Absolute quantification of selenoproteins and selenometabolites in lung cancer human serum by column switching coupled to triple quadrupole inductively coupled plasma mass spectrometry

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Abstract

One of the most important causes of the high mortality rate and low life expentancy of lung cancer is the detection at advanced stages. Thus, there is an urgent need for early diagnosis and the search of new selective biomarkers. Selenium is an important constituent of selenoproteins and a powerful antioxidant able to protect against cancer. In this work, the absolute quantification of selenium in selenoproteins and the total content in selenometabolites has been performed for the first time in serum from lung cancer patients (LC) and healthy controls (HC). To this end, a method for the simultaneous speciation of selenoproteins using size exclusion chromatography (SEC) and affinity chromatography (AF) with detection by ICP-QQQ-MS, and quantification by isotopic dilution (IDA) (SEC-AF-HPLC-SUID-ICP-QQQ-MS) was developed to determine the selenium concentration in eGPx, SEPP1 and SeAlb, as well as total selenometabolites, to find alterations that may serve as biomarkers of this disease. In the same way, a method based on anion-exchange chromatography coupled to ICP-QQQ-MS was developed to quantify selenometabolites (SeCys2, SeMeSeCys, SeMet, selenite and selenate) in the same LC and HC serum samples. The results showed that the averaged concentrations of selenium in eGPx, SeAlb and selenite were significantly

higher in LC patients (LC (eGPx: 21.24 ± 0.77 ng g^{-1}; SeAlb: 49.56 ± 3.16 ng g^{-1} and Se(IV): 6.20 ± 1.22 ng g^{-1}) than in HC group (eGPx: 16.96 ± 0.53 ng g^{-1}; SeAlb: 38.33 ± 2.66 ng g^{-1} and Se(IV): 3.56 ± 0.55 ng g^{-1}). In adittion, the ratios between selenoproteins and selenometabolits have been calculated for the first to study their potential use as LC biomarkers. The rates eGPx/SEPP1, SEPP1/SeAlb, eGPx/Se(IV) and SEPP1/Se(IV) were significantly different between LC and HC groups.

Keywords:

Lung cancer, serum, isotopic dilution, selenoproteins, selenometabolites, selenocystine, selenium, ICP-QQQ-MS.

1. Introduction

Lung cancer (LC) is one of the ten most common causes of death causing 1.3 million deaths per year in the world [1,2]. Moreover, the 5-year survival period only comprises 15% due to the diagnosis at advanced stages [3–5], which highlight the need for an early detection to increase this rate [6–9]. On the other hand, selenium has been claimed as one of the most important essential elements in cancer research due to its chemopreventive role related to its antioxidant function via glutathione peroxidase selenoprotein (GPx). Previous works have affirmed an increase of GPx in tissues from LC patients to overcome the oxidative stress caused by free radicals [10]. On the other hand, selenium also plays essential roles inhibiting angiogenesis [11], improving the immune response [12], controlling the unnecessary proliferation of cells [13] and protecting the body against the toxic effect of heavy metals [14]. Several studies have shown the key role of selenium in cancer indicating that selenium supplements reduce the mortality associated to LC [15] and consequently, there is a decrease in the risk of LC in populations with a diet rich in selenium [16]. On the other hand, there is a very narrow limit between the essential and toxic dose for the organism. A study in cell cultures, evidenced that low concentrations of selenium are necessary for cell growth, while moderate and high concentrations inhibit it inducing apoptosis in malignant cells at concentrations that do not affect the viability of normal cells [17].

The active center of selenoproteins (with selenocysteine (SeCys incorporated by a specific codon, e.g. glutathione peroxidase (eGPx), selenoprotein P (SePP1)) and selenoenzymes is SeH-, but Se can also be present in selenium containing proteins (e.g. selenoalbumin (SeAlb) with selenomethionyl residues), inorganic selenium, selenoaminoacids and methylated selenium [18]. Some selenium species are

transformed into selenide, an intermediate metabolite in the synthesis of selenoproteins. The most abundant selenium specie in the bloodstream is SEPP1, which has been considered as a good indicator of Se status in humans. Moreover, SEPP1 also serve as transporter of selenium, and previous research suggested the protective role in human astrocytes and endothelial cells from oxidative damage [19]. eGPx is the most important selenoenzyme against oxidative stress acting as a catalyst for the reduction of lipid hydroperoxide and extracellular hydrogen peroxide [20]. A relationship has also been described between the altered expression of selenoproteins and cancer risk [21]. In this sense, an allelic variant of GPx1 gene, has been linked to a high risk of LC [22]. Also, several polymorphisms in the selenoprotein gene of 15 kDa (SELENOP15) have been related to increased susceptibility of several cancers [23] such as LC [24].

On the other hand, serum is the most useful biofluid for selenium determination since it responds quickly to changes in selenium status, which in turn correlated with dietary intake or physiological disorders. The concentration of selenium in human serum is about 90 ng g^{-1}, with the following relative abundance of the species: SePP1>SeAlb>eGPx>Na_2SeO_3 [25]. In spite of many authors have reported activities or concentrations of some selenoproteins in several cancers such as, prostate [26–28], renal [26], breast [29], colorectal [30] and laryngeal cancers [31], few studies are related to the determination of selenium in selenoproteins in LC patients [16]. On the other hand, the most commonly used analytical techniques for the determination of selenoproteins concentrations in cancer patients have been based on immunoluminometric sandwich assay [26,27], radioinmmunoassay [29], colorimetric enzyme-limled immonoassay [30] or Enzyme-Linked ImmunoSorbent Assay (ELISA) [16]. Likewise, SEPP1 has been determined in 372 lung cancer patients using the ELISA method demonstrating that there is a decrease in the risk of lung cancer in populations with high levels [16]. However, one of the most powerful approaches for selenium determination is inductively coupled plasma mass spectrometry (ICP-MS), which allows the sensitive and precise absolute quantification of selenoproteins and selenometabolites by heteroatom tagged proteomics. In this sense, selenoproteins and selenometabolites have been previously determined by coupling size exclusion (SEC) and affinity chromatography (AF) to ICP-MS in human serum of healthy people [32,33] as well as using isotopically enriched seleno-peptides and high performance liquid chromatography (HPLC) coupled to ICP-MS [34]. In addition, previous work based on the simultaneous quatification of SEPP1, eGPx, SeAlb and the inorganic species selenite and selenate, in serum from human and mice using species-unspecific isotope dilution (SUID) and ICP-MS have also been described [35] .

However, triple quadrupole ICP-MS (ICP-QQQ-MS) has never been previously applied for the absolute quantification of selenoproteins and selenometabolites in LC patients. The main advantage of using ICP-QQQ-MS is selectivity obtained by the efficient elimination of interferences in comparison with either standard single quadrupole (Q) mode or ICP-MS with collision/reaction cell. This is very important for the measurement of selenium in biological samples. Likewise, the signal of ^{80}Se in oxygen mode ($^{96}SeO^{+}$) would overlap with signals of $^{96}Ru^{+}$, $^{96}Zr^{+}$, $^{96}Mo^{+}$ in a single quadrupole ICP-MS, but it is overcame by ICP-QQQ-MS because these interferences are rejected in the first quadrupole.

On the other hand, inorganic and organic low molecular mass molecules of selenium can have important implications to human health including anticancer properties [36]. Organic selenium compounds, mainly selenoaminoacids, are usually less toxic than inorganic forms. In addition, selenium toxicity depends on multiples factors and the dose and the compound forms are very critical parameters [37]. However, some works have reported the use of inorganic selenium compounds in cancer therapy [36].Dietary supplements of selenium are based mainly in inorganic forms such as sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), selenide (Se^{-2}) and Se^0. Although, some multivitamins can also include organic species of selenium including selenomethionine (SeMet), selenocysteine (SeCys), methyl-selenocysteine (MeSeCys) and selenized yeast (Se-yeast) rich in SeMet. [38]. On the other hand, determinations of SeMet, selenomethylselenocysteine (SeMeSeCys), selenocystine ($SeCys_2$), selenite and selenate in human serum have been described in literature using different chromatographic methods such as anion-exchange chromatography or reverse phase chromatography [39], but few studies have reported concentrations of selenometabolites in serum from LC patients, although most of them have studied the selenium supplementation related to cancer prevention and therapy [36].

In this work, the concentration of selenium as selenoproteins (eGPx, SELENOP, SeAlb) including the total content of selenometabolites have been determined in serum from lung cancer (n=48) and healthy controls (n=39) using a column switching system based on two size exclusion chromatography columns (SEC) and two affinity columns (AF). The absolute quantification of selenoproteins and selenometabolites was carried out using species-unspecific isotopic dilution analysis (HPLC-SUID-ICP-QQQ-MS). In addition, an anion-exchange chromatography (AEC) has been optimized to determine and quantified the amount of selenium as low molecular mass selenometabolites such as SeMet, selenomethylselenocysteine (SeMeSeCys), selenocystine ($SeCys_2$), Na_2SeO_3 and Na_2SeO_4.

2. Materials and methods.

2.1. Study population.

Blood samples were collected from lung cancer patients (LC) and healthy people (HC) at the Pneumology Area of Juan Ramón Jiménez Hospital (Huelva, Spain). The blood samples were obtained by venipuncture of the antecubital region, after 8 hours of fasting, and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. Immediately, samples were cooled and protected from light for retracting the clot during 30 minutes. After centrifugation (2000 *g* for 10 minutes) serum samples were frozen at -80°C until analysis. A total of 87 serum samples (48 from LC and 39 from HC) were collected for the analysis. The study was performed in accordance to the principles contained in the Declaration of Helsinki and approved by the Ethical Committee from Juan Ramón Jiménez Hospital and University of Huelva. In addition, all people gave informed consent for the extraction of peripheral venous blood. Table 1 shows clinical characteristics of patients (LC) and healthy controls (HC).

Table 1. Clinical features of lung cancer patients (LC) and healthy controls (HC).

Characteristics	LC	HC
Number of samples	n=48	n=39
Age (years)	64 ± 10	58 ± 13
Sex (M/W)	38/10	18/21
Histology		
NSCLC	42	-
SCLC	6	-
Smoking habits		
Smokers	12	0
Ex-smokers	31	23
Non smokers	5	16
Comorbidities		
AHT (%)	58	49
Asthma (%)	5	0
DM (%)	23	27

NSCLC: Non-small cell lung cancer, SCLC: Small cell lung cancer, M: Men, W: Women, LC: Lung Cancer, AHT: Arterial Hypertension and DM: Diabetes Mellitus.

2.2. Standard solutions and reagents

Human serum certified reference material (CRM) BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Enriched ^{74}Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK). Sodium selenate, sodium selenite, selenomethionine, selenocystine and selenomethylselenocysteine standards were purchased from Sigma Aldrich, (Steinheim, Germany). Ammonium

acetate and citric acid were used to prepare the mobile phases for SEC-AF-SUID-ICP-QQQ-MS and AEC-SUID-ICP-QQQ-MS respectively. Both reagents were purchased from Sigma Aldrich, (Steinheim, Germany). Methanol optima LC/MS grade was supplied by Fisher Scientific (Loughborough, Leics. U.K).

2.3. Instrumentation

The absolute quantification of selenoproteins, selenometabolites and the total content of selenium were performed by inductively coupled plasma mass spectrometry equipped with a triple quadrupole using the Agilent 8800 Triple Quad (Agilent Technologies, Tokyo, Japan), with oxygen of high-purity grade (>99.999%), and pure hydrogen gas (>95%) as reaction gases. Instrumental conditions were optimized using a Tuning aqueous solution containing Li, Co, Y and Tl at 1 μg L^{-1}. Nickel sampling and skimmer cones were employed, with a sampling depth of 10 mm. The forward power was set at 1550 W, and the gas flow rates were fixed at 15 L min^{-1} and 1.08 L min^{-1} for plasma and carrier gas, respectively. Selenium was determined using a mixture of H_2 (2 ml min^{-1}) and O_2 (40%). Isotopes monitored were ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se y ^{80}Se with a dwell time of 0.3 s per isotope. Chromatographic selenoproteins separation was performed using an HPLC model 1260 Infinity Quaternary LC (Agilent Technologies) using two 5 ml HiTrap ® Desalting Columns (GE Healthcare, Uppsala, Sweden) connected to two affinity columns, with stationary phases of heparin-sepharose (HEP-HP) and blue-sepharose (BLU-HP), both purchased from GE Healthcare, Uppsala, Sweden. The separation of low molecular mass molecules of selenium was carried out with an anion-exchange column Hamilton PRP-X100, 10 μm, I.D. × L 4.1 mm × 250 mm using the same model of HPLC (HPLC 1260 Infinity Quaternary LC (Agilent Technologies).

2.4. Total selenium determination

Total selenium content of serum was determined in diluted samples as previously described [40]. To this end, serum was five-fold diluted with ultrapure water and 0.1 μg ml^{-1} of Rh was added as internal standard. Samples were filtered using 0.45 μm pore size filters of PTFE before the analysis by ICP-QQQ-MS.

2.5. Selenoproteins speciation by size exclusion-affinity chromatography hyphenated to species-unspecific isotope dilution ICP-QQQ-MS (SEC-AF-SUID-ICP-QQQ-MS).

The absolute quantification of selenoproteins has been performed by a previously optimized method SEC-AF-HPLC-SUID using a single quadrupole ICP-MS [41], although in this work we have used a triple quadrupole ICP-QQQ-MS as a detector. The

separation of analytes was carried out by the on-line coupling of two size exclusion (HiTrap® Desalting) connected to two affinity columns by means of a six-way switching column valve. The stationary phases of the AF columns were heparine-sepharose (HEP-HP) and blue-sepharose (BLU-HP). SEPP1 has affinity for heparine-sepharose stationary phase and blue-sepharose, while SeAlb has affinity only for blue-sepharose [27]. The combination of two HiTrap Desalting columns increases the resolution of the chromatographic separation and allows separating the peak of eGPx from selenometabolites, which are not retained in the AF columns. The chromatographic arrangement based on SEC-AF-HPLC was connected to the nebulizer of ICP-QQQ-MS using a T piece where post-column ^{74}Se is also introduced to carry out the dilution analysis. Samples were centrifugated to remove suspended particles and 100 µl of serum were injected into the system SEC-AF-ICP-QQQ-MS directly. Mobile phase A (0.05 M of ammonium acetate, pH:7.4) and mobile phase B (1.5 M of ammonium acetate, pH:7.4) were used for the chromatographic analysis at a flow rate of 1.3 ml min^{-1}. The acquisition time was adjusted to 40 minutes.

In a first step (Position A), mobile phase A is pumped through the system during 12 minutes eluting eGPx and selenometabolites, which are not retained in the affinity columns, at the time of 3 and 5 minutes respectively. Subsequently, the gradient change to mobile phase B for the elution of SEPP1 at 20 minutes and in this moment, the valve is switch to Position B to isolate the BLUE-HP column, which retains SeAlb. Immediately, the valve is switched on to Position A for the elution of SeAlb, which elutes at 25 minutes with mobile phase B. Finally, mobile phase A is passed through the system for equilibration during 15 minutes before the next injection. Figure 1 illustrates the instrumentation and both positions used in the analysis. A human serum reference material certified in total selenium (BCR-637) was used to validate the methodology.

2.6. Speciation of low molecular mass molecules of selenium by anion-exchange hyphenated to species-unspecific isotope dilution ICP-QQQ-MS (AEC-SUID-ICP-QQQ-MS).

Specific selenometabolites were determined and quantified by AEC-SUID-ICP-QQQ-MS. Samples were ultra-filtered using filters of 10 kDa (Amicon Ultra 0.5 ml Centrifugal Filters, Ultracel 10K, Merck Millipore, IRL) to remove the proteins, and the filtrate was placed in 100 µl inserts for the chromatographic analysis. The analytical procedure was based on the methodology used by Cuderman et al [42], with several modifications. In brief, two mobile phases of different concentration of citric acid were used for the anion-

exchange chromatography. Mobile phase A contained 3 mM of citric acid with 2 % of MeOH, and mobile phase B contained 10 mM of citric acid with 2 % of MeOH were used for this purpose. The pH of both mobile phases was adjusted to 4.8 with ammonium hydroxide. The chromatographic method starts from 100% A to 25% A during 7 minutes, 6 minutes in constant gradient at 25 % A, 1 minutes from 25% A to 100% B, 8 minutes in constant gradient at 100 % B and 3 minutes from 100%B to 100% A to recondition the system. The acquisition time lasts a total of 25 minutes. The flow rate was set at 0.5 ml min^{-1}. Figure 2 shows the characteristic chromatogram for SeMet, SeMeSeCys, $SeCys_2$, selenite and selenate with the operational conditions applied. Elution order was SeCys2 (4.45 min), SeMeSeCys (5.67min), Na_2SeO_3 (8.11 min), SeMet (9.44 min) and Na_2SeO_4 (17.42 min). Repeatability studies were carried out to validate the methodology.

2.7. Quantification of selenium in selenoproteins and selenometabolites by SUID-IDA.

The intensity of selenium isotopes were converted to mass flow chromatograms for the quantification of selenium species in serum samples, using mathematical equation of isotopic dilution and the ratio $^{78}Se/^{74}Se$. Figure 3 show the mass flow chromatogram of selenoproteins (a) and selenometabolites (b) corresponding to a serum sample from a LC patient. Finally, the resulting areas from mass flow chromatograms were integrated using Origin 8.0, OriginLab, Corporation software. The final concentrations were calculated dividing the areas between the amounts of sample introduced (0.1 g).

2.8. Statistical Analysis.

Statistical calculations were made in STATISTICA 8.0 software (StatSoft, Tulsa, USA). Parametric methods were used since most of the variables showed a skewed distribution (checked by normal probability plots) and variances were not homogeneous (checked by Levene´s test). One-way ANOVA followed by Tuckey test was carried out for pairwise comparisons to find differences between groups. Only p values below 0.05 were regarded as statistically significant. Finally, to evaluate the specificity and sensitivity of metabolites altered by the disease, ROC (receiver operator characteristic) curves were applied to the dataset and species with “area under the curve” (AUC) higher than 0.75 were considered as relevant in the progression of LC.

3. Results and discussion.

3.1. Validation of SEC-AF-SUID-ICP-QQQ-MS and AEC-SUID-ICP-QQQ-MS methodologies.

The speciation methodology has been validated using a CRM of human serum (BCR-637) certified for total Se content (81 ± 7 ng ml^{-1}). In addition, this CRM material was spiked with SeMet to test the recovery as well as the resolution of IDA-SEC-AF-ICP-QQQ-MS for selenometabolites and eGPx, which usually co-elutes with other methods using only one SEC column [43]. On the other hand, the BCR-637 certified material was also fortified with a mixture of selenometabolites (10 ng Se g^{-1}, each), namely: SeMet, SeMeSeCys, $SeCys_2$, selenite and selenate in order to evaluate the chromatographic resolution by IDA-SEC-AF-ICP-QQQ-MS. Since, no efficient separation of these species was attained (Figure 4A), the approach AEC-ICP-QQQ-MS was used for selenometabolites (Figure 4B).

Parameters of quality, such as method repeatability, reproducibility and accuracy were determined for both methods. Precision was evaluated by determining the repeatability (extracting and analyzing 10 different aliquots of the reference material in the same day) and reproducibility (extracting and analyzing 3 different aliquots of the reference material in three different days, Table S1, Supplemetary Material). To this end, aliquots of serum certified reference material were fortified with 10 and 50 ng of Se of each selenometabolite (SeMet, SeMeSeCys, $SeCys_2$, selenite and selenate) per g of serum for SEC-AF-ICP-QQQ-MS and AEC-ICP-QQQ-MS, respectively. On the other hand, a serum sample was chosen randomly to assess the precision of both methods. Figure 4 illustrates the typical chromatograms obtained for selenoproteins (Fig.4A) and selenometabolites (Fig4B) after the analysis of a spiked serum sample using both approaches. Table 2 shows the repeatability in terms of relative standard deviation for selenoproteins and selenometabolites including the detection limits of both SEC-AF-SUID-ICP-QQQ-MS and AEC-SUID-ICP-QQQ-MS methodologies.

	Human Serum BCR-637 (81 ±7 ng ml^{-1}) + 10 ng of Se of each selenometabolites per g of serum n=10		Human Serum BCR-637 spiked with a mixture of selenometabolites (50 ng of Se of each selenometabolites per g of serum) n=10		Random Serum Sample (ng g^{-1} of Se) n=10		LOD (ng Se g^{-1})
Selenium species	Concentration (ng g^{-1})	%RSD	Concentration (ng g^{-1})	%RSD	Concentration (ng g^{-1})	%RSD	
SEC-AF-SUID-ICP-QQQ-MS							
eGPx	12	3	-	-	6	2	0.2
Se-MTB	45	4	-	-	2	0.5	0.1
SEPP1	61	7	-	-	68	5	0.4
SeAlb	14	3	-	-	21	4	0.4

Total Se*	105	9	-	-	97	6	0.3
AEC-SUID-ICP-QQQ-MS							
SeMet	-	-	45	2	<LOD	-	0.1
SeMeSeCys	-	-	52	2	<LOD	-	0.1
$SeCys_2$	-	-	54	3	0.8	0.3	0.1
Na_2SO_3	-	-	46	4	0.4	0.1	0.1
Na_2SO_4	-	-	48	3	0.3	0.1	0.1

Table 2. Repeatability in terms of %RSD. Detection limits of selenium species. Se-MTB: selenometabolites, LOD: limit of detection. *Total content of selenium determined by SUID-ICP-QQQ-MS.

The concentration of different selenium species present in BCR-637 are not certified (only total selenium), but they are in good agreement with other results values reported in the literature [25,43]. On the other hand, accuracy of the method SEC-AF-SUID-ICP-QQQ-MS, determined in the spiked sample ((spiked-determined/spiked)x100), was close to 10 %, (n=10). In the same way, accuracy of selenometabolites (SeMet, SeMeSeCys, $SeCys_2$, selenite and selenate) ranged from 4 to 10 % (n=10) with AEC-SUID-ICP-QQQ-MS. The repeatability associated to % RSD is in the range of 3-9 % for selenoproteins and 2-3 % for selenometabolites. The random serum sample showed %RSD values from 0.5-6 % for selenoproteins and 0.1-0.3% for selenometabolites. Finally, low LODs (Table 2) were determined for selenoproteins and selenometabolites by both SEC-AF-SUID-ICP-QQQ-MS and AEC-SUID-ICP-QQQ-MS methodologies being in the range of 0.1-0.4 ng g^{-1}.

3.2. Absolute quantification of selenoproteins by SEC-AF-SUID-ICP-QQQ-MS.

The isotopic dilution equation was used to calculate the concentration of selenoproteins in the BCR-637 and serum samples from LC and HC patients. The analysis of LC and HC serum samples reveals that SEPP1 accounts for the highest content of selenium in both studied groups, followed by SeAlb, eGPx and finally, selenometabolites. In addition, the percentage of SEPP1 was lower in LC (50%) than in HC group (55%), while the percentage of SeAlb against the total was higher in serum from LC (27%) than in HC group (23%). The content of eGPx (LC: 15%, HC: 16%) and total content of selenometabolites (LC and HC: 7%) were very similar in both groups. Figure 5 illustrates the percentages of each selenoprotein against the total sum of species. Table 3 shows the concentration of selenoproteins in the serum of HC and LC patients. The sum of selenocompounds was higher in LC patients (166.00 ± 7.36 ng g^{-1}) than the HC group (144.47 ± 6.17 ng g^{-1}). Altered activities and expressions of some selenoproteins including SEPP1 have been reported in LC studies suggesting an association to cancer

risk. However, the absolute quantification of selenium content and selenoproteins in this disease are rarely described [44]. In our study, SEPP1 was the protein with highest selenium concentration in both HC (76.74 ± 3.72 ng g^{-1}) and LC (82.08 ± 4.41 ng g^{-1}) serum samples, increasing in the cancer group, although one-way ANOVA test did not show significant differences. Takata et al [45] concluded that plasma SEPP1 concentration was positively associated with lung adenocarcinoma risk, although they only found a non-significant and slightly lower SEPP1 concentration in LC people compared to HC by ELISA. On the contrary, eGPx and SeAlb showed significant differences in selenium concentrations ($p< 0.05$) from LC (eGPx: 21.24 ± 0.77 ng g^{-1}; SeAlb: 49.56 ± 3.16 ng g^{-1}) and HC (eGPx: 16.96 ± 0.53 ng g^{-1}; SeAlb: 38.33 ± 2.66 ng g^{-1}) being also higher in serum from LC patients (Table 3) presenting fold changes (LC/HC) of 1.25 for eGPx and 1.30 for SeAlb. In-Jae et al [46] reported significant and lower GPx3 levels in serum from LC patients compared to a control group, increasing in patients with metastasis. However, the role of GPx3 in carcinogenesis is not clearly elucidated. On the other hand, no previous SeAlb levels have been reported in serum from LC studies in literature, although studies in colorectal cancer have described increase levels of SeAlb in the cancer group [47]. Finally, selenometabolites content was the lowest concentration of selenium in both, LC and HC serum samples. Although there were not significant differences between groups, the total concentration of selenium in selenometabolites from LC serum (10.71 ± 0.13 ng g^{-1}) was slightly higher in HC group. Areas under the curve (AUC) of the receiver operating characteristic curves (ROC) are also showed in Table 3. AUC values close to 0.75 are related to good biomarker for clinical purposes [48]. The AUC value of SeAlb (AUC= 0.67) was higher than other selenoproteins (eGPx (AUC= 0.64), SEPP1 (AUC= 0.55)), semetabolites (AUC= 0.63), and the total sum of species (AUC= 0.63)) being close to the good value AUC=0.7.

Concentrations (ng of Se g^{-1})	**eGPx**	**Se-metabolites**	**SEPP1**	**SeAlb**	**TOTAL Sum**
HC group					
Average	16.96	10.71	76.74	38.33	144.74
SEM	0.53	0.13	3.72	2.66	6.17
MAX	27.60	15.37	116.20	80.45	186.74
MIN	2.60	0.44	17.00	5.64	26.29
LC group					
Average	21.24	13.12	82.08	49.56	166.00
SEM LC	0.77	0.17	4.41	3.16	7.36

MAX LC	31.30	15.75	124.90	84.35	196.97
MIN LC	2.85	0.46	17.95	5.45	26.71
FC (LC/HC)	1.25	1.22	1.07	1.30	1.17
p value	0.03	N.S.	N.S	0.02	N.S
AUC	0.64	0.63	0.55	0.67	0.63

Table 3. Averaged concentrations of selenium in form of selenoproteins in serum from HC and LC groups including SEM (standard error of mean), MAX (maximum) and MIN (minimum) values. FC: Fold change, p-value (statistical pairwise comparisons by one way ANOVA test) and AUC values of ROC curves. LOD: Limit of detection. N.S.: Non-Significant.

3.3. Absolute quantification of selenometabolites by AEC-SUID-ICP-QQQ-MS.

Generally, the most abundant selenometabolite detected by AEC-SUID-ICP-QQQ-MS in serum samples from LC and HC groups was selenite followed by selenate and $SeCys_2$. The selenometabolites SeMet and SeMeSeCys were not detected in serum. The distribution of selenium of these selenometabolites can be observed in Figure 5 where the percentages of selenite and selenate were higher (44% and 36% respectively) in LC patients than the percentages in HC group (39% and 34% respectively). On the contrary, only a 20% of selenium in form of $SeCys_2$ was presented in LC serum, while the selenium content of this selenometabolite in HC group reached 27 % versus the total. Table 4 shows the selenium concentration of selenometabolites previously commented.

Concentration (ng of Se g^{-1})	SeCys2	SeMeSeCys	Se (IV)	SeMet	Se (VI)	TOTAL Sum
			HC group			
Average	2.49	<LOD	3.56	<LOD	3.15	9.20
SEM	0.24	<LOD	0.55	<LOD	0.53	1.01
MAX	3.68	<LOD	7.70	<LOD	4.30	15.68
MIN	0.00	<LOD	0.00	<LOD	0.00	0.00
			LC group			
Average	2.76	<LOD	6.20	<LOD	5.16	14.12
SEM	0.25	<LOD	1.22	<LOD	1.15	2.07
MAX	3.12	<LOD	7.60	<LOD	6.54	17.26
MIN	1.12	<LOD	<LOD	<LOD	<LOD	2.09
FC(LC/HC)	1.11	-	1.74	-	1.64	1.53
p-value	N.S	-	0.003	-	N.S	N.S
AUC	0.53	-	0.62	-	0.59	0.61

Table 4. Average concentrations of selenium in form of $SeCys_2$, SeMeSeCys, Se(IV), SeMet and Se(VI) in serum from HC and LC groups including SEM (standard error of mean), MAX (maximum) and MIN (minimum) values. FC: Fold change, p-value (statistical pairwise comparisons by one way ANOVA test) and AUC values of ROC curves. LOD: Limit of detection. N.S.: Non-Significant.

Selenite concentrations were significantly higher (p=0.003) in serum from LC patients (6.20 ± 1.22 ng g^{-1}) than the HC group (3.56 ± 0.55 ng g^{-1}) showing the higher AUC value in ROC curves (AUC=0.62) against other selenometabolites (AUC(SeCys2)=0.53 and AUC(Se(VI))=0.59). We also found increased, but non-significant levels of selenium in form of $SeCys_2$ and selenate in the LC group (Table 3). It is well known that selenite and selenate are usually used by the liver for the synthesis of selenoproteins and the excretion of methylated metabolite, being their body absorption quite different for both selenocompounds [49]. There are not many antecedents about levels of selenite and selenate in serum from LC patients, although some studies have described the use of sodium selenite and sodium selenate as drugs in cancer treatment and chemopreventive agents [50–52]. On the other hand, the presence of $SeCys_2$ instead of SeCys in serum is due to the high reactivity of SeCys, which could damage cells, and consecuently, cells store selenium in the less reactive oxidized form, $SeCys_2$ [53]. In our study, selenium levels in form of $SeCys_2$ were similar in both groups (LC: 2.76 ± 0.25 ng g^{-1}, HC: 2.49 ± 0.24 ng g^{-1}). In addition, no significant differences were found between LC and HC samples. As well as selenite and selenate, $SeCys_2$ has been studied for chemotherapeutic purposes. Fan et al [54] demonstrated that SeCys2 could enhance auranofin (AF)-induced A549 human lung adenocarcinoma cell apoptosis in vitro and in vivo through synergetic inhibition of selenoprotein thioredoxine reductasa 1 (TrxR1). Moreover, the citotoxicity of this selenometabolite has been studied for several tumors including lung breast, cervical, liver and melanoma cancers [55].

3.4. Total selenium in serum from LC and HC patients and mass balance.

The concentrations of total selenium in serum from LC and HC samples were determined by ICP-QQQ-MS with the conditions described previously. The selenium content in serum from LC patients (157.91 ± 5.48 ng g^{-1}) was higher than the concentration in serum from HC group (130.48 ± 6.07 ng g^{-1}). These concentrations were compared to the sum of selenium species determined in the speciation analysis by SEC-AF-SUID-ICP-QQQ-MS showed a relative standard deviation lower than 8 % in both LC and HC groups. On the other hand, the mass deviation between the total content of selenium in

selenometabolites determined by SEC-AF-SUID-ICP-QQQ-MS, and the sum of selenometabolites determined by AEC-SUID-ICP-QQQ-MS were lower than 11 %.

3.5. Inter-selenoproteins and selenometabolites ratios

It has been reported that selenoproteins are interrelated, because Se bound to albumin (SeAlb) is assumed to be transported to the liver for new synthesis of SEPP1 and eGPx that are then released into the bloodstream [25]**.** On the other hand, as previously reported, the ratios between several elements have resulted to be important biomarkers of lung cancer, which reflect the existence of an interconnected homeostasis in lung cancer [56]. In this case, the ratios between selenoproteins and selenometabolites were calculated to determine their relationships and their potential use as biomarkers. Table 4 reported the average of these ratios for HC and LC groups.

Ratios	HC Group		LC Group		FC (LC/HC)	p_ANOVA	AUC
	Average	SEM	Average	SEM			
eGPx/SEPP1	0.14	0.01	0.16	0.01	1.16	0.04	0.72
eGPx/SeAlb	0.39	0.04	0.33	0.02	0.84	N.S.	0.60
SEPP1/SeAlb	3.04	0.36	2.07	0.12	0.68	0.04	0.69
eGPx/SeCys2	13.67	1.43	17.19	1.91	1.26	N.S.	0.58
eGPx/Se(IV)	8.86	0.68	5.82	0.84	0.66	0.03	0.65
eGPx/Se(VI)	11.44	1.46	9.78	1.63	0.86	N.S.	0.50
SEPP1/SeCys2	59.43	5.80	63.04	7.79	1.06	N.S.	0.56
SEPP1/Se(IV)	33.62	2.91	21.44	3.03	0.64	0.01	0.65
SEPP1/Se(VI)	34.73	3.62	31.83	4.56	0.92	N.S.	0.55
SeAlb/SeCys2	24.00	2.98	30.46	3.55	1.27	N.S.	0.63
SeAlb/Se(IV)	14.40	1.63	9.68	1.50	0.67	N.S.	0.58
SeAlb/Se(VI)	24.13	6.43	21.03	4.68	0.87	N.S.	0.59
SeCys2/Se(IV)	0.61	0.08	0.37	0.05	0.62	N.S.	0.55
SeCys2/Se(VI)	1.28	0.29	1.01	0.35	0.79	N.S.	0.52
Se(IV)/Se(VI)	1.83	0.28	1.38	0.21	0.75	N.S.	0.51

Table 4. Ratios between selenoproteins and selenometabolites in HC and LC groups. SEM: Standard error of mean, FC: Fold change, p-value (statistical pairwise comparisons by one way ANOVA test) and AUC values of ROC curves. N.S.: Non-Significant.

The ratio eGPx/SEPP1 was significant different between LC and HC groups ($p<0.05$) increasing in cancer patients (LC: 0.16 ± 0.01, HC: 0.14 ± 0.01, FC=1.16). In addition, eGPx/SEPP1 was the rate with the high AUC value compared to the rest ratios (AUC=0.72) suggesting to be a good value for clinical diagnosis [48]. Moreover, SEPP1/SeAlb ratio also showed significant differences between groups ($p<0.05$) being lower in LC group (LC: 2.07 ± 0.12, HC: 3.04 ± 0.36, FC=0.68) and it presented an AUC

value closed to 0.7 (AUC=0.69). In the same way, eGPx/Se(IV) and SEPP1/Se(IV) were the only significant ratios between selenoproteins and selenometabolites, although the ROC curves analysis did not show values higher than 0.7 (Table 4).

4. Conclusions.

In this work, the absolute quantification of selenium in selenoproteins by species-unspecific isotopic dilution analysis (SUID) has been performed for the first time in serum from lung cancer and healthy controls using a column switching system based on size exclusion chromatography (SEC) and affinity chromatography (AF) with detection by ICP-QQQ-MS. Likewise, an anion-exchange chromatography coupled to ICP-QQQ-MS has been optimized for the determination of selenometabolites SeCys2, SeMeSeCys, SeMet, selenite and selenate in the same LC and HC serum samples. The analysis by SEC-AF-SUID-ICP-QQQ-MS reveals that SEPP1 accounts for the highest content of selenium in both LC and HC serum samples, followed by SeAlb, eGPx and finally, selenometabolites. In the same way, selenite represented the most abundant selenometabolite in serum followed by selenate and $SeCys_2$. SeMet and SeMeSeCys were not detected in serum. The selenium species eGPx, SeAlb, and selenite showed significant differences between LC and HC groups like the ratios eGPx/SEPP1, SEPP1/SeAlb, eGPx/Se(IV) and SEPP1/Se(IV), showing the interconnected homeostasis between selenoproteins and selenometabolites.

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Abreviations.

AEC	Anion exchange chromatography
AF	Affinity chromatography
AHT	Artesial Hypertension
AUC	Area under the curve
BLU-HP	Blue-sepharose
DM	Diabetes Mellitus

eGPx	Extracelular glutathione peroxidase
GPx	Glutathione peroxidase
HC	Healthy control
HEP-HP	Heparine-sepharose
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-QQQ-MS	Inductively coupled plasma mass spectrometry with tryple quadrupole
IDA	Isotopic dilution analysis
LC	Lung cancer
NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
Se(IV),	Selenite
Se(VI),	Selenate
SeAlb	Selenoalbumin
SEC	Size exclusion chromatography
SeCys	Selenocysteine
SeCys2	Selenocystine
SeMeSeCys	Selenomethylselenocysteine
SeMet	Selenomethionine
SEPP1	Selenoprotein P
SUID	Species-unspecific isotopic dilution
TrxR1	Thioredoxin Reductase

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Figure Captions:

Figure 1. Scheme of the instrumentation based on SEC-AF-SUID-ICP-QQQ-MS. Chromatographic conditions: mobile phase A 0.05 M ammonium acetate; mobile phase B 1.5 M ammonium acetate. Gradient: 0-12 min 0% B, 12-25 min 100% B, 25-40 min 0%. Flow rate: 1.3 mL min^{-1}.

667 Figure 2. Superimpossed chromatograms of Se^{78} and Se^{80} signals obtained from the
668 injection into the AEC-SUID-ICP-QQQ-MS system of the standards: SeMet,
669 SeMeSeCys, $SeCys_2$, selenite and selenate, at 100 ng Se L^{-1}.

670 Figure 3. Mass flow chromatograms of a serum sample using the ratio $^{78}Se/^{74}Se$ by SEC-
671 AF-SUID-ICP-QQQ-MS for selenoproteins and using AEC-SUID-ICP-QQQ-MS for
672 selenometabolites.

673 Figura 4. A) Superimpossed ^{78}Se chromatogram of selenoproteins in a non-spiked and
674 a spiked serum sample with 10 ng Se g^{-1} of each: SeMet, SeMeSeCys, $SeCys_2$, selenite
675 and selenate. B) Superimpossed ^{78}Se chromatogram of selenometaboliotes in a non-
676 spiked in a spiked serum sample with 50 ng g^{-1} of SeMet, SeMeSeCys, $SeCys_2$, selenite
677 and selenate

678 Figure 5. Selenium distribution in selenoproteins and selenometabolites in human serum
679 of LC and HC groups.
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