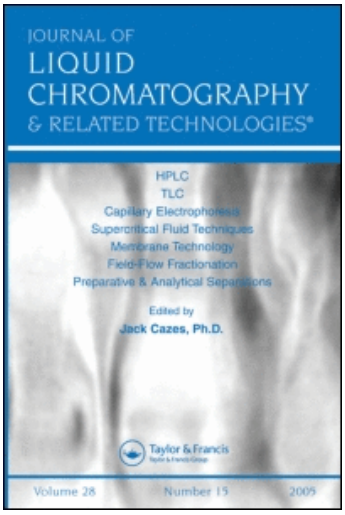

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Busto, O. , Miracle, M. , Guasch, J. and Borrull, F.(1997) 'Solid Phase Extraction of Biogenic Amines from Wine Before Chromatographic Analysis of Their AQC Derivatives', Journal of Liquid Chromatography & Related Technologies, 20: 5, 743 — 755

To link to this Article: DOI: 10.1080/10826079708014139

URL: http://dx.doi.org/10.1080/10826079708014139

J. LIQ. CHROM. & REL. TECHNOL., 20(5), 743–755 (1997)

SOLID PHASE EXTRACTION OF BIOGENIC AMINES FROM WINE BEFORE CHROMATOGRAPHIC ANALYSIS OF THEIR AQC DERIVATIVES

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ABSTRACT

This paper describes a method for determining the most important biogenic amines in wines. It uses reversed phase high performance liquid chromatography (RP-HPLC) with gradient elution and fluorimetric detection, performed on the amines after automatic precolumn derivatization with 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate (AQC). Solid phase extraction (SPE) with strong cation exchanger (SCX) cartridges was used

prior to derivatization to improve the selectivity and to reduce the detection limits (LOD) of the method. The overall method was used to determine the aforementioned amines in red wines from the Tarragona region.

INTRODUCTION

Biogenic amines are alkaline, aliphatic and heterocyclic compounds whose negative presence in food has been well documented.[1-6] These compounds can be found in the raw material but, above all, they are found in the fermented products as a result of the decarboxylation of their precursor amino acids.[7,8]

In the last two decades, the determination of the content of biogenic amines in food products has become more and more important. Increasing interest in producing processed foods that meet the quality that complies with import and exportation laws have encouraged research into these areas.

Biogenic amines are compounds formed during the fermentative processing of foods. Several authors have proved that, for grapes and wines, they appear during the malolactic fermentation, which is the main reason why red wines have higher quantities than white wines.

Historically, a major problem with biogenic amines' identification and quantitation in wines has been the lack of a sensitive analytical technique. Many reports have introduced derivatizing agents and/or other sample treatments to overcome the problems that wines, especially red wines, show because of their matrix complexity. So, many analytical reagents have become popular among wine researchers. Orthophthaldialdehyde (OPA),[7,9-21] the derivatizing agent that enhances both the selectivity as well as the sensitivity of the method when working with HPLC, has been adopted as the best of the agents used, and is at present the method adopted by the *Office International de la Vigne et du Vin* (OIV) for the standard validation of an analytical method.[22] Nevertheless, various shortcomings are also described in all of the reports written on this subject, as we have pointed out in a previous paper.[23] A new derivatizing agent, AQC, overcomes most of these limitations, although its use with different samples is still being studied.[24]

Furthermore, solid-phase extraction is a useful technique that commonly cleans-up and concentrates the analytes by reducing the sample pretreatment which is normally necessary when using other techniques such as liquid-liquid extraction (LLE). To enrich aqueous amine samples, many sorbents including C_{18} and strong and weak cation exchangers, have been tested and compared.[26]

Although C_{18} is the most popular sorbent used for extracting organic compounds from aqueous samples, when amines have to be isolated, the best results are obtained by using weak cation exchangers such as Amberlites.[10,18,27]

Nevertheless, C_{18} recoveries can be improved by modifying the polarity of the amines, either by derivatizing[28] or by reacting them with suitable ion pair reagents.[25]

The aim of this work is to provide a fast analytical method for the simultaneous determination of eight undesirable biogenic amines such as histamine, cadaverine, putrescine, iso-amylamine, tyramine, β-phenethylamine, ethylamine and methylamine, all of them correlated with each other in terms of synergistic toxicity. They are isolated from the main matrix with a strong cation exchanger (SCX), eluted with an optimized solvent mixture and finally chromatographed after AQC automatic derivatization in order to test an alternative procedure to the well-known OPA derivatization.

This method has been used to determine the above-mentioned amines in several red wines from the Tarragona region.

EXPERIMENTAL

Chemicals and Reagents

The eight amines studied were: methylamine, histamine, ethylamine, tyramine, putrescine, cadaverine, β-phenethylamine, and 3-methylbutylamine (iso-amylamine), all of which were supplied by Aldrich-Chemie (Beerse, Belgium). An individual standard solution of 2000 mg L^{-1} of each amine was prepared in HPLC-grade methanol (Scharlau, Barcelona, Spain) and stored in darkness at 4°C. More dilute solutions used in the calibration and SPE studies were prepared by diluting these standard solutions with Milli-Q purified water.

The methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) used in the chromatographic and the extraction method were HPLC grade (Scharlau). The water used for more diluted solutions was of Milli-Q quality (Millipore, Bedford, MA, USA). The sodium acetate reagent (NaAc, HPLC grade) was also supplied by Scharlau. For the automatic derivatization method, the AccQ·Fluor Reagent Kit (Waters, Milford, USA) was used.

Equipment

A Hewlett-Packard (Waldbronn, Germany) model 1050 HPLC equipped with an HP 1046A programmable fluorescence detector was used to perform the chromatographic experiments. The samples were derivatized and injected with an HP Series 1050 automatic injector. Separation was performed using a thermostatted Spherisorb ODS-2 cartridge (250 x 4.6 mm I.D., particle size 5 μm) preceded by a guard-column, both heated to 65°C and supplied by Hewlett-Packard. Chromatographic data was collected and recorded on an HP ChemStation version A.01.01.

SPE experiments were performed using a Visiprep™ DL Disposable Liner Solid Phase Extraction Vacuum Manifold with 12 individual flow control valves from Supelco (Bellefonte, USA).

High-Performance Liquid Chromatographic Method

Two solvent reservoirs containing (A) 1% THF and 0.05M NaAc in Milli-Q water and (B) MeOH were used to perform the optimized gradient programme which began with 5 min of isocratic elution at 35% of MeOH followed by a 7 min linear gradient from 35 to 100% (v/v) methanol. Then, the programme had three additional minutes at 100% of MeOH as a clean-up step and then 2 min to reach the initial conditions and stabilise the corresponding mobile phase. Determination was at a flow-rate of 1 mL min^{-1}. The AQC-derivatives eluted were detected by monitoring their fluorescence using 250 nm and 395 nm as the wavelengths of excitation and emission respectively. Under these conditions, all 8 amines were eluted in less than 13 min.

Derivatization

The derivative reagent was formed by reconstituting the 6-aminoquinolyl-n-hydroxy-succinimidyl carbamate powder with 1 mL of acetonitrile. The alkalinity needed to perform the derivatization was obtained with the borate buffer supplied by Waters in its Fluor Reagent Kit (AccQ·Tag™).

The derivatization was fully automated by means of an injector program. The injection system mixed the reagents automatically, drawing the AQC reagent, the borate buffer, and the sample sequentially into the injection needle. The steps in the derivatization sequence have been specified in a previous paper.[23]

Solid-Phase Extraction

In order to simultaneously clean up and concentrate the sample, solid-phase extraction was carried out with the sulphonic acid cation exchanger (SCX cartridges, 500 mg) supplied by Varian (Harbor City, CA, USA). The solid-phase extraction consisted of a prior step to condition the cartridge by rinsing it with two fractions of 3 mL of methanol. Then, it was rinsed with two fractions of 3 mL of 0.1 mM HCl. The sample was then passed through the conditioned cartridge, washed with 3 mL of 0.1mM HCl and eluted with two fractions of 1 mL of 75 mM borate in MeOH 50 % (v/v) (2.86 g of sodium tetraborate decahydrate was dissolved with distilled water in a 100 mL volumetric flask and then 50 mL of this solution was diluted to 100 mL with MeOH). This step has been optimized according to the procedures described in the following section.

RESULTS AND DISCUSSION

After sample derivatization according to the procedure previously reported,[23] the chromatographic gradient elution was optimized to obtain good resolution between the peaks in a shorter analysis time, with no interference from the more polar compounds usually present in the analysis of real samples. Figure 1 shows the optimum separation of a 5 mg L^{-1} standard mixture of the 8 amines studied, after derivatization with the AQC reagent. All of the amines were well resolved and there was no interference from the peaks corresponding to the derivatization reagents and other system peaks. Ten identical standard samples were derivatized using this procedure. Between-day reproducibility for retention times was excellent (always lower than 1%, average R.S.D. = 0.46%).

In order to verify the linearity of the response of the different derivatives at the previously specified wavelengths for the working concentration, standard solutions of amines that ranged between 0.1 and 15 mg·L^{-1} were prepared and injected. Calibration graphs of each amine were constructed by plotting the amine peak-area against the amine concentration. Linear least-squares regression was used to calculate the slope, the intercept, the correlation coefficient (r^2) and the LODs. All the calibration lines showed ranges of linearity ($r^2 > 0.995$) between 0.20-0.50 and 10-20 mg L^{-1}. The LOD's were calculated by direct injection of the amines and ranged between 20 μg L^{-1} (β-phenethylamine and 3-methylbutylamine) and 60 μg L^{-1} (methylamine).

The method was then applied to real samples by fortifying a red wine with standard solutions. The linearity, the LODs, the repeatability, and the reproducibility were determined. Good linearities ($r^2 > 0.995$) were obtained

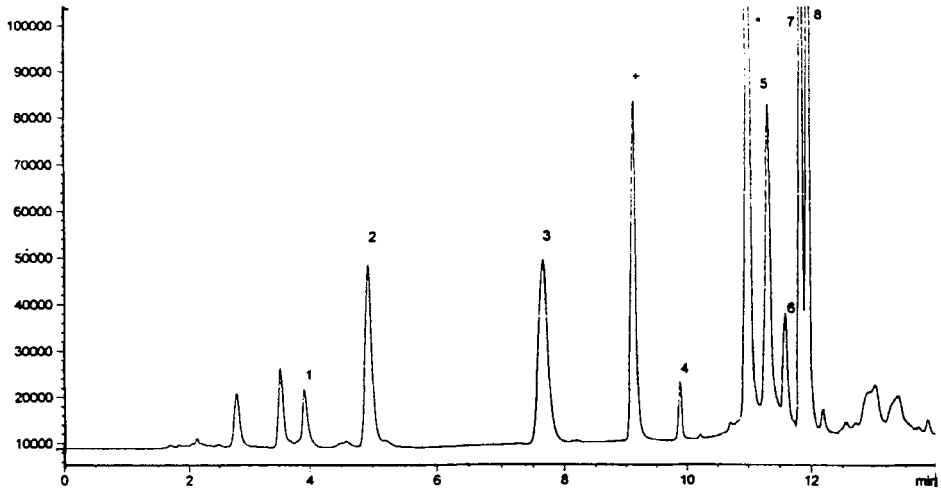

Figure 1. Optimum chromatographic separation of the AQC-amine derivatives. The standard solution of the biogenic amines was about 5 mg L^{-1}: 1 = methylamine, 2 = histamine, 3 = ethylamine, 4 = tyramine, 5 = putrescine, 6 = cadaverine, 7 = β-phenethylamine, 8 = 3-methylbutylamine. Experimental conditions given in text. (+) = peak corresponding to the excess of AQC; (*) Unknown.

between 0.5-1.0 and 10-15 mg l^{-1} with detection limits between 25 μg L^{-1} for β-phenethylamine and 3-methylbutylamine and 500 μg L^{-1} for methylamine and ethylamine. Reproducibility and repeatability of the method were determined at two concentration levels by adding 2 and 10 mg L^{-1} of a standard solution to a red wine. In both cases repeatability was lower than 8% and reproducibility less than 10%.

After many successive analyses, the peak widths of the amine derivatives increased, mainly for histamine and ethylamine, although this was temporarily avoided by flushing the chromatographic system with ACN after every ten injections.

The method was first applied to the analysis of wines which had not been subjected to any special treatment. In this case, they were only filtered through a 0.45 μm nylon membrane.

Figures 2a and 2b show an example of a red wine which was analyzed with and without standard addition about 2 mg·L^{-1} of each of the biogenic amines. It can be seen that the most polar compounds, such as amino acid

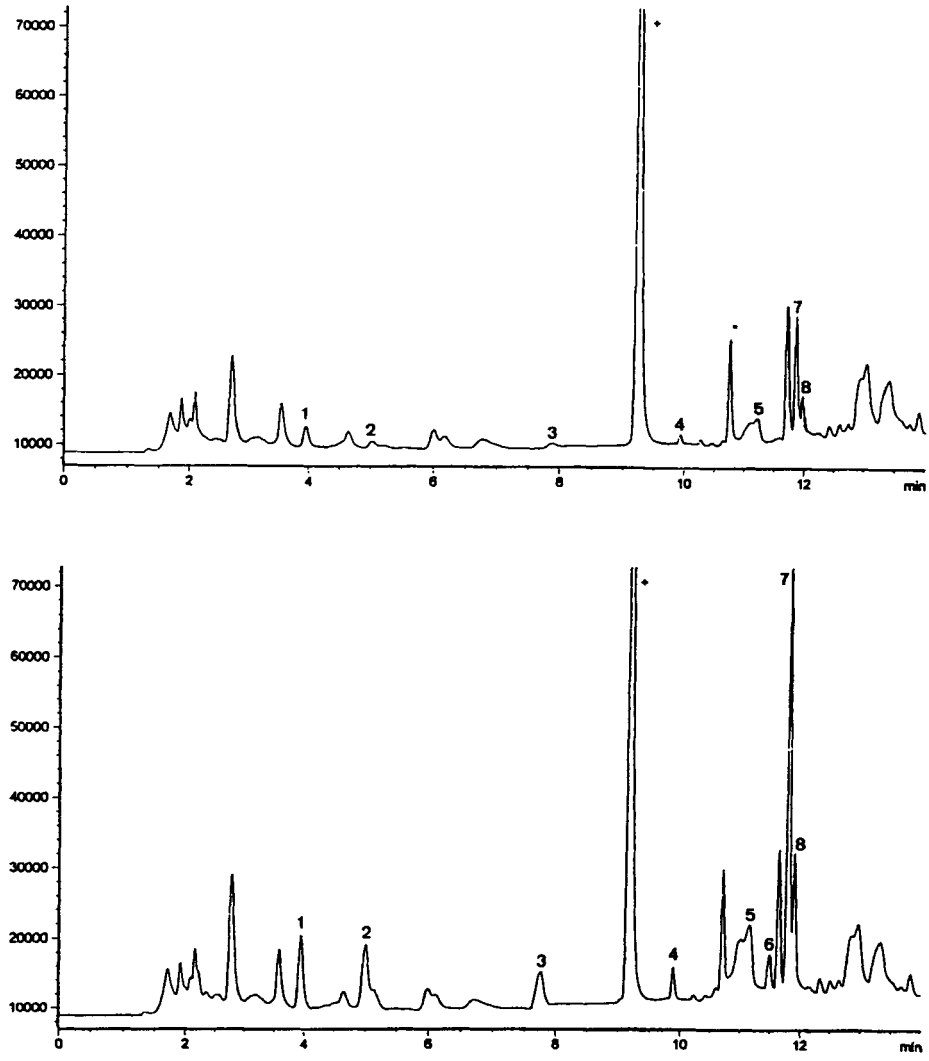

Figure 2. Chromatogram of red wine and the same sample spiked with a standard solution at a final concentration of 2 mg L^{-1} of the amines studied: a) red wine and b) red wine spiked with the standard solution. For peak assignments, see Fig. 1.

derivatives, did not interfere with respect to the peaks corresponding to the compounds studied. Figure 2a shows that histamine can be determined directly without interferences from amino acids. This is highly interesting since it is one of the limitations of the commonly used OPA. However, the rest of the

Table 1

Recovery (R) and Relative Standard Deviation (R.S.D.) of Eight Biogenic Amines Extracted from Red Wine by Solid Phase Extraction[a]

	R (%)	R.S.D. (%)
methylamine	70	8.7
histamine	100	4.1
ethylamine	85	5.1
tyramine	100	4.4
putrescine	100	4.0
cadaverine	101	3.9
b-phenethylamine	80	5.7
3-methylbutylamine	100	5.7

[a] Results from triplicate analysis of 15 ml of red wine spiked with 500 $\mu g \cdot L^{-1}$ of the amines. The baseline is determined from the same wine spiked with methanol instead of amines.

compounds can only be identified because their concentrations are near the detection limits of the method. On the other hand, tyramine cannot be identified in this sample. In order to decrease the matrix effect and to determine these amines at low concentration levels, a solid-phase extraction technique was studied. In previous papers, SPE with C_{18} cartridges was used to this end, but some tedious steps such as pH adjustment and the use of an ion-pair reagent[25] were necessary in order to obtain good results.

We reported in a previous study[21] that biogenic amines in wines can be preconcentrated using SPE with SCX as strong cation exchanger and HCl in MeOH as eluent. The recoveries ranged between 75 and 100% for all the amines except for two of the most important amines present in wines, putrescine and cadaverine, the recoveries of which were 35% and less than 10%, respectively.

To improve this extraction method, several buffer solutions and organic solvents were tested. The best results were obtained with 75mM borate solution in MeOH 50/50 v/v. Attempts to increase the percentage of methanol failed because the borate was not very soluble in methanol at concentrations higher than 50%. Furthermore, lower borate concentrations (25 mM and 50 mM) gave poor amine recoveries, even if the percentage of methanol was increased. So, the above mentioned concentration was chosen as a compromise between

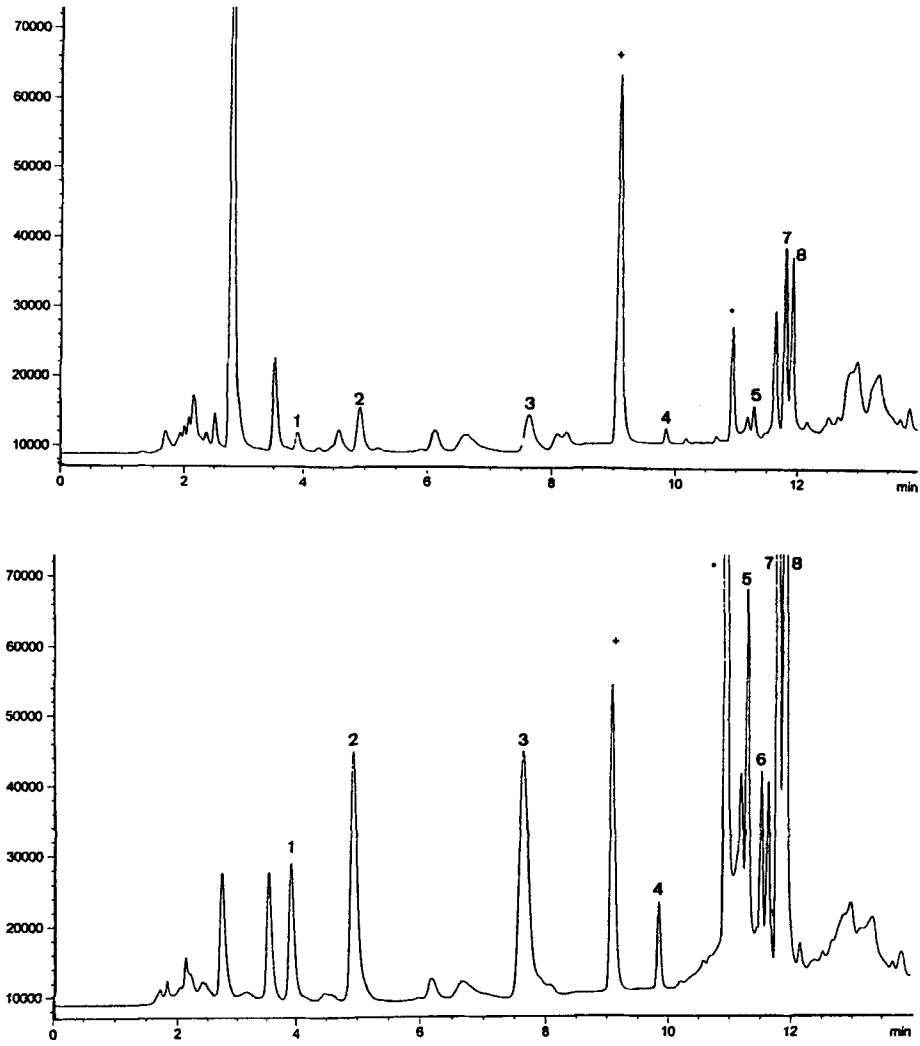

Figure 3. Chromatograms showing the difference when the SPE procedure is applied. 3*a*) Wine given in Fig. 2a after SPE; 3*b*) Same wine spiked with 0.3 mg L^{-1} and processed with the analytical procedure described. For peak assignments, see Fig. 1.

the percentage of organic solvent and the ionic strength, since neither of them can be increased without precipitating the salt. Under these conditions, the results obtained after preconcentrating 15 mL of a red wine fortified with 0.3 mg L^{-1} of each of the biogenic amines and eluted with 1 mL of the optimized

eluting solution are shown in Table 1. As can be seen, putrescine and cadaverine were now well recovered (100%). The recovery of the rest of the amines varied between 80% and 100%, except for methylamine, whose recovery was 70%. So, as well as 3-methylbutylamine and cadaverine, this method also recovered histamine, tyramine and putrescine, which are present in larger quantities in wines.

Figure 3a shows the results of the SPE applied to the wine the chromatogram of which can be seen in Fig. 2a, whereas Fig. 3b gives the chromatogram resulting from the same treatment of the fortified wine. There is a decrease in the most polar peaks that appeared at the beginning of the chromatogram as well as the decrease in other system peaks that interfere in the determination of the analytes. In this wine, in addition to the histamine, methylamine, putrescine, cadaverine, β-phenethylamine and 3-methylbutylamine could also be easily determined after SPE. On the other hand, ethylamine coelutes with another peak, which this treatment removes, although tyramine cannot be identified.

The LOD's of the method were determined using the S/N ratio = 3 for all compounds in a fortified red wine.[23] The LOD's determined ranged between 20 $\mu g\ L^{-1}$ for β-phenethylamine and 3-methylbutylamine and 0.1 $mg\ L^{-1}$ for methylamine. The repeatability was close to 10% for all the amines and the reproducibility was close to 15%.

Attempts to decrease the detection limits result made sample preparation more difficult because an off-line concentration method, such as rotary evaporation of the eluates or concentration under nitrogen steam, had to be added. Experiments to evaporate samples under vacuum failed to recover most of the amines analysed. Neither did concentration under nitrogen steam increase the efficiency of the treatment.

This method was applied to fifteen red wines belonging to different varieties from Tarragona. Results obtained for the SCX concentration are shown in Table 2.

CONCLUSIONS

The HPLC system described enables biogenic amines to be determined in wines with good sensitivity and specificity. By means of automatic pre-column AQC-derivatization and fluorescence detection, appropriately coupled with a prior SCX extraction, eight amines can be quantified at low levels. SCX cartridges again proved to be adequate to clean up the wine samples, while

Table 2

Analysis of 15 Red Wines[a]

Name	Concentration Range	Median
histamine	5.3 - 7.8	6.3
putrescine	3.3 - 4.8	4.4
3-methylbutylamine	0.1 - 0.2	0.1
methylamine	0.7 - 0.8	0.8
ethylamine	0.2 - 0.6	0.5
cadaverine	0.5 - 0.7	0.6
b-phenethylamine	0.5 - 0.6	0.6
tyramine	ND	-

[a] Concentrations in mg L^{-1}. Results from SCX extraction of the wines.

retaining biogenic amines, so enabling the samples to be simultaneously concentrated. Recoveries have been improved in comparison with other studies and the concentration step has been optimized, allowing these compounds to be detected at low levels with no interference from other compounds such as amino acids.

ACKNOWLEDGEMENT

The authors wish to thank CICYT (project ALI 94-0505) for financial support given.

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Received April 10, 1996
Accepted August 15, 1996
Manuscript 4148