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# Development and validation of a rapid method for enzymatic digestion of HBOCs utilizing microwave irradiation

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## Introduction

Increased oxygen-carrying capacity through the use of blood substitutes could help elite athletes to lengthen endurance capacity and improve their performance. For this reason haemoglobin-based oxygen carriers (HBCOs) have been prohibited in sport since January 2000 [1]. LC-MS/MS methods currently used by accredited anti-doping laboratories for the confirmation of HBOCs, are based on the analysis of peptide composition following enzymatic digestion [2-3]. The process is generally time consuming (5 h-12 h), a critical factor during drug testing for major international events. Here we present a procedure to speed up the enzymatic digestion step of three HBOCs (Hemopure®, Polyheme® and Oxyglobin®) using microwave irradiation, technology already used in our laboratory to speed up urine sample pre-treatment (derivatization, hydrolysis and extraction steps) in anti-doping analysis [4]. We specifically consider the effects of temperature, incubation time and amount of reaction reagents used in the digestion process and the pattern of tryptic peptides generated was compared with that obtained by the reference method (12 h incubation, 37 °C), presently used in our laboratory.

## Experimental Section

*Chemicals and Reagents*

Hemopure® and Oxyglobin® were provided by Biopure Corporation (Cambridge, MA). Polyheme® was provided by Northfields Laboratories. Water was from a MilliQ water purification system (Waters, Italy). Trypsin used for tryptic digestion, carbonic anhydrase used as internal standard (ISTD), human and bovine hemoglobin were supplied by Sigma-

Aldrich (Milano, Italy). Acetic acid, trifluoroacetic acid, ammonium bicarbonate and acetonitrile were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy).

*Microwave and heating block conditions*

Digestion of proteins was conducted in a temperature controlled, single beam microwave oven for organic synthesis, MARS5 (Microwave Apparatus CEM Corporation, Matthews, NC). This microwave has a maximum power output of 1200 W and a nominal frequency of 2.45 GHz. The protein was treated with the procedure described in a previous paper [3], and the digestion products were sampled at intervals of 10, 30, 60, 120, 180 and 240 min. The actual reaction solution temperatures during and after microwave irradiation were measured with a temperature probe inside a reference sample in the microwave. This was to establish the maximum temperature reached during the irradiation and to determine whether the temperature was constant. The same procedure was carried out using a heating block; in this case temperatures were monitored using a mercury thermometer placed in an adjacent sample well. Enzyme digestion experiments were carried out at 37 °C, 45 °C, and 55 °C and the sample aliquots were collected at intervals of 10, 30, 60, 120, 180 and 240 min.

*LC-MS/MS conditions*

All LC-MS/MS experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy). Reversed-phase liquid chromatography was performed using a Zorbax 300SB C18 column (2.1 × 50 mm, 3.5 μm). The solvents were: water containing 0.2 % acetic acid and 0.02 % TFA (eluent A) and acetonitrile containing 0.02 % acetic acid and.0.02 % TFA (eluent B). The gradient program started at 5 % B increasing to 100 % B in 9 min. The column was flushed for 1 min at 100 % B and finally re-equilibrated at 5 % B for 4 min. Data was acquired using an Applied Biosystems (Applied Biosystems, Monza, Italy) API4000 triple-quadrupole instrument with positive electrospray ionization. The ion source was operated at 550 °C, the applied capillary voltage was 5500 V and selective reaction monitoring (SRM) experiments were performed employing collision-induced dissociation (CID). The mass and SRM transitions monitored for the five methods developed here are all presented in Table 1.

**Table 1:** Peptide composition of Human Hb, Bovine Hb and HBOCs considered in this study after tryptic digest and transitions selected for the LC-MS/MS analysis.

| Hb Chain | MW (Da) | Molecolar ion peptide formed | | Peptide formed after tryptic digest (amino acid sequence) | (Bovine Hb) Hemopure® and Oxyglobin® | (Human Hb) Polyheme® | Transition selected (m/z) |
|---|---|---|---|---|---|---|---|
| | | $(M+2H)^{2+}$ (m/z) | $(M+2H)^{3+}$ (m/z) | | | | |
| β | 1275 | ***638**** | 426 | LLVVYPWTQR | Yes | Yes | 638/638 |
| α | 1530 | ***766**** | 511 | VGAHAGEYGAEALER | Yes | Yes | 766/766 |
| α | 2367 | 1185 | ***790**** | AVEHLDDLPGALSELSDLHAHK | Yes | No | 790/790 |
| α | 1834 | ***918**** | 612 | TYFPHFDLSHGSAQVK | Yes | Yes | 918/918 |
| β | 2059 | ***1030**** | 687 | FFESFGDLSTPDAVMGNPK | No | Yes | 1030/1030; 1030/547 |
| β | 2090 | ***1046**** | 698 | FFESFGDLSTADAVMNPK | Yes | No | 1046/1046;1046/120 1046/267 |

***Peptide selected for the LC-MS/MS method***

## Results and discussion

This preliminary study demonstrates that it is possible to use microwave-irradiation to speed up the enzymatic digestion step of Hemopure®, Polyheme® and Oxyglobin® without sacrificing sensitivity and specificity. Power, temperature, incubation time, amount of denaturing reagents (acetonitrile) and protease-to-protein ratio were optimized to achieve an increase in digestion efficiency and a decrease in hydrolysis time without degradation/breakdown of the proteins.

The repeatability of digestion recovery (CV% <10 for all target compounds), obtained by analyzing 20 different plasma samples spiked with the three HBOCs separately, was comparable with the results obtained by the reference method. Other observations obtained by upgrading the reference digestion procedure are reported: (i) the rapid increase in the reaction temperature is only partially responsible for the accelerated rate of digestion. Investigation of three different temperatures (37 °C, 45 °C, and 55 °C), in both the microwave and heating block, showed only a small increase in digestion efficiency; (ii) after subjecting HBOCs to 60 min of microwave irradiation without enzyme we could detect only intact protein (data not shown). Thus, microwave irradiation enhances the enzymatic digestion of proteins but does not induce degradation/breakdown of the protein in the absence of a protease; (iii) the observed different rates of digestion between Hemopure®, Oxyglobin® and Polyheme® appear to be dependent on the different protein structures; (iv) the LOD for the three HBOCs studied is 0.3 mg/mL. These LODs seem to be adequate in detecting administration in blood samples for up to five days; a reasonable period for doping purposes, where their use would be at competition with infusion just prior to an event.

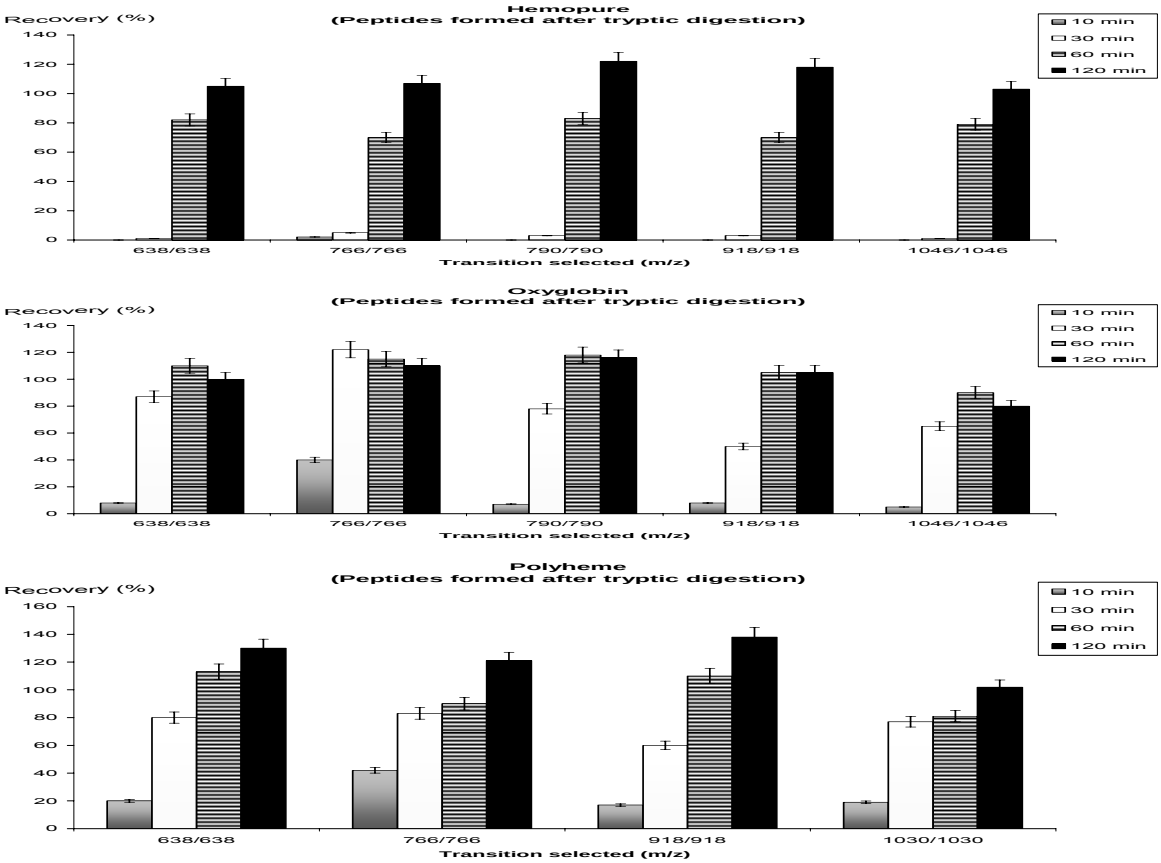


**Figure 1**: Tryptic digestion recovery, in plasma sample spiked with HBOCs at 1 mg/mL, using microwave irradiation sampled at 10, 30, 60 and 120 min for the three HBOCs considered. Results are relative to control sample digested for 12 h at 37 °C.

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