High sensitive Amino Acid Analysis by Capillary HPLC

Precolumn derivatization with 6-Aminoquinolyl-N-hydroxysuccinimidyl-carbamate (AQC)

J. Naidanow *, L. Minárikova **, G. Barka ***, P. Földi * / ****

Introduction. In the past years, methods for pre-column derivatisation and subsequent separation via reversed phase chromatography have become increasingly popular at the expense of traditional methods for the analysis of amino acids. The classical method for amino acid analysis involves the separation of unmodified amino acids via ion exchange chromatography and their reaction with ninhydrin at high temperature in a mixing coil [1], which enables their facile identification and quantification. An advantage of this approach is its high resolution in addition to its robustness. For example, not only all 42 physiological amino acids, but also amino sugars can be analysed in clinical samples. Unfortunately, this method is relatively insensitive and, furthermore, generally requires 1 to 2 hours for a single analysis. Even though the substitution of fluorescamine for ninhydrin increases the sensitivity of the traditional method of post-column derivation, it has not been able to replace ninhydrin [2].

Compared to the post-column derivatisation methods, analytical methods of pre-column derivatisation with subsequent separation of amino acids via reversed phase HPLC are generally faster and more sensitive. Nonetheless, derivatisation with neither 5-(dimethylamino)-1-naphthalenesulfonylchloride (DANSYL Cl) [3], 4-(dimethylamino)-azobenzene-4'-sulfonyl chloride (DABSYL-Cl) [4] nor phenyl isothiocyanate (PITC) [5] have been able to supplant the traditional methods. Some of the reasons for this include the production of undesired by-products and the relatively low reactivity of DANSYL- and DABSYL-Cl, as well as the labor-intensive derivatisation methodology or the necessity for multiple evacuation steps for the removal of excess PITC [6].

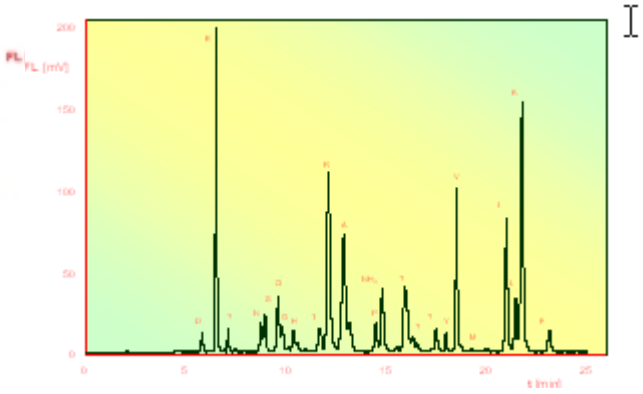
The pre-column derivatisation of amino acids with o-phthaldialdehyde (OPA) and a thiol, such as 3-mercaptothanol or ethanethiol, is one of the more modern techniques available today [7]. Special advantages of this method include high sensitivity, with a lower detection limit ≥10fMol, and the shortest analysis times [8]. However, serious drawbacks of derivatisation with OPA are the facts that it does not react with secondary amines like proline or hydroxyproline and its adducts are unstable [9]. Nonetheless, the extremely sensitive analysis of the D- and L-enantiomeres of amino acids in foodstuffs via fully automatic pre-column derivatisation with OPA and N-isobutyryl-L-cysteine and subsequent separation and quantification of the stereoisomers via HPLC is of special interest [10, 11].

To circumvent the problems associated with the analysis of protein hydrosylates, cerebral spinal fluid, serum, sea water and other difficult samples, B. Josefsson et al. employed the derivatisation of amino acids with 9-fluorenylmethoxycarbonyl chloride (FMOC) in 1983 [12, 13]. The resulting FMOC adducts, normally employed in peptide synthesis reactions, were characterized by their low limit of detection, ≤ 50 fMol, as well as their long-term stability, ≥ 24 h. The excess, free FMOC, which is necessary to ensure a complete reaction but which disturbs the analysis, can be inactivated in both manual and automated derivatisation reactions quite simply by the addition of 1-aminoadamantane [14]. Another, particularly prominent advantage of this method is the fact that the substitution of FMOC with his chiral analogue, (+)-1-(9-fluorenyl)ethyl chloroformate, under otherwise identical reaction and similar separation conditions, permits the simple analysis of sample mixtures of enantiomeric amino acids and amines [15].

While each of these briefly described methods has not only advantages, but also disadvantages when compared to each other, to date it has been necessary to select the individually optimal method for each and every specific analytical problem. Given that the investigation and diagnosis of, for example, Alzheimer's and other fatal, in many cases genetically determined diseases [16] as well as the analysis of the proteome requires ever increased sensitivity as a result of the often minimal sample amounts available, the experiments reported in this manuscript were designed to further develop the already described standard and analytical HPLC methods employing pre-column derivatisation of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (AQC) [17]. Goals for the new method include robustness, simple manual and automatic handling and sensitivity at least equal to that obtained via derivation with OPA or FMOC. This latter feature means that the new method would be compatible with nano and capillary HPLC and thus fulfill the requirements for a modern analysis of proteomes [18].

Materials and Methods. The analysis of yeast extracts and human plasma and biopsy material was performed with an HPLC system consisting of the following components: Delta-Chrom SDS150 (master) and SDS15s (slave) high pressure gradient pumps (Watrex, Prague, Czech Republic), Midas Autosampler (Spark, Holland), 1040 HP Fluorescence Detector (Agilent Technologies), and EZ Chrom datastation (Scientific Software, USA).

An HPLC system from Thermo Finnigan (Darmstadt, Germany) was used for microbore and narrowbore chromatography. This system consisted of a model P 4000 HPLC pump, a model AS 3000 autosampler/column thermostat, a model Spektra Focus UV detector (flow cell: 1.3 µl volume, 3 mm pathlength) and a model FL 2000 fluorescence detector (flow cell: 3 µl volume, 2 mm pathlength). Data acquisition and system control were performed with software from Thermo Finnigan running on a PC equipped with an AMD Athlon 1000 MHz microprocessor.



Stationary phase: WATREX Amino Acid-AQC 5 µm; **Column:** 250 x 4 mm with 20 x 4 mm guard column; **Eluent A:** 100 mM Na-acetate buffer pH 6.4; **Eluent B:** 70 % ACN / Water = 60 / 40 30 %; **Gradient:** 5-18% B (0-4 min), 18-19% B (4-10 min), 19-35% B (10-16 min), 35-40% B (16-24 min); 40-60% B (24-31 min); **Flow rate**: 1,5mL/min; **Lin. vel:** 1.99; mm/sec; **Pressure:** 20-24 MPa; **Temperature:** 37°C; **Detection (Fl):** 254 exc.- 395 em; **Flow through cell:** 16µL.; **Injection:** 20µL; **Sample:** Yeast cell extract,

Fig. 1 Analysis of a yeast extract

Nano- and capillary columns were run with an HPLC syste from Sunchrom (Friedrichsdorf, Germany) which consisted of a model MicroPro capillary HPLC pump (syringe volume 2 ml) and either a model Upchurch Scientific M-435 manual microinjection valve or a model Endurance autosampler for automatic derivatisation and a Spectraflow model 501 UV detector (flow cell: 45 nl volume, 1 cm pathlength). SunChrom Chromstar Software ver. 6.0 was used to control the system and collect the data.

All HPLC eluents were continuously gassed with helium.

Yeast extracts were prepared from 2 or 3 yeast colonies resuspended in 3 ml water. The amino acids were extracted according to the method of Y. Ohsurni et al. as modified by D.P. Gent und J.C. Slaughter [19, 20]. Human colon biopsy samples were prepared by incubating 10-30 mg of colon tissue in 200 µl water at 50°C for 10 min. The preparation was briefly centrifuged. Thereafter, 100 µl of the supernatant was supplemented with 400 µl of acetonitrile and intensively mixed on a Vortex mixer. The sample was clarified by centrifugation at 10, 000 x g for 10 min and the supernatant was used for analysis.

Amino acid standards were purchased from Serva (Heidelberg, Germany), HPLC grade solvents and analytical grade buffer substances were from Merck (Darmstadt, Germany). Narrowbore (150 x 2 mm) and capillary (150 mm x 300 µm) columns packed with GROM Sapphire C18 or C8 matrices (3 µm particle size) were kindly provided by Grom Analytik + HPLC (Rottenburg-Hailfingen, Germany). The analytical HPLC column (WATREX 250 x 4 mm Amino Acid-AQC, 5 µm, with a 20 x 4 mm guard column) and the derivatisation reagent AQC were from WATREX. All amino acid standards and other samples were derivatized according to a protocol supplied by WATREX [21].

Results and discussion. Since the declared goal of the experiments described in this study was the development of a simply implemented and reproducible method for the analysis of amino acids in proteome research, initial efforts concentrated on the search for a suitable, commercially available stationary phase, which could also be packed in capillary columns. For these reasons, initial, standard scale analyses employed WATREX DeltaChrom™-AQC columns (250 x 4 mm, 5 µm particle size) (Figs. 1, 2).

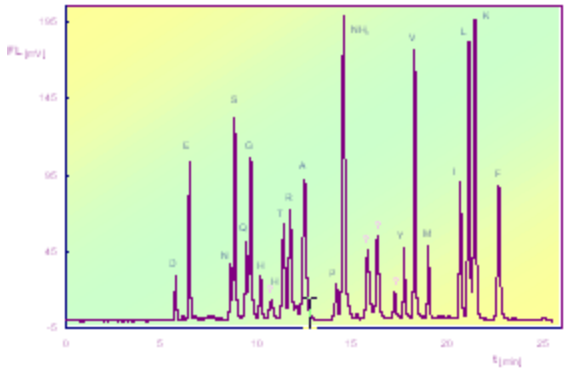


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Fig. 2 Amino acid analysis in cancer research-biopsy from human colon tissue.

However, after a screening of additional phases (results not shown) and in order to increase the sensitivity of the analysis, only narrowbore and capillary columns (150 x 2 mm or 300 µm) filled with GROM Sapphire C18, 3 µm particles were used. Even though the chromatography conditions were optimized at 15 cm column length, 3 µm particle size, elution buffer 50 mM Na-acetate, pH 5.75 or pH 6.0 and column temperature of 45°C, it was not possible to reduce the run time with the GROM Sapphire C18 phase to below 60 min. (Fig. 3). Simple substitution of a GROM C8 column in this system resulted in a re-

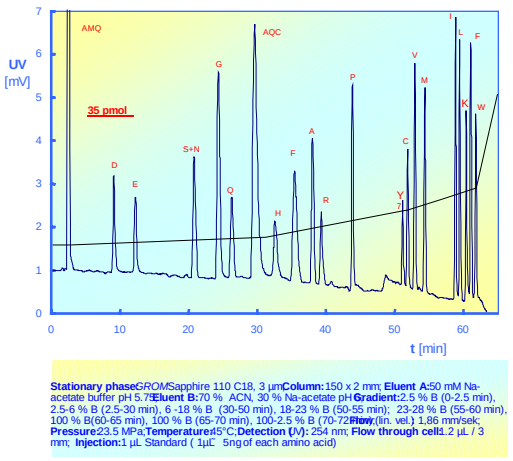

Fig. 3 Optimized separation conditions and results obtained with a C18 phas (*GROM* Sapphire 110 C18-3µm).

duction of the run time by ≥ 20 min (Fig. 4). No further attempts were made to reduce the run time by substituting shorter length columns, since it became apparent that a point of diminishing returns had been reached between speed of analysis and the robustness of the method for routine applications.

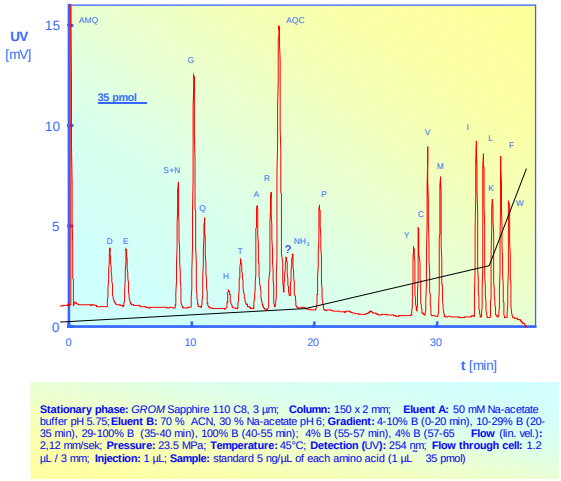

Fig. 4 Optimized separation conditions and results obtained with an octyl (*GROM* Sapphire 110 C8-3µm).

In order to validate the suitability of this analysis method for laboratories without access to fully automated sample handling systems, which in

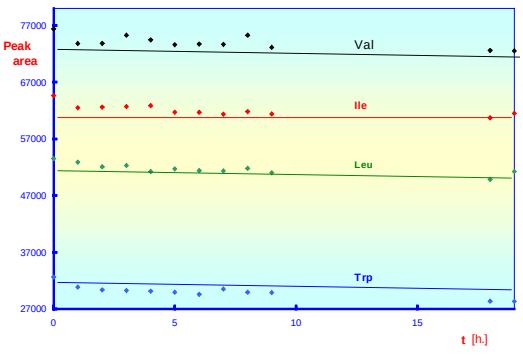

Fig. 5 Stability test of the indicated amino acid-AQC adducts stored at 21°C for the times

turn requires the optimization of various aspects of manual derivatisation including complete mixing of µl quantities of sample, reagents and buffers, the stability of the amino acid-AQC adducts was investigated at both 4°C and at room temperature. It was found that these were stable for at least 15 h at room temperature (Fig. 5) and for more than 14 days at 4°C (Fig. 6).

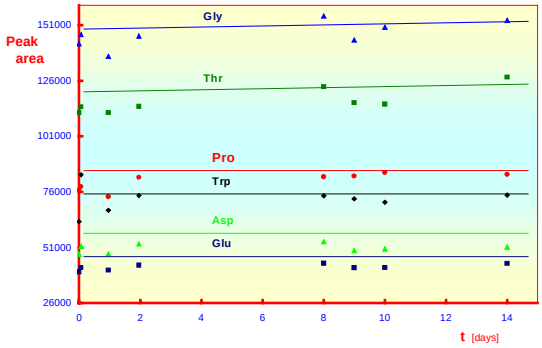

Fig. 6 Stability test of the indicated amino acid-AQC adducts stored at 4°C for the times indicated before chromatography as described in the legend to Fig.3.

As can be seen in Figs. 7 and 8, fluorescence detection of the amino acid-AQC adducts was about 50 to 100 times more sensitive than UV detection. Such a result is of course not limited to amino acid analysis and is dependent on both the illuminated volume and the pathlength of the flow cell employed, but not on the dimensions of the HPLC column. Unfortunately, no commercially available fluorescence detectors are presently on the market which have flow cells appropriate for capillary and nanobore HPLC. Likewise, laser-induced fluorescence detectors with the necessary excitation wavelength of 250 nm are also not available. Nonetheless, the use of a 300

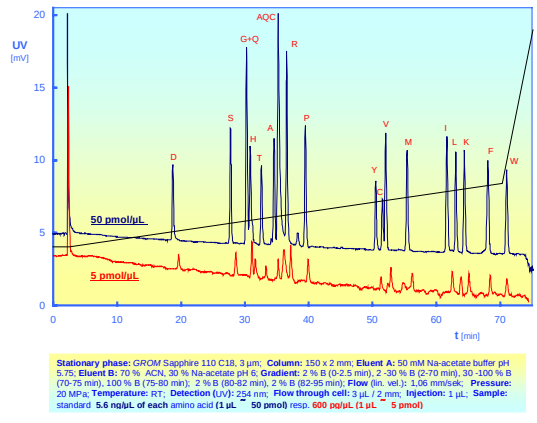

Fig. 7 **Lower limit of detection of amino acid-AQC adducts with a 2 mm ID** narrowbore HPLC column and UV detection.

µm ID capillary column and a suitable UV detector in place of a 2 mm ID narrow bore column and fluorescence detector results in the same lower limit of detection in the analysis of amino acids, namely ca. 100 fmol (Fig. 9). Under the same conditions, replacement of the capillary column

with a nanobore HPLC column with a 50 µm ID would result in a reduction of the lower limit of detection by a factor of ca. 40.

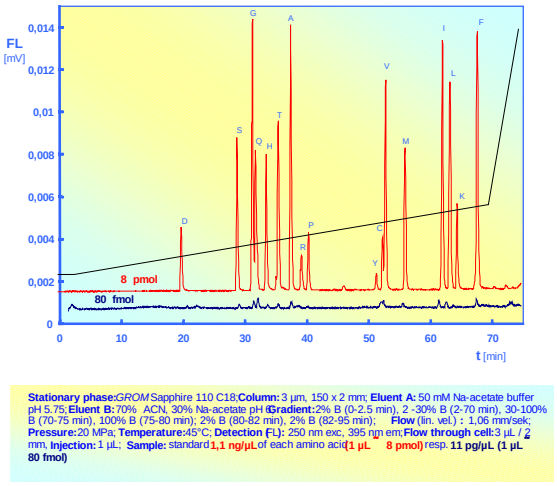

Fig. 8 Lower limit of detection of amino acid-AQC adducts with a 2 mm ID narrowbore HPLC column and fluorescence

In addition to its extreme sensitivity, the requirement for minimal sample amounts and the very small volumes needed for injection of a capillary HPLC column are extraordinary advantages of this method when applied to proteome analysis. With a suitable autosampler, the smallest samples can be derivatized and injected automatically, thus taking full advantage of this method (Fig. 10). The precision of this modern method, which encompasses not only the

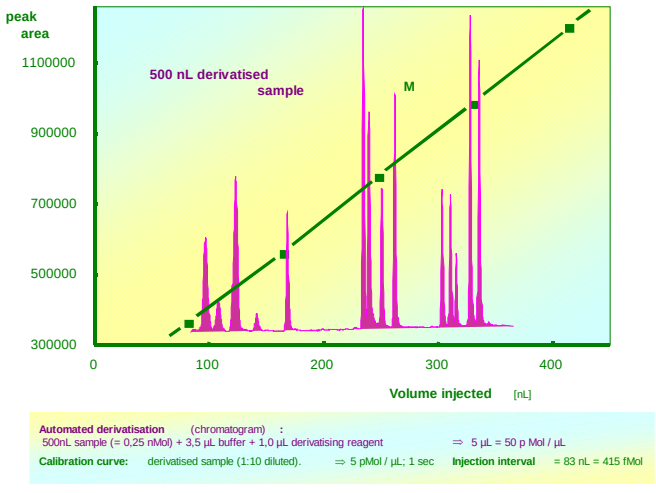

Fig. 10 Automatical Derivatisation of amino acids in the nano scale - calibration curve for the quantitative determination of methionine -

automated but also the manual pre-column derivatisation of amino acids with AQC and the subsequent separation of the AQC adducts via capillary or nanobore HPLC, is similar to the other more classical methods and therefore supports the notion of the superiority of the AQC method as a result of its simpler handling.

Summary. The method described in this study employing AQC as pre-column derivatizing reagent for the analysis of amino acids is clearly distinguished from the other briefly mentioned, more classical methodologies on the basis of its simple handling and execution. This is primarily a

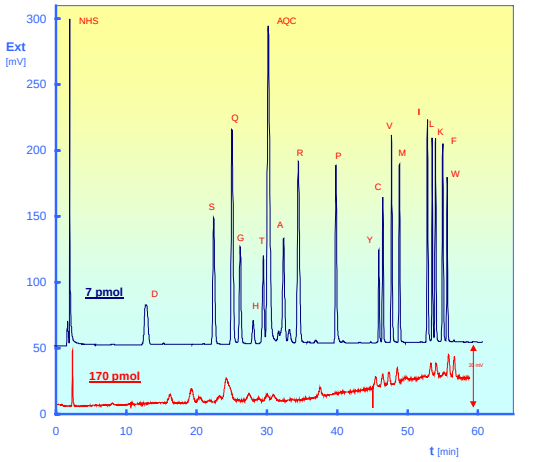

Fig. 9 Lower limit of detection of amino acid-AQC adducts with a capillary HPLC column and UV detection.

function of the fact that the amino acid-AQC adducts are substantially more stable than the derivatives produced with other reagents or methods. With UV detection of AQC adducts, the lower limit of detection can be reduced by more than 200-fold simply by using a 300 µm ID capillary column in place of a 4.0 or 4.6 mm ID analytical column. In other words, reducing the column diameter dramatically increases the sensitivity of the assay. Further improvements are also possible if nanobore HPLC columns (≤ 100µm ID) are employed. The sensitivity achieved the method of pre-column derivatisation with AQC with separation on a capillary column and UV detection is at least as high as that obtained with analytical columns and fluorescence detection of OPA or FMOC derivatives. UV detection offers a considerable advantage over fluorescence detection in that tryptophan can also be quantified with high sensitivity. This is not possible with fluorescence detection due to the high intramolecular quenching observed.

Given the high sensitivity observed on the one hand and the simple sample handling on the other, the results obtained in direct comparison to the other methods of amino acid analysis clearly demonstrate the superiority of this method employing derivatisation of the amino acids with AQC.

Perspectives. The search for an economical laser-induced fluorescence detector continues. With such an instrument, provided that it is equipped with a corresponding nano-flow through cell, it should be possible to improve the sensitivity of the analysis of amino acids via pre-column derivatisation to include the lower atomole

level. While this extremely low level of detection seems entirely possible from a technical viewpoint, it remains to be seen whether it will be of practical use or whether a point of diminishing returns has finally been reached due to problems associated with contamination and nanoscale sample handling.

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