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Letter to the Editor

Determination of homocysteine by gas chromatography-mass spectrometry following treatment with chloroformates: a comment

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Dear sir:

The recent paper of Myung et al. [1] follows derivatization approach used in the earlier paper of Sass and Endres [2] i.e., the one-step amino acid derivatization with a chloroformate, but the nowadays popular solid-phase microextraction (SPME) of the analytes from the reaction medium is offered optionally to the liquid-liquid microextraction (LLME). Passing the fact that SPME makes the procedure unnecessary lengthy and more liable to variabilities, there are some erratic conclusions and questionable steps which should not be pursued. As a result, the procedure can be hardly recommended as a reliable alternative to the current methods.

First, confusing and erratic statements were made regarding reactivity of various chloroformates. Based on responses (peak areas, Fig. 1) of the particular propyl esters treated with ethyl through to butyl and isobutyl chloroformates it was concluded that the former reagent were less reactive than the latter ones because of larger peak areas found upon treatment with the two latter (*sic*!). Moreover, the legend to the Fig. 1 points confusingly to 'Comparison of the reactivity of the alkoxycarbonyl propyl ester derivatives' instead of (wanted) that of the reagents. Apart from the fact that reagents with shortest alkyls, i.e. the methyl and ethyl chloroformates, exert evidently highest reactivity [3], the following is to object to *Results and discussion*: (a) As the peak areas in the Fig. 1 concerns it is not specified if they were obtained via SIM or SCAN mode; (b) The peak areas can hardly reflect efficiency of the chloroformate treatment since the responses not necessarily correspond to the molar concentrations; fragmentation of the different derivatives does not need to be identical, abundance of the selected monitored ion may differ from derivative to derivative; (c) The questionable figure, thus, merely reflects responses of the different alkoxycarbonyl propyl esters obtained under the defined conditions. Next to this, neither the legend to Fig. 6 nor the text do inform explicitly whether the EI mass spectra correspond to ethoxycarbonyl propyl esters of the particular compounds.

Second, reaction medium for treating the analytes with a chloroformate [1,2] is far from optimum for an undisturbed derivative formation because of the presence of trichloroacetic acid (TCA, final concentration 6%) and dithiothreitol (DTT, about 1.1%). Even when TCA is not esterified due to its high acidity and the molar excess of pyridine suffices to initiate the esterification of the analytes, the sulfhydryl groups of DTT consumpt portion of the reagent. Due to this, lower derivatization yields

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occur and several extraneous peaks appear on the chromatogram when detected with flame ionization, which is clearly not recognized under employment of SIM-MS detection. Under such conditions it seems to be rather surprising that constant and reproducible derivatization yields are declared.

Third, composition of the reaction medium and presence of chloroformate in the extraction solvent are a mere copy of conditions optimized for derivatization of protein amino acids with ethyl chloroformate in presence of ethanol [4]. In that case the 1%-addition of reagent to chloroform aimed to improving reaction yields of asparagine and aspartic acid, i.e., there was no relevance to the S-containing amino acids at all. Reaction conditions employed with reagents and alcohols of higher alkyls [5] were in [1,2] not reflected at all.

Forth, TCA and pyridine are partially transferred into chloroform during extraction. As a result, a massive white residue of TCA-pyridine salt remains in a vial after evaporation of the organic phase. Being dissolved in chloroform and subsequently injected into the instrument it results in formation of a huge broad peak that requires a longer time of temperature programmed operation to elute. Even when this escapes monitoring by means of SIM-MS, it does not prevent an untimely deterioration of the capillary column performance.

Fifth, a rapid poisoning of the capillary column and injector must occur due to the fact that lipids were not removed from plasma prior LLME or SPME of the derivatized analytes [1,2]. This could be simply done by exposing the plasma supernatant to hexane extraction [6] or by using cation exchange chromatography along with plasma homocysteine determination [7]. Employing SPME for uptake of derivatized analytes in plasma supernatants leads, undoubtedly, to co-adsorption of the lipids at the polyacrylate fiber. The influence of this factor is apparently not taken in account. Simplification of the methodology by omitting the lipid removal causes simultaneously its shortened lifetime.

Sixth, some words to *Calibration and limit of detection* [1] eventually. The method is presented as precise and sensitive, despite of given detection limit of <5 μmol/l for homocysteine and the others S-containing amino acids. However, such a limit is close to the mean concentration of the analyte of interest in plasma of controls. Erratic must be also statement that 'the calibration curve was linear over the range 5–50 μmol/l for homocysteine and cysteine and 40–400 μmol/l for methionine'. In a better case the values for the two latter amino acids, regarding their plasma abundance, were interchanged. The concluding sentence of this paragraph, i.e. 'The method would be equally suitable for the determination of the total homocysteine', is apparently fully misleading. What was determined, then?

(*Note*: The determination of homocysteine by means of gas chromatography following derivatization with chloroformates is a part of a 3-year project (1998–2000) granted by Internal grant agency of Ministry of Health of the Czech Republic under No. 4848-3).

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