Research Article

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Bioanalysis

# Absolute quantification of 35 plasma biomarkers in human saliva using targeted MS

**Background:** Although the use of human saliva for diagnosing disease has been known to be of great clinical potential, few attempts have been made so far to develop its use. In this work, we developed an MRM-MS approach for 35 plasma biomarkers using human saliva in a clinical environment. **Methods & results:** A 30-min micro LC–MS/MS run in MRM mode was conducted in order to quantify the 35 plasma proteins in human saliva. Sample preparation procedures were performed in quadruplicate and analyzed in duplicate. Results show that 32 of the 35 plasma proteins were quantified in human saliva using calibration curves in the 2- log10 dynamic ranges with excellent linearity. **Discussion/conclusion:** Our MRM method is compatible with routine measurements in daily clinical practice.

**Keywords:** biochemistry • clinical chemistry • MS • proteomics • saliva

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Although saliva is not generally regarded as one of the most interesting biological fluids, the fact that it can be sampled using simple, noninvasive methods [1] makes it an interesting alternative to blood for diagnostic purposes [2–4]. In addition, this approach has the advantages of being cheap, easy to perform and less stressful to patients than other biological fluids such as blood. Saliva is a complex biological fluid which is involved in a wide range of biological processes, and its potential for the diagnosis of local and systemic diseases is growing since 10 years [5].

Thanks to the use of bottom-up proteomic approaches, more than 2200 salivary proteins [6] have already been identified and some of them have been described as classified as potential clinical biomarkers. Approximately 25% of them are plasma components, and the remainder originate from endogenous salivary glands and desquamated epithelial cells. It has been suggested that some salivary proteins could serve as biomarkers signaling the presence of head and neck tumors and malignant oral diseases [7]. The use of this highly efficient noninvasive approach to monitor the onset and progression of diseases is of great potential interest. Appropriate sensitive multiplex methods are now urgently required for this purpose, in addition to data on the specific salivary biomarkers corresponding to systemic and local disorders/diseases.

Although immunobased tests are being widely used to quantify proteins in biological fluids, they are not very suitable for use on saliva because of the strong matrix effects which are mainly induced by the presence of high molecular weight proteins such as mucins in this complex fluid. In this context, targeted MS methods, which are known to be highly specific, sensitive, robust and multiplexable methods, provide a useful means of testing this particular fluid and overcoming the difficult problems associated with matrix effects. To implement this approach on human saliva, an MS quantifying mode called the multiple reaction monitoring (MRM) mode was combined with stable isotope dilution MS (SID-MS) methods. MRM is an MS approach which was developed several decades ago for quantifying small molecules in the context of clinical chemistry and has been applied to proteins for about 10 years [8]. MRM is generally applied using

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triple quadrupole (QqQ) mass spectrometers usually available at clinical laboratories. The quantitation performed with MRM is based on distinctive proteotypic peptides from proteins of interest. The specificity of the MRM assay is based on the possibility of isolating an analyte (such as proteotypic peptide) by determining three molecular characteristics: the retention time, the precursor ion mass (Q1 *m/z*) and the fragment ion mass (Q3 *m/z*). The combination between the precursor ion mass and the fragment ion mass z, which is highly specific, is called a transition.

In the present study, we have translated a method utilized for plasma proteins to human saliva: 35 plasma proteins were quantified in saliva using 35 stable isotope standard (SIS) peptides as molecular surrogates for the endogenous analogues. Based on these internal standardization procedures, the data obtained were normalized and adjusted to account for the matrix effects, ion suppression and the variability of the instruments performance. Previous authors have established that SIS peptides or protein [9,10] can be used in MRM-based quantitative proteomic workflows for blood biomarker analysis [11,12], but this method has never been applied to the analysis of human saliva.

The results obtained using this innovative, multiplex, fast and robust targeted MS approach show for the first time, the feasibility and the validity of this method for the absolute quantification of 35 biomarkers in human saliva.

## Experimental section

### Ethical approval & human participants

The saliva specimens used here originated from an officially registered biobank with the reference number # DC-2008–417. This biobank contains anonymized samples provided by participants (most of them are dental students) who signed an ethically approved informed consent form. Whole saliva specimens were collected from 20 nonsmoking adult volunteers (ten males and ten females) ranging from 20 to 26 years of age These individuals showed no signs of gingivitis, periodontal disease, active dental caries, oral lesions or any other oral or systemic conditions liable to affect the whole-saliva composition.

### Saliva samples

To minimize the circadian effects, saliva specimens were all collected between 9:00 and 11:00 a.m [13]. Prior to the sampling procedure, participants rinsed out their mouths three-times with water. To induce salivary production, they were asked to chew neutral and citric acid impregnated Salivette® cotton swabs for exactly 60 s. Each of the participant's salivary flow rate was calculated on a milliliter per minute basis. Saliva specimens were centrifuged for 2 min at a rate of 1000 × *g* to yield clear saliva, which was aliquoted into 500 µl samples in LoBind tubes and stored at -80°C before being analyzed. The saliva protein concentrations were determined using by colorimetric protein assay (BCA Protein Assay Kit, Thermo Scientific Pierce, USA) using bovine serum album (BSA) as standard. In order to minimize any bias possibly due to individual variations, two pools of saliva (neutral saliva and acid saliva) collected from 20 individuals were prepared. The results of the MRM quantification procedure were checked on individual salivary samples (n = 6).

### SIS proteotypic peptides

The SIS peptides were purchased by MRM proteomics as PeptiQuant™ Performance Kit optimized for Agilent 6490 mass spectrometer (Standard Flow). All SIS peptides contained a heavy isotope form of an arginine ($[^{13}C_6]$ or $[^{13}C_6, ^{15}N_4]$) or lysine ($[^{13}C_6]$ or $[^{13}C_6, ^{15}N_2]$) amino acid residue (Cambridge Isotope Laboratories, Andover, MA, USA) at the C-terminus. SIS peptide concentrations were adapted to reflect the endogenous concentrations. 35 SIS peptides were measured: afamin, α-1-antichymotrypsin, α-1B-glycoprotein, α-2-antiplasmin, angiotensinogen, anti-thrombin-III, apolipoprotein A1, apolipoprotein A-II, apolipoprotein A-IV, apolipoprotein B-100, apolipoprotein C-I, apolipoprotein E, β-2-glycoprotein I, ceruloplasmin, clusterin, coagulation factor XII, complement C3, complement C4-B, complement component C9, complement factor B, complement factor H, fibrinogen α-chain, fibrinogen β-chain, gelsolin, haptoglobin, hemopexin, heparin cofactor II, inter-α-trypsin, kininogen-1, plasminogen, retinol-binding protein 4, serum albumin, transthyretin, vitamin D-binding protein and vitronectin.

### Proteomic workflow

Fifty microliters of saliva were used as the starting material. Saliva proteins were precipitated with 200 µl ethanol at -20°C overnight. Samples were then centrifuged (at 17,000 × *g* for 5 min at 4°C) and the supernatants were removed. Salivary protein pellets were resuspended with 20 µl urea 8 M in water and transferred to 96-well plates before performing an automated reduction/alkylation/digestion/clean-up using the BRAVO AssayMap (Agilent) platform. Briefly, 30 µl of denaturation solution (20 mM DTT, 100 mM Tris pH 8.5) was added to each well and incubated for 1 h at 37°C under agitation. Alkylation was then performed by adding 6 µl of alkylant solution (400 mM iodoacetamide, 1M Tris pH11) at 37°C for 30 min. Before the digestion step, samples were diluted with 210 µl of 20 mM Tris pH 8.5 + 2 mM DTT. Protein digestion

was then carried out at 37°C overnight after adding 0.5 µg trypsin, and the digestion was stopped by adding 15 µl formic acid (pH <4). The peptides generated were desalted using C18 AssayMap tips in line with the manufacturer's instructions. Samples were then transferred to lobind tubes (Eppendorf, Germany), dried on/in a vacuum concentrator (Labconco, Kansas City, MO, USA) and resuspended using 20 µl of 2% acetonitrile/0.1% formic acid/97.9% water for 10 min under agitation. The sample preparation procedures were performed in quadruplicate.

## MRM method

Tryptic peptides were separated by performing LC on a 1290 liquid chromatography system (Agilent Technologies). Peptides were resolved using a reverse-phase column (RRHD Eclipse plus C18, 2.1 × 150 mm, 1.8 um) at 400 µl/min. A 30-min multistep gradient was performed, starting with 2.7% of solvent B (10% water, 90% acetonitrile and 0.1% formic acid), which was increased to 9.9% after 2 min, 17.1% after 15 min, 26.1% after 22 min and 40.5% after 25 min. After 27 min, the column was flushed for 2 min with 81% of solvent B and again for 3 min with 2.7% of solvent B.

Peptide analyses were carried out on a QqQ MS system (6490, Agilent technologies) equipped with an Agilent Jet-Stream ESI interface in the positive ion mode. The ESI source was set as follows: capillary tension 3500 V, nozzle voltage 300 V, nebulizer 30 psi, gas flow rate 15 l/min, gas temperature 150°C, sheath gas flow rate 11 l/min, sheath gas temperature 250°C. The MS was performed in dynamic MRM mode with a retention window of 1.5 min and a maximum cycle time fixed at 700 ms. One peptide per protein and three transitions per peptide were studied as defined in the MRM kit used (Supplementary Table 1). MRM analyses were performed in duplicate.

## Calibration curve generation

The stable isotope standard (SIS) peptide panel used, which was purchased from MRM proteomics (Victoria, CANADA), was composed of 35 high abundance blood proteins listed in Supplementary Table 1. As noted in this table, 12 of these proteins were already US FDA approved or FDA cleared analytes in human plasma or serum, and appropriate clinical plasma immunoassays are currently available in most clinical laboratories [12]. One heavy precursor peptide per protein and three daughter ions were monitored [14]. Calibration was established using stable isotope-labeled standard peptides spiked into the saliva samples prior to LC–MS/MS analysis. Calibration curves were generated using heavy peptide standards using seven different peptide concentrations adapted for each SIS peptide (Table 1). Calibration curves were drawn up in triplicate on all the peptides targeted.

## Data analysis

Bioinformatic data analysis was performed using Skyline 2.6 software. All calibration curves and sample replicates were loaded into the software database. The automatic peak detection method used was tested by performing manual inspection. Areas were exported to excel files in order to draw up calibration curves and quantify sample proteins.

## Results & discussion

The 2000 proteins described in human saliva are of various origins: endogenous proteins secreted by salivary glands (the parotid, submandibular and sublingual glands and minor salivary glands) and exogenous proteins originating from gingival crevicular fluid bacteria lysates, desquamated epithelial cells, oral bacterial and blood [15]. Blood proteins can enter the saliva as the result of various processes such as active transport, passive intracellular diffusion and extracellular ultrafiltration processes [16]. Blood proteins account for up to 27% of the salivary proteins and it has been suggested that 40% of them may constitute suitable biomarkers for detecting the presence of diseases such as cancer, cardiovascular disease and stroke [17].

### Effects of salivary sampling methods on the protein concentration

Whole saliva specimens were sampled from 20 healthy subjects and pooled in order to minimize the effects of individual variability. We used here stimulated saliva because it corresponds to our common clinical procedure for samples collection as it is more rapid and less constraining than resting saliva collection. In addition, this sampling can be performed using a validated medical device (Sarstedt collection device). Salivary samples were processed in quadruplicate and analyzed in duplicate as described above (Figure 1). The salivary flow rates measured using the two stimulating devices (chewing stimulated saliva vs acid stimulated saliva) showed that the use of acidic swabs induced a dramatic increase of the salivary flow rate (2.46 ml/min ± 0.76 vs 4.98 ml/min ± 0.75, respectively ($p < 0.05$). The average protein concentrations also differed: they amounted to 1.35 µg/µl ± 0.85 in the case of the chewing stimulated saliva versus 0.86 µg/µl ± 0.19 ($p < 0.05$) in that of the acid stimulated saliva. The low protein concentration present in the acid stimulated saliva was probably due to the sample being more highly diluted [18]. It was previously reported that acidic stimulation preferentially involves the parasympathetic nervous system since the salivary flow rate was found to be much greater

Table 1. Thirty-five targeted plasma proteins quantified in human saliva.

| Quantified protein in saliva | US FDA approved | Linear concentration range pg/ml | Linear response ($R^2$) | LOD pg/ml | LOQ pg/ml | Concentration in saliva (pg/ml) | Concentration in plasma (µg/ml) | Clinical relevance |
|---|---|---|---|---|---|---|---|---|
| Afamin | | 16–807 | 0.9963 | 5.5 | 9.2 | 60.1 | 5.7 | |
| α-1-antichymotrypsin | | 10–1046 | 0.9776 | 12.7 | 18.4 | 165.3 | 187.7 | Lung and liver disease, inflammation |
| α-1B-glycoprotein | Yes | 56–2786 | 0.9930 | 69.4 | 84.6 | 1156.5 | 39.8 | Inflammation, malignant neoplasms, oral squamous cell carcinoma |
| α-2-antiplasmin | Yes | 26–663 | 0.9915 | 12.0 | 17.8 | 38.4 | 261.6 | Fibrinolysis, hemostatic balance in the oral cavity |
| Angiotensinogen | | 10–515 | 0.9910 | 5.3 | 29.0 | 128.9 | 60.6 | OSCC |
| Antithrombin-III | Yes | 18–1833 | 0.9886 | 24.2 | 154.6 | 989.5 | 355.9 | Congenital AT deficiency |
| Apolipoprotein A1 | Yes | 12–12,206 | 0.9893 | 194.0 | 229.0 | 295.7 | 1079.5 | Coronary artery disease,peridontal disease |
| Apolipoprotein A2 | | 4–752 | 0.9813 | 10.6 | 25.9 | 16.5 | 116.8 | Obesity, cardiovascular risk |
| Apolipoprotein A4 | | 4–188 | 0.9527 | 4.8 | 13.6 | 13.9 | 49.5 | |
| Apolipoprotein B | Yes | 92–1863 | 0.9861 | 17.2 | 58.4 | 118.6 | 172.9 | Coronary atherosclerosis, metabolic syndrome |
| Apolipoprotein C1 | | 0.2–18 | 0.9395 | 16.2 | 70.3 | 0.0 | 1.2 | Primary Sjögren's syndrome |
| Apolipoprotein E | | 6–240 | 0.9828 | 3.4 | 8.4 | 80.1 | 8.2 | Alzheimer's disease |
| β-2-glycoprotein 1 ou ApoH | | 25–1017 | 0.9790 | 30.1 | 85.3 | 162.9 | 104.2 | Sjögren's disease |
| Ceruloplasmin | Yes | 41–1059 | 0.9771 | 66.8 | 115.7 | 399.7 | 123.7 | Wilson's disease, liver disease, chronic disseminated periodontitis |
| Clusterin | | 17–1789 | 0.9617 | 1.0 | 2.8 | 761.9 | 152.4 | Alzheimer's disease |
| Coagulation factor XII | | 2–214 | 0.9877 | 0.9 | 2.3 | 761.9 | 124.3 | |
| Complement C4B | | 23–2283 | 0.9786 | 50.1 | 111.8 | 2433.8 | 169.5 | Immunologic diseases including lupus erythematosus, chronic active hepatitis, OSCC |
| Complement C9 | | 28–733 | 0.9936 | 23.6 | 71.2 | 426.9 | 129.7 | Immunologic diseases including lupus erythematosus, serum sickness |
| Complement factor 3 | | 96–4795 | 0.9786 | 98.6 | 164.7 | 23.9 | 33.6 | OSCC |

FDA biomarker status, linear concentration range (pg/ml), linear response ($R^2$), detection limit (LOD, pg/ml), quantification limit (LOQ, pg/ml), concentration in human saliva (pg/ml), concentration in human plasma (µg/ml) and clinical relevance.
OSCC: Oral squamous cell carcinoma.

Table 1. Thirty-five targeted plasma proteins quantified in human saliva (cont.).

| Quantified protein in saliva | US FDA approved | Linear concentration range pg/ml | Linear response ($R^2$) | LOD pg/ml | LOQ pg/ml | Concentration in saliva (pg/ml) | Concentration in plasma (μg/ml) | Clinical relevance |
|---|---|---|---|---|---|---|---|---|
| Complement factor B | | 2–251 | 0.9927 | 6.8 | 12.0 | 44.3 | 95.1 | Head and neck squamous carcinoma |
| Complement factor H | | 16–677 | 0.9919 | 4.6 | 47.3 | 51.1 | 123.7 | |
| Fibrinogen-α chain | Yes | 1–446 | 0.9791 | 10.1 | 34.2 | 1.8 | 3115.8 | Fibrinogen deficiency, diagnosis of disseminated intravascular coagulation, OSCC |
| Fibrinogen-β chain | Yes | 34–3441 | 0.9696 | 462.7 | 2301.9 | 70.0 | 300.1 | Fibrinogen deficiency, diagnosis of disseminated intravascular coagulation, OSCC |
| Gelsolin | | 4–233 | 0.9583 | 10.9 | 23.9 | 659.5 | 49.2 | Oral cancer |
| Haptoglobulin | Yes | 53–10,578 | 0.9775 | 215.2 | 228.8 | 1308.4 | 324.0 | OSCC |
| Hemopexin | Yes | 36–8919 | 0.9902 | 201.9 | 222.5 | 2703.2 | 290.1 | OSCC |
| Heparin cofactor II | | 3–272 | 0.9876 | 2.3 | 11.8 | 10.3 | 50.8 | |
| Inter-α-trypsin inhibitor | | 0.2–773 | 0.9823 | 17.6 | 58.1 | 70.9 | 22.0 | Gastric cancer |
| Kininogen | | 9–847 | 0.9942 | 1.0 | 1.0 | 543.6 | 82.4 | Advanced colorectal adenoma and colorectal cancer |
| Plasminogen | Yes | 6–143 | 0.9950 | 3.6 | 7.2 | 31.5 | 200.0 | Thrombotic risk |
| Retinol-binding protein 4 | | 14–364 | 0.9649 | 2.9 | 6.4 | 151.6 | 9.5 | Metastatic oral cancer |
| Serum albumin | Yes | 154–38,585 | 0.9911 | 481.3 | 584.5 | 114,180.1 | 76,032.3 | Nutritional status, blood oncotic pressure, renal disease, gingivitis or periodontitis |
| Transthyretin | | 17–838 | 0.9949 | 25.7 | 43.1 | 250.2 | 123.9 | OSCC |
| Vitamin D binding protein | | 14–1360 | 0.9881 | 38.5 | 43.1 | 698.1 | 104.8 | Colorectal cancer in the prostate, lung, colorectal and ovarian cancer |
| Vitronectin | | 63–635 | 0.9299 | 55.4 | 72.4 | 309.6 | 165.5 | Breast cancer |

FDA biomarker status, linear concentration range (pg/ml), linear response ($R^2$), detection limit (LOD, pg/ml), quantification limit (LOQ, pg/ml), concentration in human saliva (pg/ml), concentration in human plasma (μg/ml) and clinical relevance.
OSCC: Oral squamous cell carcinoma.

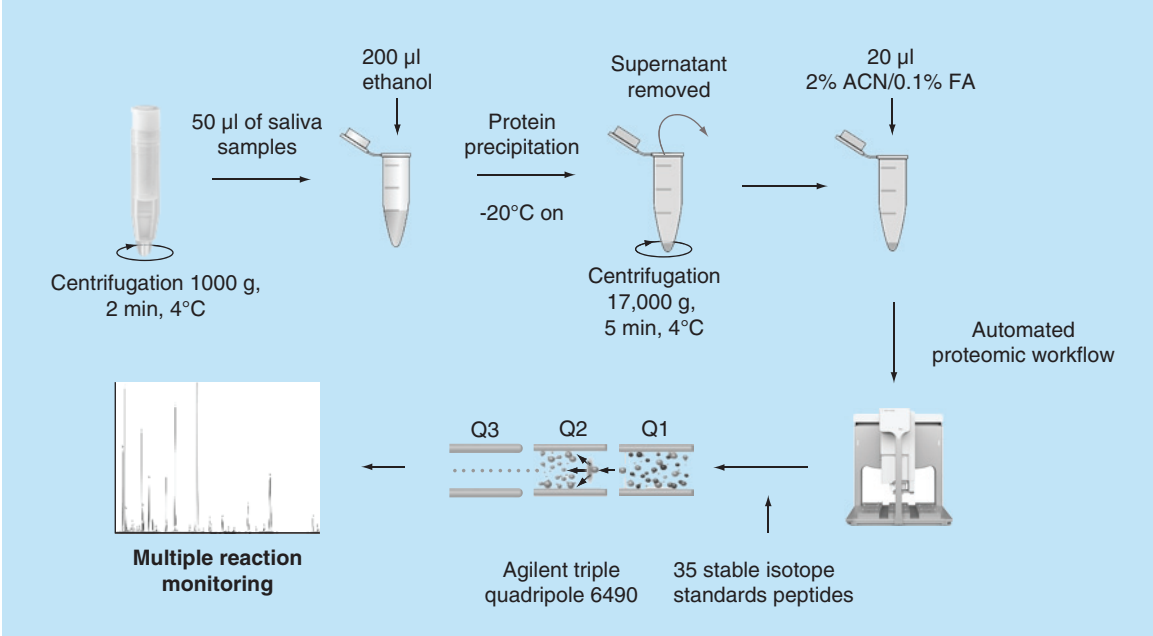



**Figure 1. Schematic diagram of MRM MS workflow for the quantification of 35 plasma biomarkers in human saliva.**

in response to parasympathetic stimulation than to sympathetic stimulation [19].

## Multiplexed MRM protein quantitation

The quantitation performed with MRM is based on the establishment of 35 calibration curves obtained for the 35 stable isotope-labeled standard (SIS) peptides in saliva matrix. This plays an indispensable role in interference testing while correcting for any sample losses incurred during analysis following its addition. As a control, we inspected the synthetic (SIS) and the endogenous (natural, NAT) peptides for their strict coelution and similar peak shapes. Moreover, we checked the relative intensities of the three selected transition for a given peptide that should remain constant. If relative intensities exhibit significant changes, it indicates the presence of an interfering chemical species. In this work, we considered that targeted peptides have passed the interference screening test if the coefficient of variance (CV) of the relative abundances of the three transitions per peptide was <25%. The MRM transitions of each of the 35 plasma proteins were therefore investigated in order to confirm their presence in the saliva tested. The observed discrepancies mainly rely on the diffusion of plasma protein through the buccal mucosa (Figure 2). For retention time, values for the 32 peptides had CV of less than 1.0% and average CV% was 0.47%, which indicates that the retention times for all 32 peptides are reproducible.

Results show that 32 of the 35 existing plasma proteins were quantified in the chewing stimulated human salivary pool, whereas only 12 proteins were quantifiable in the acid stimulated saliva pool, where the overall protein concentrations were significantly lower than in the chewing stimulated saliva. The significant differences observed between the effects of the two kinds of stimulation suggest that acidic swab stimulated saliva is not suitable for MRM quantitation purposes. Besides, ethanol precipitation of the salivary proteins seem to be the most suitable and simplest procedure for quantifying the maximum number of protein biomarkers when performing MRM.

In order to obtain an accurate MRM assay, the analytical variability (MRM in duplicate) and the total variability (sample preparation in quadriplicate) of each of the 35 peptides studied were determined. Analytical variability ranged from 0.32% in the case of the vitamin D binding protein to 55.43% in that of the fibrinogen-β chain. In the four sample preparations studied, the total variability ranged from 1.9% in the case of apolipoprotein A-I to 35% in that of the fibrinogen-β chain peptides. Only three of the 35 proteins studied showed a variability higher than 20%, and these were only present in very low concentrations. These results confirm that the procedure used to obtain the salivary samples was reproducible. Based on these results, 32 proteins out of the 35 studied were still quantifiable using MRM with an average analytical variability of 4.6% and a total variability of 8.5%. Apolipoprotein C-1, the fibrinogen-β chain and the fibrinogen-α chain could not be quantified because of the weakness of the corresponding MRM signals and the great analytical variability observed. MRM absolute quantitation was verified using individual saliva samples (n = 6) and showed similar results

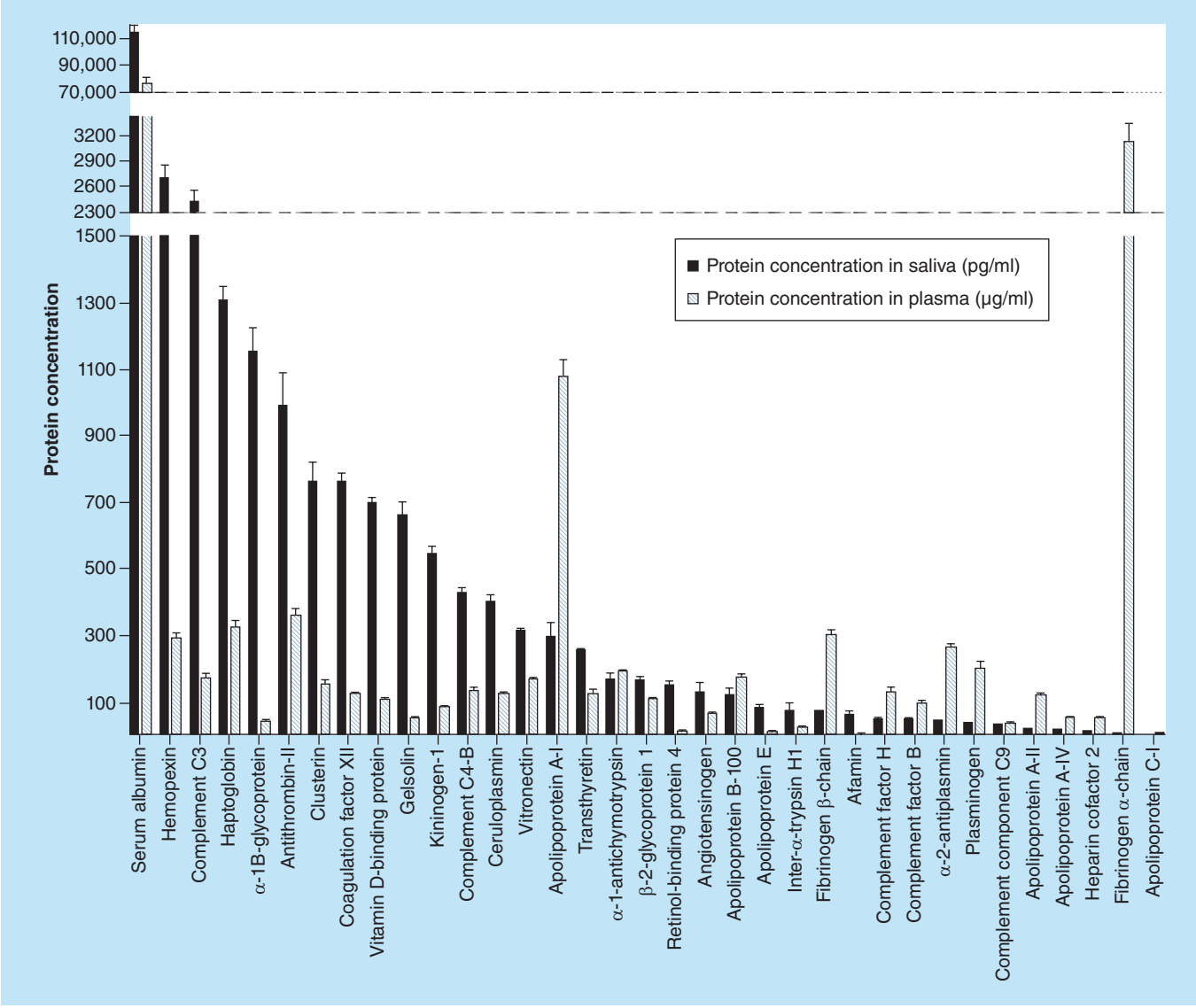



**Figure 2. Comparison between the concentrations of the 35 proteins quantified in neutral stimulated saliva and human plasma in descending order of abundance.**

(data not shown). Interindividual variations were calculated for the 32 plasma proteins ranging from 32.7% for the vitronectin to 103.1% for the apolipoprotein A-2. As expected, highly abundant plasma protein in salivary samples (Hemopexin, Albumin, complement C-3 or vitronectin) exhibited lower interindividual variation (from 32.7 to 37.9%) whereas lower abundant ones as apolipoprotein A-2 and A-4 reached 90.3 to 103.1% CV.

## Method validation

All 32 peptides selected were then individually tested by performing salivary quantification. Calibration curves were drawn up in duplicate by spiking two different salivary pools of SIS peptides (see examples in Figure 3). Each calibration curve was performed using seven concentrations corresponding to the SIS peptide studied. Analyses were performed in duplicate. The area obtained for each SIS peptide peak was then plotted versus the theoretical concentrations. Linear regression fitting was then performed, resulting in $R^2$ values ranging from 0.996 in the case of afamin protein to 0.929 in that of vitronectin. The detection limit of the 32 SIS peptides in saliva was computed based on a signal/noise ratio = 3. Interestingly, the linear dynamic range for the 32 peptides was greater than 2 log10, which shows that these peptides were promising targets for developing salivary clinical MRM assays.

## Plasma proteins quantified using MRM in human saliva

In this study, the 35 plasma proteins selected included several major plasma proteins such as albumin and

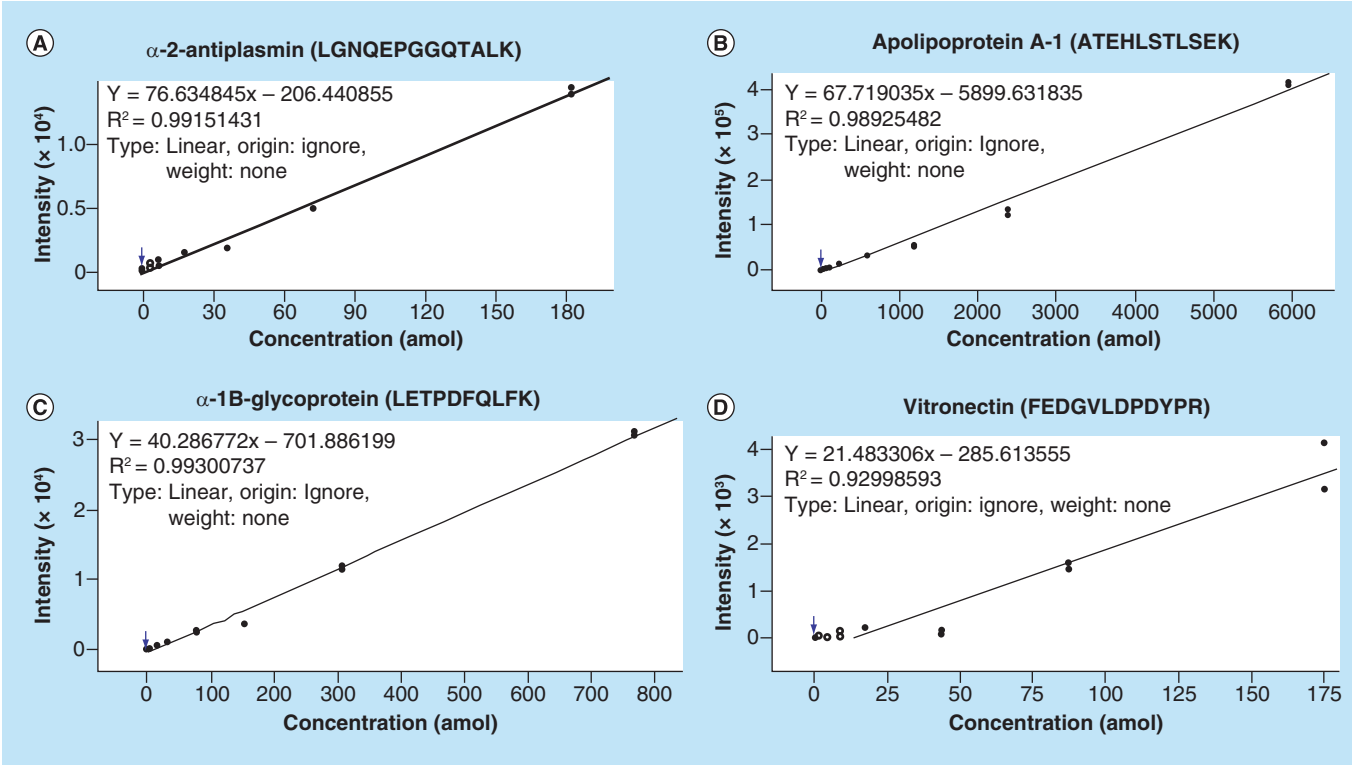


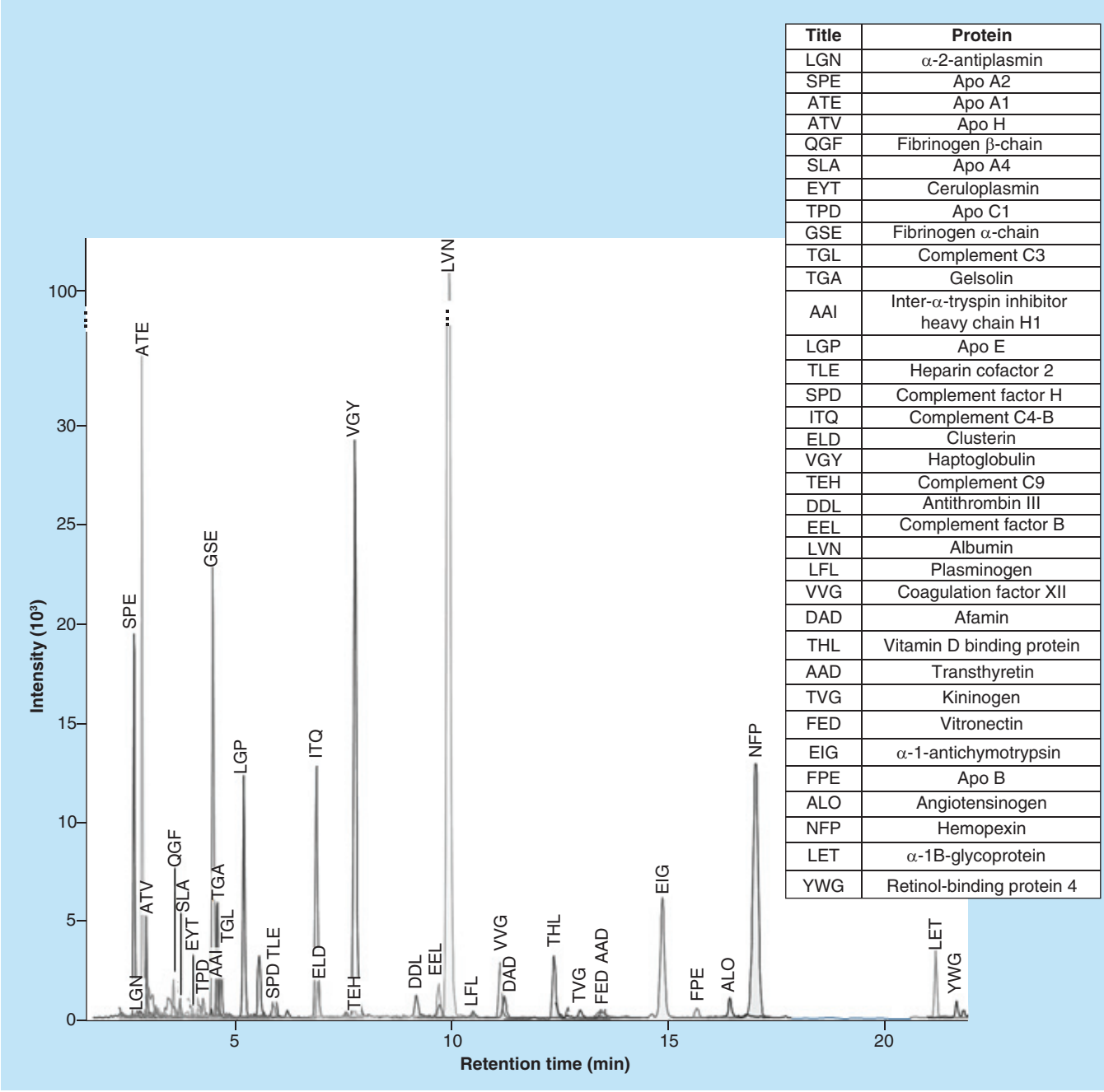

**Figure 3. Examples of calibration curves obtained in the case of (A) α-2-antiplasmin (group 1, oral cancer), (B) apolipoprotein A-1 (group 2, oral pathologies), (D) vitronectin (group 3, nonoral cancer) and (C) α-1B-glycoprotein (group 4, nonoral inflammatory diseases).**

transthyretin (Table 1), and 31 of the proteins have been proposed for use as plasma biomarkers for detecting specific pathologies. Based on classical top down proteomics, 16 of them have in fact been reported to be plasmatic or salivary biomarkers associated with specific oral pathologies such as oral cancer, Sjögren's syndrome or periodontitis. α-1B-glycoprotein, for example, is a putative biomarker of breast cancer in plasma [20] and of oral squamous cell carcinoma in saliva [21].

The proteins detected in our multiplex saliva assay can be divided into four groups. The first group consisted of proteins reported to be possible biomarkers of oral cancer: α-2-antiplasmin [22], complement component C9 [23], fibrinogen-α and -β chains [24], gelsolin [25], haptoglobin [21], hemopexin [21], retinol-binding protein 4 [26] and transthyretin [21]. The second group of proteins were associated with noncancerous oral pathologies/diseases: apolipoprotein A-I [27], β-2-glycoprotein 1 [28], ceruloplasmin [29] and serum albumin [30]. Apolipoprotein A-I is a potential biomarker of periodontal disease in gingival crevicular fluid (plasma exudate), whereas ceruloplasmin may be a biomarker of chronic disseminated periodontitis in saliva. Serum albumin, which is the main protein present in plasma and was the most highly concentrated protein in the present salivary panel, has been reported to be a good biomarker of gingivitis and periodontitis [31].

The third group of proteins identified here consisted of nonoral cancer blood biomarkers: gastric cancer in the case of inter-α-trypsin inhibitor heavy chain H1 [32], colorectal cancer in that of kininogen-1 [33], lung cancer in that of vitamin D binding protein [34] and breast cancer in that of vitronectin [35]. The fourth and last group consisted of proteins associated with inflammatory (α-1B-glycoprotein), immunologic (complement C3, complement C4-B), cardiovascular (apolipoprotein A-I, apolipoprotein B-100) and neurological diseases (apolipoprotein E, clusterin) (Table 1).

Interestingly, the concentrations of the 35 proteins showed different patterns of distribution between blood and saliva. Albumin was found to be the most highly concentrated protein in both serum and saliva, but no fibrinogen-α chain was detected in saliva whereas it was highly concentrated in blood. The salivary concentrations of the other 33 proteins determined using MRM differed considerably from the blood concentrations, as shown in Figure 4. This may be attributable to the fact that most of the plasma proteins enter the saliva via the tight junctions of the oral mucosal epithelium, or via transcellular (passive intracellular diffusion and active transport) or paracellular (extracellular ultrafiltration) routes, depending on their physicochemical properties (molecular weight, hydrophobicity) [1,36].




| Title | Protein |
|---|---|
| LGN | α-2-antiplasmin |
| SPE | Apo A2 |
| ATE | Apo A1 |
| ATV | Apo H |
| QGF | Fibrinogen β-chain |
| SLA | Apo A4 |
| EYT | Ceruloplasmin |
| TPD | Apo C1 |
| GSE | Fibrinogen α-chain |
| TGL | Complement C3 |
| TGA | Gelsolin |
| AAI | Inter-α-tryspin inhibitor heavy chain H1 |
| LGP | Apo E |
| TLE | Heparin cofactor 2 |
| SPD | Complement factor H |
| ITQ | Complement C4-B |
| ELD | Clusterin |
| VGY | Haptoglobulin |
| TEH | Complement C9 |
| DDL | Antithrombin III |
| EEL | Complement factor B |
| LVN | Albumin |
| LFL | Plasminogen |
| VVG | Coagulation factor XII |
| DAD | Afamin |
| THL | Vitamin D binding protein |
| AAD | Transthyretin |
| TVG | Kininogen |
| FED | Vitronectin |
| EIG | α-1-antichymotrypsin |
| FPE | Apo B |
| ALO | Angiotensinogen |
| NFP | Hemopexin |
| LET | α-1B-glycoprotein |
| YWG | Retinol-binding protein 4 |

**Figure 4. Extracted ion chromatogram corresponding to all 35 MRM assays on 35 salivary proteins in a single 30-min LC–MRM/MS run.**

## Conclusion

In this study, the feasibility and the potential value of targeted MS as a tool for performing the absolute quantification of a panel of protein biomarkers in human saliva were established for the first time.

It has been recognized for more than 20 years that saliva is a biological fluid of particular interest because it can be collected using noninvasive methods. It has many other advantages in comparison with blood: it is easier to handle during diagnostic procedures, safer for operators and can be sampled without inducing any stress in the patients. Although saliva is not yet being widely used in clinical practice for the surveillance of diseases, mainly because of the lack of clinical data, it is certainly a highly desirable goal in the field of healthcare.

The results obtained here show that a targeted MS approach can yield a fast, sensitive, reproducible and multiplexed analysis of salivary biomarkers of interest for detecting oral pathologies (such as OSCC, peri-

odontitis and Sjögren's syndrome) and nonoral pathologies (such as cancer and inflammatory, metabolic and immunological diseases). This quantification approach could be used to confirm the relevance of these putative biomarkers in human saliva, especially for detecting oral cancer, which is a serious public health issue. Further large statistic studies would be necessary to confirm the potential clinical utility of saliva. In addition, albumin and transthyretin, which are thought to be blood biomarkers of nutritional status, could be monitored in saliva much more easily than in blood. The development of MRM platforms in clinical environments would improve the detection and follow-up of many diseases and possibly make it possible to monitor patient's general state of health over time. Since MRM technology is already available at many clinical laboratories, it could be used to perform new clinical tests on saliva samples in the immediate future and then re-engage the interest of saliva in the clinical use.

## Future perspective

Since many years, the use of human saliva represents a potential useful biological fluid for diagnosis. Saliva sampling is noninvasive, stress free and may be an alternative to blood sampling. Lately, thanks to high-throughput proteomics approaches, more than 2400 proteins have been identified in human saliva, demonstrating the great complexity of its composition. The use of quantitative MS method based on multiple reaction monitoring (MRM) to conduct research on human saliva samples represents the future of salivary research. Thanks to these new developments, quantitative proteomic workflow using human saliva in a clinical environment is highly applicable. Our approach is compatible with routine measurements in daily clinical practice and could be used to perform new clinical tests on saliva samples in the immediate future.

### Acknowledgements

The authors would like to specially thank Jessica Blanc for revising the manuscript.

### Financial & competing interests disclosure

This work was supported through the AOI SALIVALZ (AOI CHRU MONTPELLIER 2011). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.15.228

### Executive summary

- Use of human saliva for diagnosing disease is of great clinical potential.
- Development of a targeted MS (MRM-MS) approach for the quantitation of 35 plasma biomarkers using human saliva in a clinical environment.

**Methods**

- A 30-min micro-LC–MS/MS run in MRM mode was developed.
- Simple salivary sample preparation procedures.

**Results & discussion**

- Results obtained show that a targeted MS approach can yield a fast, sensitive, reproducible and multiplexed analysis of salivary biomarkers.
- MRM approach is compatible with routine measurements in daily clinical practice.

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