tumors, some analytical techniques have been developed to complement the NMR data.

Regardless of the analytical technique used, a major problem is to avoid the autolysis of the biopsied tissue during the analysis. The autolysis is due to the activity of proteolytic enzymes which continue in the brain after death increasing the level of most amino acids which are constituents of proteins. In fact, it has been demonstrated [7–9] that the free amino acid profile of brain tissues strongly modifies with the time when the biopsies are left at the temperatures of the refrigerators. Some amino acids are remarkably stable while others are rapidly altered in a few minutes. On the other hand, it has been demonstrated that little change in levels of amino acids takes place in the maximum period of 30 s that elapsed between first biopsy incision and immersion in liquid nitrogen [7–9]. For this reason, a suitable extraction protocol was developed [7–12], i.e., the tissues excised at biopsy are promptly homogenized and deproteinized with 5–10% cold perchloric acid (PCA), and the aqueous extracts are analyzed (see Experimental).

The classical analytical technique uses automated amino acid analyzers [7–9,13,14]. However, this method requires an expensive dedicated equipment due to the post-column derivatization, long assay times and large sample volumes. More recently, reversed-phase high-performance liquid chromatography (RP-HPLC), which employs pre-column derivatization procedures of the amino acids, has been developed. The most popular procedure uses a derivatization with *o*-phtalaldeyde (OPA) [15,16]. Major problems with this protocol are the instability of the derivatives which require an automated on-line derivatization system that allows rapid and exact reaction times. Moreover, secondary amino acids [17] and other very important amino acids, such as glycine (Gly) and threonine (Thr), could not be detected with this technique [4,15,16], while poor quantitative results are obtained for alanine (Ala). Other derivatizing reagents are available, such as 9-fluorenylmethyl-chloroformiate (FMOC-Cl) [18], 4-dimethyl-aminoazobenzene-sulfonyl chloride (DABSCl) [19]. Significant interference of these reagents with their derivatives and the formation of multiple derivatives are observed [20–22] and only two papers on the determination of physiological amino acids in brain specimens are reported [19]. Interestingly, another protocol is in principle available which permits the assay of primary and secondary free-amino acids. This protocol employs a pre-column derivatization of the amino acids with phenylisothiocyanate (PITC) [17,23]. We have adapted this protocol to specimens of human brain tumors. Only one report on the determination of a selected number of amino acids, namely, glutamic acid (Glu), aspartic acid (Asp), glutamine (Gln), Gly and γ-aminobutyric acid (Gaba), in rat brains is reported in the literature [24]. Here we describe the results of the analysis of five selected biopsies: two meningiomas, two oligodendrogliomas and one glioblastoma.

## 2. Experimental

### *2.1. Reagents*

Analytical-reagent grade acetonitrile and methanol were obtained from Lab-Scan. Sodium acetate trihydrate, disodium hydrogenphosphate, sodium dihydrogenphosphate and hydrochloric acid (HCl) were obtained from Fluka. Ethylenediamminetetracetic

acid disodium salt (EDTA), phosphoric acid and PCA were purchased from Aldrich. Triethylamine (TEA) and PITC were obtained from Pierce. High-purity water was obtained by a Milli-Q purification system (Millipore, Milford, MA, USA) which was fed with a supply of previously deionized water. Basic (Product NA-6282), neutral and acid (Product NA-6407) physiological amino acid standard solutions were obtained from Sigma. Volumes of 10–40 μl were used for derivatization. Derivatization was performed in Pyrex tubes.

### 2.2. Clinical material

To minimize the influence of ischemia and of autolysis, all the specimens were extirpated at surgery and immediately (<20 s) frozen in liquid nitrogen and stored at −80°C. Parallel samples were examined with the routine histo–pathological diagnosis and classified according to World Health Organization (WHO) classification [25]. Single biopsies were obtained at craniotomy from five adults. The indication at surgery were the following: two meningioma tumors (specimen M1, patient 1, site of biopsy: parietal lobe; specimen M2, patient 2, site of biopsy: left parietal lobe), three tumors of glial origin, namely, a glioblastoma (specimen G, patient 3, site of biopsy: frontal lobe) and two oligodendrogliomas (specimen OL1 and OL2 patients 4 and 5, respectively, site of the two biopsies: frontal lobe). The oligodendroglioma specimens consisted either of tumor tissue or of peritumoral grey matter tissue which had to be sacrificed in order to remove the deep-seated tumor. After removal, the biopsies were trimmed so that any apparent peritumoral non-infiltrated tissue was separated from the specimens. Both tumor tissues (OL1T and OL2T, patients 4 and 5, respectively), and the apparent normal tissue (OL1N, patient 4) were prepared for the derivatization according to the PCA extraction protocol.

### 2.3. Tissue preparation and extraction

The frozen tissues were homogenized with an Ultra Turrax T-8 under liquid nitrogen. 7% PCA (3 ml PCA/g tissue) was added to the homogenized tissue and the mixture was stirred at 0°C for 1 h. The solid phase was separated, and the liquid was centrifuged at about 4000 *g*/min at 4°C. The aqueous layer was treated with 3 *M* potassium hydroxide (KOH) to pH 8.5–9.5, stored at 0°C for 8 h, centrifuged to eliminate potassium perchlorate ($KClO_4$) and lyophilized.

### 2.4. Derivatization of amino acids with PITC

An exact amount (1–10 mg) of lyophilized specimen was dissolved in 0.1 *M* HCl, sonicated for 20 min, and passed through 0.45-μm HV Millipore filter from Waters. Volumes of 10–40 μl were mixed with an exact amount of $MetSO_2$ and dried under a vacuum (60–80 mTorr; 1 Torr=133.322 Pa). The samples were reconstructed for derivatization with 20 μl of 1 *M* sodium acetate–methanol–TEA (2:1:1) solution, dried at 80 mTorr, and dissolved in 20 μl of derivatization solution (methanol–water–TEA–PITC, 7:1:1:1). The derivatization of both primary and secondary amino acids occurred in 20 min at 25°C and produced the corresponding phenylthiocarbamoyl (PTC) derivatives. The samples were then re-dried until a constant vacuum of 60 mTorr was obtained. Finally, the dried samples were dissolved in 200 μl of phosphate buffer (pH 7.40). After sonicating for a few seconds the samples were injected.

### 2.5. Calibration studies of Sigma standard solutions

A set of standards was prepared by mixing equimolar amounts of basic, neutral and acid standard solutions (41 metabolites) and an exact amount of methionine sulfone ($MetSO_2$, external standard) and the mixture was used for calibration. The mixture was diluted with 0.1 *M* HCl to cover a wide range of possible final concentrations (50, 80, 120, 150, 200, 250, 350, 640 pmol injected). The standard solutions were dried under a vacuum (60–80 mTorr) and reconstructed for derivatization.

### 2.6. Calibration studies of Sigma standard solutions in the biological PCA extracts

As we have reported in the Introduction, the tissues excised at biopsy are promptly homogenized and deproteinized with an aqueous solution of cold

PCA, and the aqueous extracts are analyzed to avoid the autolysis of the tissue during the analysis. For this reason, the preparation of calibration curves by standard addition methods directly on the brain matrix samples cannot be performed because possible deviations of the slopes of the calibration curves might be attributed either to an interference of the matrix with the derivatization chemistry or to an effect of the autolysis. However, it is in principle possible that the metabolites such as lactate, inositol, succinate, *N*-acetylaspartate, phosphocreatine, creatinine, choline, glycerophophorylcholine and phosphorylcholine, which are still present in the PCA extracts, may interfere with the derivatization chemistry. Thus, we have extended the validation of the PicoTag method by preparing calibration curves by the usual standard addition methods in the biological PCA extract of the oligodendroglioma sample (OL2T).

An exact amount of lyophilized PCA extract of the oligodendroglioma OL2T (9.0 mg) was dissolved in 0.1 *M* HCl and mixed with an exact amount of $MetSO_2$ (173.2 pmol injected) and the mixture was used for calibration. The mixture was divided into seven aliquots. Six standards were prepared from six aliquots by adding an exact amount of academic Sigma basic and neutral and acid standard solutions to each aliquot (50, 100, 140, 220, 300, 380 pmol each amino acid injected, Cyst2=25, 50, 70, 110, 150, 190 pmol, and Gln=0.00 pmol, respectively). The 0–380 pmol interval covered the range of possible final concentrations of the amino acids quantified in our samples. The only exception was Glu which ranged between 500–1000 pmol. The six standards and the aliquot corresponding to the real sample OL2T were dried under vacuum, reconstructed for derivatization, and injected in duplicate.

### *2.7. Recovery of the physiological amino acids contained in the Sigma basic, neutral and acid standard solutions under conditions which mimic the tissue extraction protocol*

In three experiments, equimolar amounts of basic, neutral and acid Sigma physiological amino acid standard solutions (0.29, 0.25, 0.083 μmol each amino acid, respectively, Cyst2=0.145, 0.125, 0.042 μmol) were mixed and the resulting solutions were diluted to 625 μl with water. After PCA (7.5%, w/w, 3 ml) was added, each solution was stirred at 0°C for 1 h and the liquid was centrifuged at about 4000 *g*/min at 4°C. The aqueous layer was treated with 3 *M* KOH to pH 8.5–9.5, stored at 0°C for 8 h, centrifuged to eliminate $KClO_4$ and lyophilized. Each residue was dissolved in 625 μl, sonicated for 20 min, and passed through 0.45 μm HV Millipore filter from Waters. Volumes of each solution containing 0.093, 0.080 and 0.027 μmol each amino acid (Cyst2=0.047, 0.040, 0.013 μmol), respectively, were diluted with an equimolar amount of $MetSO_2$. Thirty μl of each sample was dried under a vacuum (60–80 mTorr). The samples were reconstructed for derivatization and injected in duplicate (700, 600, 200 pmol each amino acid injected).

### *2.8. Chromatographic system*

The HPLC system consisted of system of two 600 solvent-delivery pumps, a Rheodyne 7725i manual injector, a UV–Vis Model 486 UV detector set at 254 nm, a column heater set at +46±1°C (all Waters components, Millipore). A Waters Millennium 2015 chromatography manager system ver. 2.15 was used to control the system operation and collect data. All separations were generated on a Water PicoTag column (30 cm×3.9 mm) operating at a flow-rate of 1.0 ml/min. Samples were injected in volumes of 20 μl. The mobile phase consisted of a gradient of two eluents kept under a blanket of argon. Eluent A was an aqueous buffer of 0.07 *M* sodium acetate containing 2.5% acetonitrile and 1 ppm EDTA titrated to pH 6.50 with 10% glacial acetic acid. Eluent B was an organic phase consisting of acetonitrile–water–methanol (45:40:15). The gradient employed in the separation started with eluent B rising from 3 to 34% in ca. 60 min. After a washing step of 10 min with 100% B, the column was re-equilibrated for 20 min with 100% A. A constant flow-rate of 1 ml/min was maintained.

### *2.9. Assay procedure*

In order to determine the precision and accuracy of the method, each specimen obtained from the PCA extractions was divided into three aliquots. Each aliquot was dissolved in 0.1 *M* HCl. Volumes of

20, 30, 40 μl of the aliquots were used for the derivatization. The samples containing the PTC-amino acids were stored as a dry powder under inert atmosphere at −20°C and assayed within five days to minimize technical pitfalls. In fact, it is reported that the PTC-amino acids slowly degrade to the corresponding phenylthiohydantoin amino acids when left in solution for several hours at room temperature [26]. A blank and a standard were run as a first daily injection and at intervals of five samples. Each sample was run in duplicate.

### *2.10. Calculations*

Data collection, integration and peak-identification were done with a Waters Millennium 2015 chromatography manager system.

## 3. Results and discussion

### *3.1. Analytical reproducibility of the Sigma standard solutions, their recoveries in the biological PCA extracts, and under conditions which mimic the tissue extraction protocol*

The chromatogram (Fig. 1) of an equimolar mixture (100.0 pmol injected) of 41 metabolites contained in the Sigma basic and acidic and neutral physiological amino acid standard solutions and of the external standard $MetSO_2$, after derivatization with PITC, demonstrated the excellent signal-to-noise ratio, the detection limit of the PITC-amino acids being at 1–4 pmol levels. However, because the presence of background impurities contributed from solvents, chemicals and glassware, the quantification of real samples was considered at levels of 20.0 pmol. Interference deriving from the reagents, derivatized ammonia and other components are shown in the blank chromatogram of Fig. 1. In particular, two reagent peaks at retention times ($t_R$) of 47.8 min (Reag 1) and 49.40 min (Reag 2) were present. Reag 2, which eluted closely to cystine (Cys), caused problems in the quantification of this amino acid. Some interference is also due to component peaks 1 and 2 which eluted between Thr and Ala and only caused problems with the analyses at low pmol levels (<50) of the two amino acids. For our purposes the following metabolites, even fully identified in the chromatogram, were not quantified: creatinine (Cr), ammonium chloride ($NH_4^+$), β-aminoisobutyric acid (Baib), carnosine (Carn), sarcosine (Sarc), α-amino-*n*-butyric acid (Aab), δ-hydroxylysine (HyLys1 and HyLys2), cystine (Cys), and urea. Cr eluted with the eluent front. Baib and Carn, which are occasionally detected in trace amounts in brain tumors, eluted very closely and could not be accurately resolved. Aab could not be resolved from oxidized glutathione (GSO) since their retention times are identical. δ-Hydroxylysine, a metabolite which was only occasionally detected in trace amounts, was not monitored because its profile was separated into two eluting peaks (HyLys1 and HyLys2). Similarly, cystathionine was separated into two eluting peaks deriving from a mixture of four stereoisomers, namely, D,L-*allo*-cystathionine (Cyst1) and D,L-cystathionine (Cyst2). The profiles of L-cystathionine (Cyst2), and that of Gln, another important metabolite not present in the Sigma standard solutions, as well as their standard curves, were determined using individual crystalline substances. Fifteen serial dilutions of Sigma standard samples in 0.1 *M* HCl containing an equimolar amount of $MetSO_2$ were derivatized and analyzed according to the procedure described in Experimental. Final sample concentrations were in the range of 50–400 pmol injected. The response of each amino acid was normalized to $MetSO_2=1.00$. Table 1 lists the amino acids studied in the same order as the corresponding peaks on the chromatograms, the mean $t_R$ and their standard deviations $(SDs)_1$ in minutes, the relative response (RR) and the $(SD)_2$, the slopes ($b_0$) in terms of μV $pmol^{-1}$ units, and the regression coefficients ($r^2$) of the standard curves. Good recoveries were found for most of the amino acids. The average relative standard deviations (RSDs), which were in the range from 3.2% (proline, Pro) to 7.3% (histidine, His), are consistent with the mean RSD (±4.5%) of the Sigma standard solutions. Major deviations were found for phosphoserine (pSer) (11.8%), Thr (11.7%), Ala (11.1%), Aab (8.8%) and isoleucine (Ile, 11.6%). The correlation coefficients ($r^2$) were in the range of 0.9931 (pSer, Met) to 0.9991 (ornitine, Orn) and the intercepts were not significantly different from zero. These results were consistent with that found in the analy-

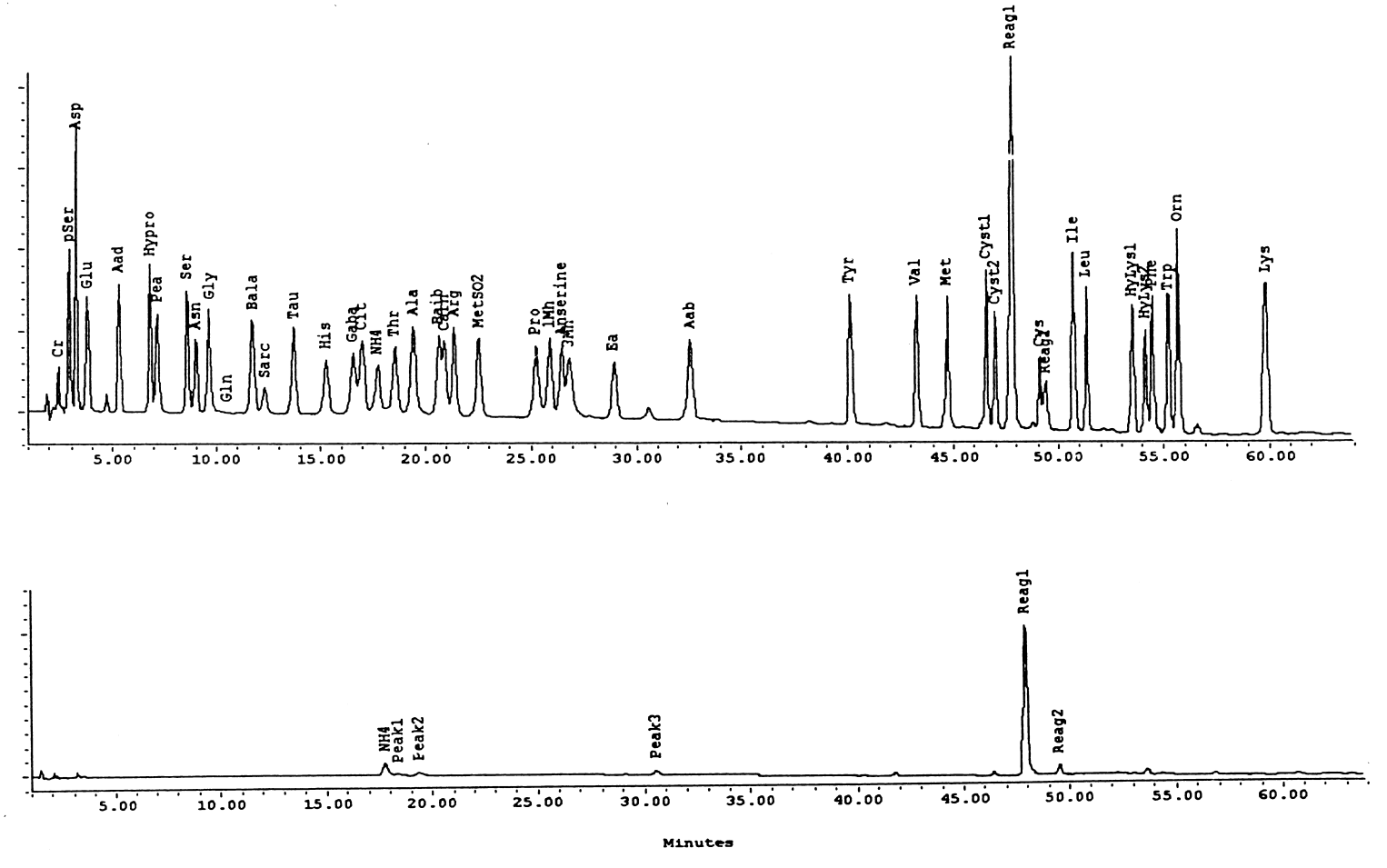


Fig. 1. Chromatograms at 254 nm showing (A) the PTC-amino acids of the Sigma basic and acid and neutral standard solutions (100 pmol each amino acid for injection, Cyst2=50 pmol) and the PTC-Met$SO_2$ (100 pmol). (B) Hydrolysis blank.

Table 1
Mean retention time ($t_R$) and standard deviation $(SD)_1$, relative response (RR) and standard deviation $(SD)_2$, correlation coefficients ($r^2$), and slopes of the calibration curves ($b_0$) of various PTC-amino acids (AAs) obtained from the derivatization of Sigma basic, neutral and acid standard solutions

| AA | $t_R$ $(SD)_1$ | RR[a] $(SD)_2$ | $r^2$ | $b_0$[b] |
|---|---|---|---|---|
| pSer | 2.95 (0.09) | 0.908 (0.118) | 0.9931 | 619 |
| Asp | 3.28 (0.13) | 0.982 (0.053) | 0.9990 | 939 |
| Glu | 3.76 (0.15) | 0.977 (0.053) | 0.9975 | 650 |
| Aad[c] | 5.33 (0.26) | 0.996 (0.050) | 0.9981 | 612 |
| Hypro | 6.82 (0.32) | 0.992 (0.055) | 0.9965 | 686 |
| Pea | 7.17 (0.36) | 0.976 (0.066) | 0.9974 | 590 |
| Ser | 8.62 (0.44) | 0.988 (0.057) | 0.9981 | 659 |
| Asn | 9.03 (0.49) | 0.995 (0.040) | 0.9937 | 437 |
| Gly | 9.65 (0.49) | 0.993 (0.034) | 0.9989 | 641 |
| Gln | 10.29 (0.53) | 0.991 (0.030) | 0.9992 | 670 |
| Bala[d] | 11.75 (0.57) | 0.993 (0.037) | 0.9967 | 680 |
| Tau | 13.70 (0.73) | 0.991 (0.046) | 0.9993 | 659 |
| His | 15.27 (0.76) | 0.982 (0.073) | 0.9947 | 540 |
| Gaba | 16.58 (0.78) | 0.995 (0.040) | 0.9978 | 580 |
| Cit[e] | 17.02 (0.82) | 0.985 (0.048) | 0.9960 | 722 |
| Thr | 18.58 (0.91) | 0.938 (0.117) | 0.9861 | 577 |
| Ala | 19.45 (0.96) | 1.020 (0.111) | 0.9946 | 667 |
| Arg[f] | 21.38 (1.10) | 1.005 (0.022) | 0.9972 | 685 |
| Pro | 25.25 (1.21) | 0.993 (0.032) | 0.9989 | 681 |
| 1Mh[g] | 25.90 (1.12) | 0.996 (0.040) | 0.9988 | 695 |
| Anserine | 26.43 (1.08) | 0.981 (0.061) | 0.9942 | 639 |
| 3Mh | 26.87 (1.03) | 1.008 (0.057) | 0.9937 | 685 |
| Ea | 29.01 (0.85) | 0.984 (0.064) | 0.9944 | 548 |
| Aab | 32.90 (0.81) | 0.952 (0.088) | 0.9718 | 844 |
| Tyr[h] | 40.15 (0.43) | 0.993 (0.037) | 0.9983 | 722 |
| Val | 43.27 (0.35) | 0.996 (0.033) | 0.9976 | 705 |
| Met[k] | 44.73 (0.39) | 0.994 (0.069) | 0.9931 | 717 |
| Cyst2 | 47.00 (0.46) | – | 0.9980 | 1250 |
| Ile | 50.75 (0.29) | 0.971 (0.116) | 0.9710 | 800 |
| Leu | 51.42 (0.37) | 0.993 (0.032) | 0.9972 | 710 |
| Phe[i] | 54.53 (0.48) | 0.992 (0.055) | 0.9972 | 730 |
| Trp[j] | 55.25 (0.54) | 1.004 (0.031) | 0.9986 | 819 |
| Orn | 55.72 (0.72) | 1.005 (0.043) | 0.9991 | 1260 |
| Lys[l] | 59.88 (0.95) | 1.010 (0.062) | 0.9972 | 1330 |

[a] The relative response is normalized to $MetSO_2=1.00$.
[b] In $\mu V\ pmol^{-1}$ units.
[c] α-Aminoadipic acid.
[d] β-Alanine.
[e] Citrulline.
[f] Arginine.
[g] 1-Methylhistidine.
[h] Tyrosine.
[k] Methionine.
[i] Phenylalanine.
[j] Tryptophan.
[l] Lysine.

ses of PCA extracts of blood samples [23] and demonstrated good linear response. The only exceptions were Aab ($r^2=0.9718$), Ile ($r^2=0.9710$) and Thr ($r^2=0.9861$). For Gln and Cyst2 the intercepts were not significantly different from zero and the correlation coefficients were 0.9992 and 0.9980, respectively. It is worth noting that the literature [23] reports that Gln and asparagine (Asn), are converted into Glu and Asp, respectively, in a time dependent fashion when stored at −20°C in PCA solutions. Control experiments performed on pure Gln and Asn, under conditions which mimic the PCA extraction protocol, showed that these metabolites resist hydrolysis when the samples were neutralized with KOH as soon as they were extracted. In fact, the amount of Glu and Asp formed after hydrolysis was ≤4.0%. Differently from that reported in the literature [23], no variation in the retention times was observed for glutamate and aspartate after the PCA extracts were treated with KOH. The control of the ''PicoTag'' procedure in terms of accuracy and precision in three different solvents, i.e., water, hydrochloric acid and PCA through physiological standards prepared from 20 single amino acids of different normality is described in the literature. Comparable results were obtained in the three solvents [23].

Two superimposed chromatograms are reported in Fig. 2, namely, the chromatogram of the specimen OL2T (A) and the chromatogram (B) of a standard containing a mixture of OL2T, $MetSO_2$, and 41 metabolites of the Sigma standard solutions (300 pmol each amino acid injected, Cyst2=150 pmol, Section 2.6), demonstrating that the presence of additional metabolites in the biological extract does not affect the $t_R$ of the derivatized amino acids. As an exception, a slight variation in the $t_R$ of Aab was noticed when the amount of the academic standard solution was increased, this suggesting that a different amino acid, probably oxidized glutathione, is present in the biological extract.

The results of the analyses of the standards described in Section 2.6 are shown in Table 2. The good linear response of the calibration curve of each amino acid is demonstrated by the correlation coefficient $r^2$ which exceeded 0.99. The only exceptions were Aab ($r^2=0.9737$) and Ile ($r^2=0.9710$). The following information are also reported in Table 2:

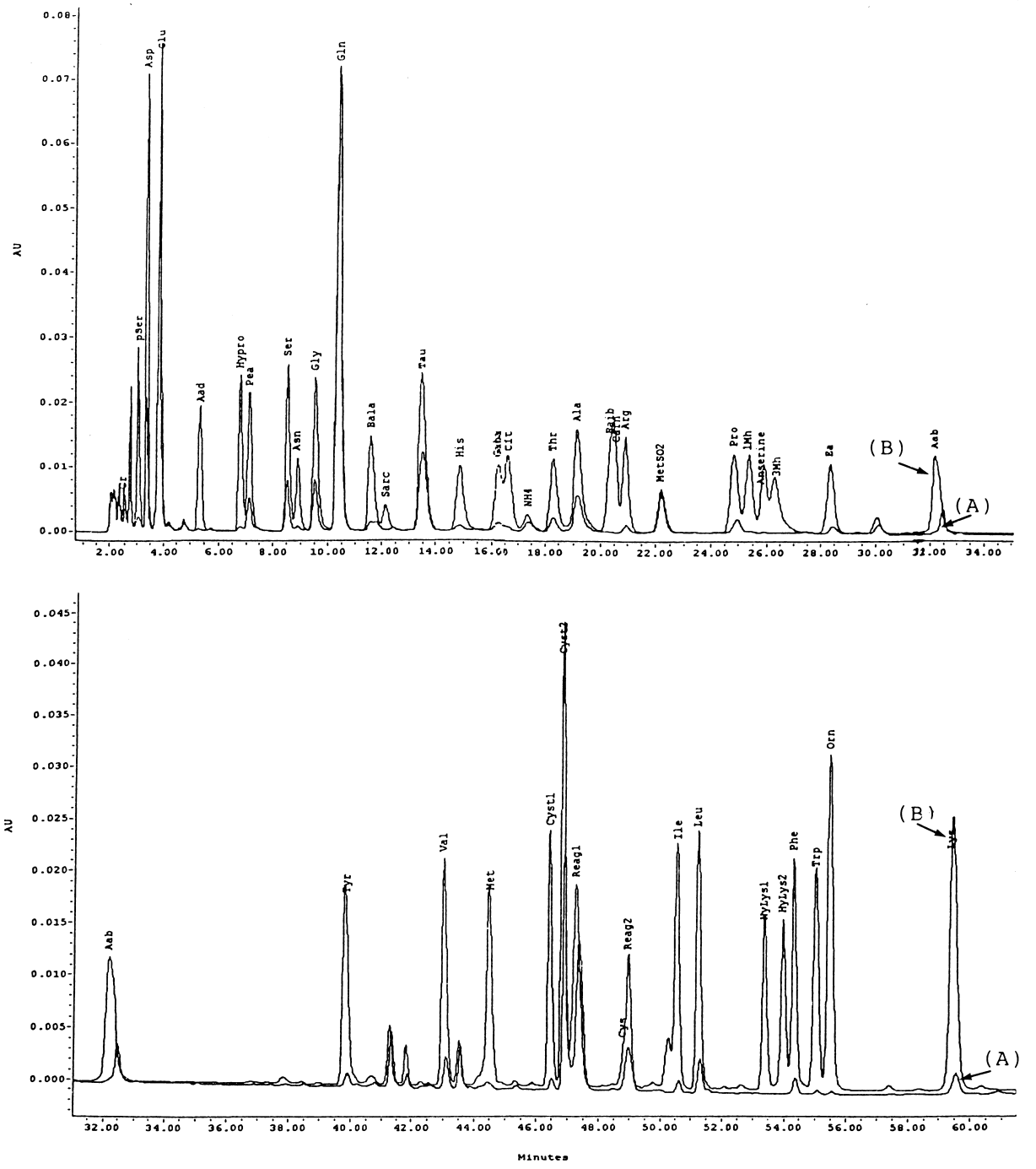


Fig. 2. Chromatograms at 254 nm showing (A) the PTC-amino acids of the specimen OL2T. (B) The PTC-amino acids obtained after derivatization of a standard solution prepared by mixing 1.29 mg of the specimen OL2T, exact amounts of Sigma basic and acid and neutral standard solutions (300 pmol each amino acid injected, Cyst2=150 pmol), and $MetSO_2$ (173.2 pmol injected).

Table 2
Intercept ($pmol_0$) and RSD (%), mean differential recovery (Rec, %) and mean SD (%), slopes ($b_0$)[a] and RSD (%), response linearity ($r^2$) of the calibration curves obtained from the six standards described in Section 2.6, and amino acid content (pmol) of the real sample OL2T

| AA | $pmol_0$±RSD | Rec, %±SD | $b_0$±RSD | $r^2$ | pmol[b, c] |
|---|---|---|---|---|---|
| pSer | 34.46±13.9 | 104.4±5.4 | 564±8.9 | 0.9967 | 29.68 |
| Asp | 128.24±1.6 | 99.7±2.3 | 949±1.1 | 0.9985 | 130.25 |
| Glu | 719.81±1.2 | 99.6±1.4 | 638±1.8 | 0.9946 | 711.35 |
| Aad | 1.09 | 102.2±5.2 | 609±0.5 | 0.9991 | <5 |
| Hypro | 3.29 | 104.0±11.6 | 687±0.2 | 0.9967 | <5 |
| Pea | 101.00±3.6 | 100.9±4.4 | 602±2.0 | 0.9957 | 104.61 |
| Ser | 125.85±3.2 | 101.1±2.6 | 625±5.2 | 0.9991 | 121.84 |
| Asn | 24.12±0.0 | 111.8±2.0 | 401±8.3 | 0.9998 | 24.11 |
| Gly | 155.41±3.1 | 96.0±1.1 | 669±4.4 | 0.9991 | 150.67 |
| Gln[d] | – | – | – | – | 1764.00 |
| Bala | 25.36±18.7 | 95.9±7.9 | 695±2.2 | 0.9956 | 20.61 |
| Tau | 387.65±1.6 | 98.3±1.2 | 668±1.4 | 0.9977 | 381.59 |
| His | 32.26±2.7 | 94.7±4.3 | 569±5.4 | 0.9970 | 31.39 |
| Gaba | 36.63±0.6 | 111.0±4.1 | 532±8.3 | 0.9900 | 36.86 |
| Cit | 23.05±5.5 | 100.4±2.1 | 713±0.1 | 0.9986 | 21.79 |
| Thr | 79.04±11.5 | 103.3±6.0 | 542±6.1 | 0.9938 | 69.91 |
| Ala | 178.72±4.1 | 101.9±2.7 | 706±5.8 | 0.9935 | 185.99 |
| Arg | 27.57±10.1 | 98.6±3.3 | 683±0.2 | 0.9985 | 24.77 |
| Pro | 57.09±1.1 | 103.9±3.2 | 645±5.3 | 0.9985 | 56.46 |
| 1Mh | 9.29±3.3 | 99.7±2.8 | 700±0.1 | 0.9996 | 9.60 |
| Anserine | 11.96±5.1 | 108.3±2.2 | 584±1.7 | 0.9996 | 12.58 |
| 3Mh | 10.72±21.7 | 110.2±4.8 | 649±5.1 | 0.9940 | 13.05 |
| Ea | 61.27±11.7 | 91.9±3.9 | 582±6.2 | 0.9949 | 54.11 |
| Aab | 117.69±7.8 | 105.3±8.9 | 719±14.8 | 0.9737 | 108.48 |
| Tyr | 15.68±12.7 | 99.0±5.2 | 748±3.6 | 0.9986 | 17.67 |
| Val | 45.82±0.3 | 100.0±2.2 | 706±0.1 | 0.9991 | 45.96 |
| Met | 16.61±8.7 | 101.5±4.1 | 720±0.4 | 0.9988 | 18.06 |
| Cyst2 | 180.92±3.5 | 96.7±1.5 | 1268±1.4 | 0.9960 | 174.56 |
| Ile | 18.84±16.0 | 117.6±4.0 | 768±4.0 | 0.9710 | 15.82 |
| Leu | 47.96±7.8 | 96.2 ±2.3 | 724±2.0 | 0.9986 | 44.20 |
| Phe | 15.40±14.4 | 98.7±5.5 | 763±4.4 | 0.9981 | 17.62 |
| Trp | 7.22±7.2 | 102.5±2.3 | 797±2.7 | 0.9997 | 6.7 |
| Orn | 1.31 | 95.5±1.8 | 1298±2.9 | 0.9996 | <5 |
| Lys | 36.60±7.6 | 100.2±2.5 | 1297±2.5 | 0.9986 | 33.82 |

[a] $b_0$=μV $pmol^{-1}$.
[b] $MetSO_2$=173.15.
[c] Total pmol=4375.47.
[d] Not present in the Sigma standard solutions.

(a) the intercepts ($pmol_0$) of the calibration curves in pmol unit; (b) the mean differential recoveries (Rec, %) of the amino acids added to the specimen OL2T; (c) the slopes of the calibration curves ($b_0$) in μV $pmol^{-1}$ units; (d) the amino acid recoveries (pmol) of the specimen OL2T. A very good correspondence is found between the intercepts and the recoveries of the amino acids of the real sample OL2T. In fact, the RSDs were in the range from 0.03% (Asn) to 11.7% (Ea) when the amount of the amino acid contained in the real sample was ≥20.0 pmol. The only exceptions were Bala (18.7%) and pSer (13.9%). A good correspondence is also found between the slopes of the calibration curves of Table 2 and Table 1. The RSDs ranged between 0.1 (Val, Cit) and 6.2 (Ea). The only exceptions were Aab (14.8), and pSer (8.9). Good mean differential recoveries (Rec, %) were found for most of the amino acids. The mean

SDs were in the range from 1.1% (Gly) to 7.9% (Bala) and were consistent with the mean RSD (±4.5%) of the academic Sigma standard solutions.

Table 3 reports the mean RR, normalized to $MetSO_2$=1.00, and the mean % SD of the physiological amino acids of three academic Sigma standard solutions after their treatment under conditions which mimic the tissue extraction protocol (Section 2.7). In most cases a very good agreement has been obtained between the RR and SD values of the amino acids recovered in the PCA extracts with the values of Table 1. The only exceptions were phosposerine (pSer) and ethanolamine (Ea). The low RR values of 0.5455 and 0.7729, respectively, indicate that these two amino acids are partially lost during the extraction.

### *3.2. Compositional analysis of the biopsies*

Fig. 3 shows a typical chromatogram of the OL1N specimen and Table 4 reports the amino acid compositional data of the derivatized biopsies OL1T, OL1N, M1, M2 and G. The results are expressed as μmol/g of biopsy and represent the mean and SD for nine different injections (see Section 2.9). A number of unidentified peaks after Aab are observed in the chromatograms, but none constantly. The amino acids with the highest concentrations are Glu, Gln, Cyst2, Tau, Gly, Ala, Asp, Pea and Gaba. The mean total amino acid content as well as the mean values for amino acid concentrations of the PCA extracts of OL1T and OL1N, M1 and M2 are comparable to the results obtained with ion-exchange chromatography [7–9,13,14] or other derivatization procedures [18,19]. Table 4 also compares the amino acid concentrations of the glioblastoma (specimen G) with the mean values [G] of 13 glioblastomas reported in the literature [14]. The amounts of most of the metabolites were comparable. In particular, the amount of Gly (1.90 μmol) was typically higher then the values reported for normal brain tissues, in the range of 0.82±0.20 [27] and 1.06±0.04 [9], while a mean value of 3.09±0.57 was found for the 13 glioblastomas [14]. Instead, the amount of the amino acids Gln (11.13 μmol) and Cyst2 (4.21 μmol) and the relative Gln/Glu value (2.9) of specimen G differed markedly from the mean values reported in the literature [14] (3.77, 1.65 and 0.90, respectively).

It is worth noting that the HPLC technique, rather than NMR is the proper tool for monitoring the Gln/Glu ratio. In fact, NMR studies showed that the resonances of GSH and Gln are in close proximity on the frequency scale, thus leading to a possible misinterpretation of the spectra [28].

Table 3
Mean relative response[a] (RR) and % mean SD of the physiological amino acids contained in three Sigma standard solutions[b] after their treatment under conditions which mimic the tissue preparation protocol

| AA | RR (SD) | AA | RR (SD) | AA | RR (SD) |
|---|---|---|---|---|---|
| pSer | 0.5455 (2.01) | His | 1.0755 (3.33) | Aab | 0.9743 (3.21) |
| Asp | 0.9817 (1.29) | Gaba | 0.9613 (0.51) | Tyr | 0.9684 (1.71) |
| Glu | 0.9529 (1.03) | Cit | 0.9279 (1.51) | Val | 0.9913 (1.67) |
| Aad | 0.9724 (1.41) | Thr | 0.9894 (0.65) | Met | 0.9936 (0.97) |
| Hypro | 0.9946 (1.24) | Ala | 0.9870 (2.47) | Cyst2 | 0.4733[c] (0.58) |
| Pea | 0.9712 (1.67) | Arg | 0.9737 (2.60) | Ile | 1.0542 (5.40) |
| Ser | 0.9852 (0.75) | Pro | 0.9845 (1.74) | Leu | 0.9994 (1.20) |
| Asn | 0.9231 (1.56) | 1Mh | 0.9824 (1.07) | Phe | 1.0091 (3.90) |
| Gly | 0.9937 (1.53) | Anserine | 0.9780 (4.63) | Trp | 0.8292 (3.58) |
| Bala | 1.0078 (0.49) | 3Mh | 0.9217 (3.18) | Orn | 1.0383 (3.12) |
| Tau | 0.9297 (0.72) | Ea | 0.7729 (6.84) | Lys | 0.9826 (3.58) |

[a] The relative response is normalized to $MetSO_2$=1.00.

[b] Solution A: 0.29 μmol each amino acid (Cyst2=0.145 μmol); solution B: 0.25 μmol each amino acid (Cyst2=0.125 μmol); solution C: 0.083 μmol each amino acid (Cyst2=0.042 μmol).

[c] RR expected=0.5000.

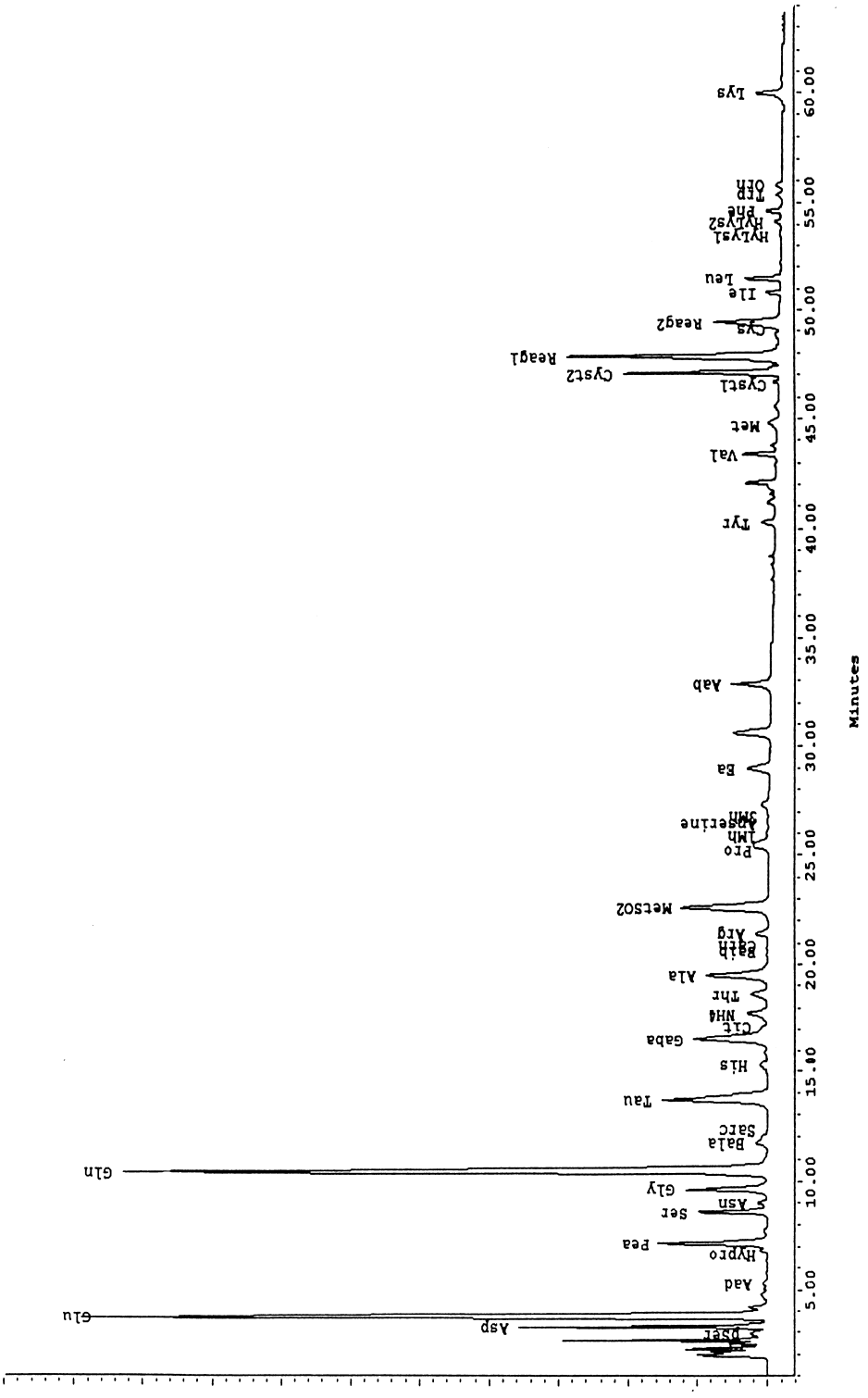


Fig. 3. Chromatogram at 254 nm of the PTC-amino acids of the specimen OL1N.

Table 4
Amino acid content (μmol/g), relative percent (%) and RSD (%) of derivatized specimens OL1T, OL1N, M1, M2 and G and mean amino acid content of 13 glioblastomas [G]±S.E.M.[a]

| AA | OL1T (%)±RSD | OL1N (%)±RSD | M1 (%)±RSD | M2 (%)±RSD | G (%)±RSD | [G]±S.E.M. |
|---|---|---|---|---|---|---|
| pSer | 0.12 (0.5)±21.9 | 0.11 (0.5)±16.3 | (0.0–0.05) | (0.0–0.05) | (0–0.05) | (0.0–0.05) |
| Asp | 0.75 (2.9)±0.6 | 0.85 (3.9)±2.1 | 0.33 (1.8)±4.6 | 1.12 (5.4)±5.1 | 0.35 (1.1)± 5.5 | 0.76±0.18 |
| Glu | 5.52 (21.6)±1.1 | 6.45 (29.3)±2.5 | 5.00 (27.1)±3.4 | 4.36 (21.0)±7.2 | 3.83 (12.0)±8.5 | 4.22±0.92 |
| Aad | (0.0–0.05) | (0.0–0.05) | 0.16 (0.9)±8.0 | 0.62 (3.0)±1.2 | 0.23 (0.7)±7.7 | nr[b] |
| Pea | 0.95 (3.7)±1.3 | 1.14 (5.2)±2.9 | 0.78 (4.3)±4.6 | 1.43 (6.9)±3.3 | 1.34 (4.2)±6.8 | 0.60±0.13 |
| Ser | 0.91 (3.6)±0.5 | 0.53 82.4)±3.5 | 0.19 (1.0)±7.0 | 0.56 (2. 7)±0.8 | 1.18 (3.7)±8.3 | 1.10±0.13 |
| Asn | 0.22 (0.8)±2.9 | 0.15 (0.7)±7.2 | 0.17 (0.9)±6.0 | 0.61 (2.9)±3.4 | 0.29 (0.9)±8.1 | nr[b] |
| Gly | 1.20 (4.7)±1.0 | 0.79 (3.6)±0.5 | 1.58 (8.6)±3.7 | 1.54 (7.4)±1.7 | 1.90 (6.0)±6.5 | 3.09±0.57 |
| Gln | 8.48 (33.2)±0.8 | 6.93 (31.4)±2.1 | 2.39 (13.0)±4.6 | 4.44 (21.4)±4.5 | 11.1 (35.0)±7.7 | 3.77±0.13 |
| Bala | (0.0–0.05) | (0.0–0.05) | 0.95 (5.1)±4.2 | 0.13 (0.6)±0.5 | (0–0.05) | nr[b] |
| Tau | 1.45 (5.7)±5.7 | 1.09 (4.9)±3.6 | 2.51 (13.6)±5.9 | 1.26 (6.1)±1.1 | 1.48 (4.7)±7.8 | 1.20±0.10 |
| His | 0.23 (0.9)±5.5 | (0.0–0.05) | 0.32 (1.8)±11.7 | 0.13 (0.6)±1.2 | 0.21 (0.7)±7.7 | 0.15±0.03 |
| Gaba | 0.69 (2.7)±5.9 | 1.21 (5.5)±3.7 | (0.0–0.05) | 0.24 (1.1)±6.4 | 0.32 (1.0)±8.1 | 0.54±0.13 |
| Cit | 0.19 (0.8)±3.4 | (0–0.05) | (0.0–0.05) | (0–0.05) | 0.07 (0.2)±5.6 | nr[b] |
| Thr | 0.44 (1.7)±7.6 | 0.23 (1.0)±9.0 | 0.11 (0.6)±12.4 | 0.24 (1.1)±0.7 | 0.87 (2.7)±6.0 | 0.73±0.14 |
| Ala | 1.03 (4.0)±2.8 | 0.86 (3.9)±2.5 | 0.83 (4.5)±4.5 | 2.41 (11.3)±11.1 | 1.47 (4.6)±7.1 | 1.92±0.36 |
| Arg | 0.25 (1.0)±5.1 | 0.09 (0.4)±5.1 | 0.19 (1.0)±9.6 | 0.18 (0.9)±0.7 | 0.19 (0.6)±6.1 | 0.17±0.07 |
| Pro | 0.24 (0.9)±4.4 | (0.0–0.05) | 1.16 (6.3)±3.9 | (1.6)±0.3 | 0.35 (1.1)±7.6 | 0.45±0.09 |
| 1Mh | (0.0–0.05) | (0.0–0.05) | 0.40 (2.1)±7.6 | (0.0–0.05) | (0.0–0.05) | nr[b] |
| Ans | (0.0–0.05) | (0.0–0.05) | (0.0–0.05) | (0.0–0.05) | 0.63 (2.0)±0.9 | nr[b] |
| 3Mh | (0.0–0.05) | (0.0–0.05) | 0.40 (2.1)±7.6 | (0.0–0.05) | (0.0–0.05) | nr[b] |
| Tyr | 0.26 (1.0)±9.3 | 0.11 (0.5)±9.9 | (0.0–0.05) | (0.0–0.05) | 0.16 (0.5)±8.7 | 0.14±0.02 |
| Val | 0.41 (1.6)±0.8 | 0.25 (1.1)±2.0 | 0.13 (0.7)±9.5 | (1.1)±1.4 | 0.43 (1.4)±8.1 | 0.37[c]±0.05 |
| Met | 0.15 (0.6)±3.9 | 0.10 (0.5)±9.4 | (0.0–0.05) | (0.5)±2.4 | 0.11 (0.3)±10.4 | 0.07±0.02 |
| Cyst2 | 1.04 (4.1)±2.2 | 0.67 (3.0)±1.3 | (0.0–0.05) | (0.0–0.05) | 4.21 (13.3)±7.3 | 1.65±0.34 |
| Ile | 0.14 (0.5)±1.9 | 0.08 (0.4)±7.0 | (0.0–0.05) | 0.04 (0.2)±1.0 | 0.11 (0.3)±5.2 | 0.12±0.02 |
| Leu | 0.44 (1.7)±0.5 | 0.22 (1.0)±2.4 | 0.13 (0.7)±4.1 | 0.25 (1.2)±1.1 | 0.31 (1.0)±6.5 | 0.27±0.04 |
| Phe | 0.11 (0.4)±8.1 | (0.0–0.05) | (0.0–0.05) | 0.13 (0.6)±1.9 | 0.13 (0.4)±5.8 | 0.15±0.02 |
| Trp | 0.054 (0.2)±0.9 | (0.0–0.05) | (0.0–0.05) | (0.0–0.05) | (0–0.05) | nr[b] |
| Orn | 0.041 (0.2)±6.9 | (0.0–0.05) | 0.11 (0.6)±10.0 | 0.11 (0.5)±2.2 | 0.06 (0.2)±5.5 | 0.11±0.03 |
| Lys | 0.32 (1.2)±2.5 | 0.18 (0.8)±5.2 | 0.38 (2.1)±4.3 | 0.31 (1.5)±1.7 | 0.31 (1.0)±7.5 | 0.46±0.10 |
| Total AAs | 25.64 | 22.03 | 18.42 | 20.77 | 31.82 | 22.02 |

[a] Ref. [5].
[b] Not reported.

## 4. Applications and conclusions

The HPLC Waters ''PicoTag'' system provides a reliable method for the assay of PCA extracts of brain tumors with high sensitivity and accuracy using very small amounts of biopsies (1–10 mg) and in a relatively short time. In fact, this protocol allows the identification and the quantification of >30 amino acids and the detection limits for most of the amino acid derivatives is ≤5 pmol. The derivatization is complete after 20 min at ambient temperature. After the reagent is removed under vacuum the PTC-amino acids can be stored as a powder under inert atmosphere at −20°C for several days. Calibration studies of Sigma standard solutions in the biological PCA extract of specimen OL2T demonstrate that the presence of additional metabolites such as lactate, choline, inositol, *N*-acetylaspartate, phosphocreatine, creatinine, phosphorylcholine, glycerophophorylcholine and succinate do not interfere with the derivatization chemistry. Interference in the analysis deriving from the reagents, derivatized ammonia and other components causes problems in the quantification of Cys and to a lesser extent of Thr and Ala at low pmol levels. PCA treatment of the metabolites failed to provide the quantitative re-

covery of pSer and Ea. Another unresolved problem concerns the quantification of oxidized glutathione (GSO) with this system because the retention time of this metabolite is identical to that of Aab. Our attempts to quantify the peak as if it were pure Aab gave relatively high values (0.5–1.5 μmol) instead of expected values <0.1 which are found both in normal and tumor tissues [7–9,14], thus suggesting the presence of significant amounts of glutathione in our specimens. The equipment required for this methodology is relatively non-expensive since it does not require an automated derivatization apparatus. It should be possible to develop with ''PicoTag'' an on-line HPLC–NMR–MS apparatus [29,30], differently from OPA or ninhydrin detection methods. This apparatus might be helpful for complete identification of individual components in such complex mixtures and of unidentified small peptides which play very important roles in the metabolism of brain tissues.

## Acknowledgements

The authors want to thank Mr. Roberto Coldani for excellent technical support in carrying out this study.

## References

[1] S. Gill, D.G.T. Thomas, N. van Bruggen, D.G. Gadian, C.J. Peden, J.D. Bell, I.J. Cox, D.K. Menon, R.A. Iles, D.J. Briant, G.A. Coutts, J. Comput. Assis. Tomogr. 14 (1990) 497.
[2] J. Urenjak, S.R. Williams, D.G. Gadian, M. Noble, J. Neurochem. 59 (1992) 55.
[3] G. Carpinelli, C.M. Carapella, L. Palombi, L. Raus, F. Caroli, F. Podo, Anticancer Res. 16 (1996) 1559, and references therein.
[4] C.L. Florian, N.E. Preece, K.K. Bhakoo, S.R. Williams, M.D. Noble, NMR Biomed. 8 (1996) 253.
[5] J. Urenjak, S.R. Williams, D.G. Gadian, M. Noble, J. Neurosci. 13 (1993) 981.
[6] J.D. de Certaines, V.A. Larsen, F. Podo, G. Carpinelli, O. Briot, O. Henriksen, NMR Biomed. 6 (1993) 345.
[7] T.L. Perry, S. Hansen, K. Berry, C. Mok, D. Lesk, J. Neurochem. 18 (1971) 521.
[8] T.L. Perry, K. Berry, S. Hansen, S. Diamond, C. Mok, J. Neurochem. 18 (1971) 513.
[9] T.L. Perry, S. Hansen, S.S. Gandham, J. Neurochem. 36 (1981) 406.
[10] I. Gutmann, A.W. Wahlefeld, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Academic Press, New York, 1974, p. 1586.
[11] W.T. Evanochko, T.T. Sakai, T.C. Ng, N.R. Krishna, H.D. Kim, R.B. Zeidler, V.K. Ghanta, R.W. Brockman, L.M. Shiffer, P.G. Braunschweiger, J.D. Glickson, Biochim. Biophys. Acta 805 (1984) 104.
[12] T.W.-M. Fan, R.M. Higashi, A.N. Lane, O. Jardetzky, Biochim. Biophys. Acta 882 (1986) 154.
[13] S. Moore, D.H. Spackman, W.H. Stein, Anal. Chem., 30 (1958) 1185.
[14] J.-M. Lefauconnier, C. Portemer, F. Chastagner, Brain Res. 117 (1976) 105.
[15] P. Lindroth, K. Mopper, Anal. Chem. 51 (1979) 1667.
[16] P. Fürst, L. Pollack, T.A. Graser, H. Godel, P. Stehele, J. Liq. Chromatogr. 12 (1989) 505.
[17] B.A. Bidlingmeyer, S.A. Cohen, T.L. Tarvin, J. Chromatogr. 336 (1984) 93.
[18] Y.-F. Cheng, N.J. Dovichi, Science 242 (1988) 562.
[19] A. Watanabe, J. Semba, A. Kurumaji, S. Kumashiro, M. Toru, J. Chromatogr. 583 (1992) 241, and references therein.
[20] J. Vendrell, F.X. Aviles, J. Chromatogr. 358 (1986) 401–413.
[21] Y. Watanabe, K. Imai, J. Chromatogr. 239 (1982) 723.
[22] S. Einarrson, B. Joseffson, S. Lagerkvist, J. Chromatogr. 282 (1983) 609.
[23] G. Buzzigoli, L. Lanzone, D. Ciciaro, S. Frascerra, M. Cerri, A. Scandroglio, R. Coldani, E. Ferranini, J. Chromatogr. 507 (1990) 85.
[24] S. Gunawan, N.Y. Walton, D.M. Treiman, J. Chromatogr. 503 (1990) 177.
[25] P. Kleihnes, P.C. Burger, B.W. Scheitauer, in: Histopatological Typing of Tumors of the Central Nervous System. WHO Blue Book, 2nd ed, Springer, 1993, p. 1.
[26] D.J. Strydom, S.A. Cohen, Anal. Biochem. 222 (1994) 19.
[27] Y. Kinoshita, H. Kajiwara, A. Yokota, Y. Koga, Neurosurgery 35 (1994) 606.
[28] J.-C. Portais, M. Martin, P. Canioni, M. Merle, FEBS Lett. 327 (1993) 301.
[29] D.L. Olson, T.L. Peck, A.G. Webb, J.V. Sweedler, in: P.T.P. Kaumaya, R.S. Hodges, (Eds.), Peptides: Chem., Struct., Biol., Proc. Am. Pept. Symp., 14th, 1996, pp. 730–731, Mayflower Scientific, Kingswinford, U.K.
[30] K. Albert, Angew. Chem. Int. Ed. Engl. 34 (1995) 641.